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Selenium

Its Molecular Biology and Role in Human Health

Third Edition

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Dedication

This book is dedicated to Drs. August Böck and Thressa Stadtman. These outstanding scientists have had a major impact on the selenium field and are responsible for key discoveries in the biochemistry and molecular biology of this fascinating element.

Dr. Böck's research provided the foundation of how selenium makes its way into selenoproteins as selenocysteine, the 21st amino acid in the genetic code, in eubacteria. In his first work in the field, he identified unique requirements for selenocysteine incorporation into protein [1]. His second publication in this area was a highly significant collaboration with Dr. Stadtman showing that the in-frame TGA codon in the formate dehydrogenase gene corresponded to selenocysteine in the protein [2]. Dr. Böck then turned his attention to solving the question how selenium was incorporated into protein, discovering genes required for the pathway, characterizing their function, and ultimately establishing the mechanism for selenocysteine biosynthesis and insertion in bacteria (see reviews and Dr. Böck's and his group's many landmark discoveries in [3–5]). All subsequent research, including that in eukaryotes and archaea, benefited from these pioneering efforts. Dr. Böck followed these major discoveries with many elegant, highly important findings that provided the groundwork for conducting selenium research in subsequent years. These latter studies are summarized elsewhere [6, 7].

Among Dr. Stadtman's many accomplishments in the selenium field, there are those that provided the foundations for selenoprotein research, selenocysteine as the selenium-containing amino acid in protein, and the mechanism of how selenium is activated for synthesizing selenocysteine. In the first of these landmark studies, she identified glycine reductase as a selenoprotein in eubacteria in 1973 [8]. Then, in 1976, she and her research group identified the form of selenium in proteins as the amino acid, selenocysteine [9]. Later, Dr. Stadtman and her group identified selenophosphate as the selenium donor in the biosynthesis of selenocysteine [10]. In addition, her group demonstrated that the UGA codon in thioredoxin reductase codes for selenocysteine rather than being a terminator [11]. The many seminal accomplishments of Dr. Stadtman highly impacted the selenium field, opened up many new doors of research and changed how we view the field.

Drs. Böck and Stadtman collaborated on several innovative studies that also had a huge impact on the selenium field. The initial of these collaborations showed, as noted above, that the TGA codon in the formate dehydrogenase gene corresponded to selenocysteine in the selenoprotein product [2]. This study suggested that UGA dictated insertion of selenocysteine into protein, and this important point was later proven in another collaborative study of Drs. Böck's and Stadtman's showing that selenocysteine was biosynthesized on its tRNA in eubacteria [12]. At the same time, Dr. Stadtman and one of us, DLH, collaborated in showing that selenocysteine was biosynthesized on its tRNA in mammalian cells [13]. These two studies demonstrated that it is selenocysteine itself that was the 21st amino acid (rather than an intermediate that was incorporated into protein and then converted to selenocysteine posttranslationally). Another very important finding that Drs. Böck and Stadtman collaborated on was the demonstration of catalytic superiority of selenocysteine over cysteine [14]. Drs. Böck and Stadtman also worked together on several studies that influenced our understanding of the role of selenium in proteins (see refs. [15–17]).

It is a great honor and privilege to dedicate this book to Drs. August Böck and Thressa Stadtman. Without their pioneering studies, the selenium field would not be as we know it today, and certainly not with the firm foundation that provides the basis on which so much of the current work relies. We are deeply indebted to them for their many major discoveries, made both independently and in collaboration.

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Foreword

Selenium (Se), a metalloid mineral micronutrient, is an essential component for the adequate and healthy life of humans, animals, archaea, and some other microorganisms. Research into Se essentially commenced with its discovery as protective factor 3 against liver necrosis in rats by Schwarz and Foltz [1] and its role in formate dehydrogenase in *Escherichia coli* by Pinsent [2]. Biochemists, nutritional scientists, molecular biologists, bioinformaticians, biologists, and physicians have since worked out basic Se metabolism and some roles of selenoenzymes. This exciting Se history is compiled in the first book chapter. Six decades later we likely know most of the biochemical players, i.e., selenoproteins, products of 25 genes in humans [3], but we are still at the very beginning of understanding their physiological roles for maintenance of human health. Classical features of Se deficiency already described in life science textbooks years ago are not yet explained at the molecular level, e.g., liver necrosis, white muscle disease, cardiac, and skeletal muscle degeneration in Keshan disease or inappropriate chondrocyte differentiation in Kashin-Beck disease.

This third edition of the book, *Selenium: Its Molecular Biology and Role in Human Health*, edited by three leading scientists in selenoprotein research, Dolph Hatfield, Marla Berry, and Vadim Gladyshev, compiles in 45 chapters, organized under four sections, representing the state of the art in this rapidly expanding area of biomedical research. Research on the essential trace element Se has made unique progress with the identification of the opal UGA stop codon and its 21st proteinogenic amino acid, selenocysteine, which expanded the universal genetic code. It is an astonishing fact that selenocysteine, the key amino acid exerting most of Se's action, is the only amino acid that cannot simply be recycled for de novo selenoprotein biosynthesis, but has to be completely degraded in an enzymatic process catalyzed by selenocysteine lyase to a reduced form of Se. This can then reenter the complex cotranslational insertion process of selenocysteine into the nascent protein chain, provided that many of structural conditions are met by its respective mRNA and a series of *cis*- and *trans*-acting translation-assisting factors are in place. This major focus of the recent efforts in Se research is covered in Part I of the book, *Selenocysteine Biosynthesis and Incorporation into Protein*. Leading experts contribute seven

chapters elaborating on components and mechanisms involved in biosynthesis of selenocysteine and selenoproteins. Details of the SECIS elements of the corresponding mRNAs and SECIS binding proteins which regulate the expression of the selenoproteome in various phyla are presented in this section. Evolutionary aspects and the degradation of selenocysteine by a dedicated lyase complete this first part.

Thirteen chapters in Part II of the book, contributed by competent selenoprotein researchers, are devoted to the biochemistry and functional aspects of selenoprotein physiology. Successful approaches combining current molecular biology and recent developments in bioinformatics revealed identity, evolution, and function of selenoproteins and their genes are reviewed in the first chapter of this section. Descriptions of selenoprotein structures and the peculiar hierarchy of Se availability for individual selenoproteins follow. The central role of selenoproteins in redox-regulation involving the thioredoxin/thioredoxin reductase system and individual members of the glutathione peroxidase family is covered in the subsequent chapters which address clinically relevant Se functions in the cardiovascular redox system, diabetes, muscular and nervous system development and their degenerative diseases, as well as in various forms of cancer. Further chapters of this section also review the tremendous recent progress in understanding the role of selenoproteins M, N, and P. Sel N is essential for muscle development and function as indicated by identification of several mutations leading to rigid spine muscular dystrophy and multiminicore disease. Sel P has been identified as the main hepatically secreted selenium distribution and transport protein in serum. Several selenoproteins are involved in quality control of protein synthesis in the endoplasmic reticulum.

The 19 chapters of Part III of this impressive book focus even more on the relationship between Se and selenoproteins in human health. The first five chapters cover the area of Se's still controversial role in cancer promotion and prevention as recently featured by the unexpected premature termination of the SELECT trial that examined the role of selenium in prostate cancer prevention. Se and selenoproteins are also involved in pathogenesis and progress of diseases such as schizophrenia, thyroid dysfunction including autoimmune diseases, impaired reproduction function in males and females including pregnancy, infections such as HIV/AIDS, and parasite-related diseases such as malaria. These topics are covered in subsequent chapters, also addressing mechanistic aspects of impaired selenoprotein synthesis and function and disturbances leading to enhanced oxidative stress. Se's role in inflammation, antioxidative defense, redox signaling, methionine sulfoxide reduction, Alzheimer's disease, and even methylmercury exposure risks are also presented. Not surprisingly, there are also clinically relevant variations in Se metabolism in males and females and important progress has been made in understanding Se metabolism in prokaryotes impacting on infectious diseases and their treatment. The last two chapters cover functional aspects of the genomics of selenoprotein and Se-related genes and review dietary sources and human Se requirements.

The final part with three chapters represents a highlight of current biomedical translational research taking advantage of novel mouse models for elucidating the role of Se and selenoproteins in health and disease. Several mouse models for

glutathione peroxidase 4's function and deficiency have provided major insight into the role of Se for mammalian development and diseases of the adult brain, the cardiovascular system, and male reproduction. The last two chapters complement molecular and functional insight into selenoproteins by discussing mouse models targeting removal or overexpression of the selenocysteine $tRNA^{[Ser]Sec}$ gene and interpret lessons learned from *Trsp* deletion in murine bone and cartilage progenitor cells and their impact on skeletal development and diseases.

This book impressively illustrates significant conceptual, methodological, and scientific changes of paradigm which occurred with novel input from bioinformatics, genome, transcriptome and proteome research, and the stringent application of mouse genetics. These powerful novel tools and the clever design and application of knockout, knockdown, knock-in and overexpression approaches of specific "selenogenes," their mutants or variants in cellular, and transgenic mouse models clearly identified molecular mechanisms related to Se action. The first molecular identifications of human phenotypes of deficient selenoprotein expression and function supported cause-effect relationships beyond previous assumptions which were based on mere correlations or observational and epidemiological studies on Se and human health and disease.

Pioneers from the first hours of Se research are still active in the field and contributed to this book together with a new generation of highly motivated and skilled researchers, who have rejuvenated the field introducing new methods, contributing novel ideas, altered paradigms, and innovative concepts such as molecular biology, genomics, bioinformatics, and developed novel drugs and agents.

Se research thus has matured and has now a firm mechanistic basis. The classical theory of selenoprotein action as antioxidative devices degrading peroxides and preventing generation of reactive oxygen and nitrogen species proved too limited. Many new questions are emerging: Are the many Se effects related to efficient differential expression of selenoprotein isoforms from a single gene? Are selenoproteins located at strategic positions controlling entire metabolic or functional pathways? Is this mediated by redox-regulation of proteins, by modulation of small molecule messengers, or both? Why do we, animals, archaea, and some microorganisms need the peculiar chemical properties of selenocysteine while other organisms, including plants and fungi, get along with cysteine alone? How are issues of deficiency, adequacy, excess, and toxicity related to specific Se forms and species? Does the genetic makeup of an individual interfere with Se uptake, metabolism, selenoproteome expression, and is this relevant for pathogenesis or treatment of major diseases?

We will convene to discuss further progress achieved at the next International Selenium Meeting in Berlin in a couple of years. Hopefully we also will soon need another edition of this illuminative book documenting these discussions and novel developments in the exciting field of biomedical Se research.

Berlin, Germany Josef Köhrle

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Preface

The selenium field is expanding at a rapid pace and has grown dramatically in the last 10 years since the first edition of *Selenium: Its Molecular Biology and Role in Human Health* was published in 2001. All aspects of selenium biology have advanced with many new approaches and insights into the biochemical, molecular, genetic, and health areas of this intriguing element. In the first edition of this book, there were 25 chapters with 46 contributors that increased to 35 chapters with 71 contributors in the second edition. In the present edition, there are 45 chapters with 96 contributors. At this pace of expansion, and provided the fourth edition of *Selenium: Its Molecular Biology and Role in Human Health* is undertaken for publication in 2016, we can envision two volumes containing 29–30 chapters per volume with more than 125 contributors.

This book addresses many of the new and exciting discoveries that have occurred since the last edition was published in 2006. The numerous selenoproteins and proteins involved in the incorporation of selenium into protein that were described in the first two editions have been further characterized, new observations made, and mutant forms of some selenoproteins have been shown to be linked to human diseases. New factors have been detected that are involved specifically in the incorporation of selenium into protein. Mouse models targeting the removal of a specific selenoprotein, or removal of all selenoproteins, have further defined the role of selenoproteins in health and development. One of these has provided a potential model for Kashin-Beck disease.

Various aspects to glutathione peroxidase 4 (GPx4) are discussed in several chapters and its targeted removal suggested that it plays significant roles in proper function of numerous tissues and organs. GPx4 is now regarded as one of the more important selenoproteins in development. A role of selenium in cancer prevention has been purported for many years but we have learned in only the last few years that there are at least three selenoproteins that appear to have roles in preventing as well as promoting cancer. A role of selenium in male reproduction has also been purported for many years and the roles of specific selenoproteins in this process are now known and their functions elucidated.

Investigators in the selenium field are now looking at selenium differences in males and females and the role of selenium in pregnancy. In addition, the biosynthetic

pathway of selenocysteine in eukaryotes and archaea has been elucidated since the last edition – selenocysteine is not only the 21st amino acid in the genetic code but it was also the last known protein amino acid whose biosynthesis had not been resolved in eukaryotes and is the only known amino acid whose biosynthesis occurs on its tRNA in eukaryotes. Very recently, sulfur was found to replace selenium in the biosynthesis of selenocysteine in eukaryotes providing a novel pathway for cysteine biosynthesis that results in the replacement of selenocysteine with cysteine in selenoproteins.

The purpose of the present edition of the book is to bring readers up-to-date with the many new discoveries in the selenium field and to inform them of our present knowledge of the molecular biology of selenium, its incorporation into proteins as selenocysteine, and the role that this element and selenium-containing proteins (selenoproteins) play in health and development. In addition to being regarded as a chemopreventive agent, several other health benefits have been attributed to selenium. It has been touted as an inhibitor of viral expression and may prevent heart disease and other cardiovascular and muscle disorders, slow the aging process, delay the progression of AIDS in HIV positive patients, and have roles in development and immune function. Thanks to the many elegant techniques developed in recent years for examining selenium metabolism and selenoproteins in greater detail, investigators are now demonstrating how this element functions at the molecular level to bring about these many health benefits.

The present book is divided into four sections. Part I is entitled *Selenocysteine Biosynthesis and Its Incorporation into Protein* and it describes in detail our current understanding of the means by which selenium makes its way into protein as the 21st amino acid in the genetic code. Also discussed in this section are some of the reasons that selenocysteine may have evolved in protein and is used in place of cysteine in selenium-containing proteins. In addition, selenocysteine lyase, an important enzyme involved in selenium metabolism, is discussed. In Part II, entitled *Selenoproteins and Selenoproteins in Health*, many of the better characterized selenoproteins are examined including those that have been shown to play roles in health as defined by studies with rodents. Other chapters in this section examine such phenomena as selenoprotein hierarchy and the evolution of selenoproteins and their functions. The focus in Part III, entitled *Selenium and Selenoproteins in Human Health*, is on the role that selenium and selenoproteins play primarily in human health, while Part IV, *Mouse Models for Elucidating the Role of Selenium and Selenoproteins in Health* emphasizes the significance that mouse models have played in assessing selenoprotein roles in development and health.

The current edition of *Selenium: Its Molecular Biology and Role in Human Health* provides a most up-to-date examination of the on-going research in the selenium field. It is an important resource for investigators in the selenium field, other scientists, students and physicians, as well as those who wish to learn more about this fascinating micronutrient.

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Chapter 1
History of Selenium Research

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Abstract Selenium research must be said to have began in 1817, when Berzelius discovered this element. The first genuine publication describing this research was published by Berzelius in 1818, in a paper where he also named the element as *Selenium*. Here, in this chapter on the history of selenium research, an attempt is made to take a "bird's-eye" view at the development of this research field since 1817 until today. The tool chosen is an analysis of the scientific literature on selenium research, thereby attempting to give an unbiased assessment of this research field. Finally, as in all assessments of historic trends, we should also ask where the future of selenium research might take us. By necessity, the answer to that question is uncertain. However, we can conclude that never before has selenium research been as vigorous and expanding as it is today, which also holds major promise for the future.

1.1 Previously Published Recollections of the History of Selenium Research

Many reviews have described the development of selenium research and the findings that have shaped current day's knowledge in the field, including personal recollections by some of the pioneers of selenium research. Just to name a few, this includes some groundwork reviews on selenocysteine by Böck [1] or Stadtman [2], reflections by Dolph Hatfield and Vadim Gladyshev on how the selenocysteine recoding of the UGA codon became the first expansion of the genetic code since its original discovery [3], and the recent narrative of "*The Labour Pains of Biochemical Selenology: The History of Selenoprotein Biosynthesis*" by Flohé [4]. In his review,

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Dr. Flohé also gave an informative chronology of a number of landmarks related to research on selenium in biology (see Table 1.1 in [4]). It would be of little use in this chapter to simply repeat information given in previous reviews on the selenium research field. The reader is therefore referred to other papers on the history of selenium research for discussions on specific details or topics of that research. Information on several aspects of selenium research is also found in other chapters of this book, which together give comprehensive up-to-date insights into most aspects of current research on selenium biochemistry and molecular biology. Here, we shall instead take a "bird's-eye" view on the history and development of selenium research, using a bibliometric analysis of the trends in selenium research literature. With this as our focus, let us begin with the very first publication on selenium as published by Berzelius.

1.2 Berzelius and the Discovery of Selenium

Jöns Jacob Berzelius (1779–1848), or "Jacob Berzelius" as he was called by his peers, was one of the most important chemists of his time. He invented the term "catalysis," he constructed the major rules of chemical notation still used, and he discovered several basic elements, among them selenium. His work has been described in several publications, among which a biography written by Dr. Söderbaum should be the most comprehensive (H. G. Söderbaum, *Jac. Berzelius, Levnadsteckning*, 3 vols., Uppsala, 1929–1931). Therein, one may read how Berzelius in 1817, studying the bottom sludge remaining from a sulfuric acid preparation, realized that there was a new element in the preparation, and how he completed his initial analyses in only 4 months. This must be viewed as a major accomplishment considering the exactness and correctness by which he described selenium in spite of his, by today's standards, rather rudimentary technology. When publishing his findings in 1818, the paper was written in Swedish and, interestingly, published in a periodical that Berzelius himself was editing together with a number of colleagues (Fig. 1.1a). It was in this article that he officially named the element *Selenium* (Fig. 1.1b) and in that very publication, he also reported on several of the typical chemical characteristics of selenium that still today underpin all work on this element. Already in his first studies, Berzelius noted the close similarities between selenium and sulfur, which obviously govern the similar properties of selenocysteine-containing proteins and those of cysteine-containing orthologues, which today is a debated and active research subject as recently discussed elsewhere in more detail (see [5] and references therein).

1.3 Bibliometric Analysis of Selenium Research Since 1945

Bibliometry is today fashionable among universities, funding agencies, and policy makers for the evaluation of research output. Many aspects of this usage of bibliometry is often flawed, as a consequence of inadequate bibliometry units, year- and

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Mineralogi.

Utgifne

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J. Afzelius, N. W. Almroth, A. Arfvedson,
J. Berzelius, H. P. Eggertz, J. af Forselles,
J. G. Gahn, W. Hisinger, P. Lagerhjelm,
M. af Pontin, E. Rothoff, N. G. Sefström,
P. Ström, E. T. Svedenstierna,
P. Walmstedt.

Sjette Delen.

STOCKHOLM,
Tryckte hos Direct. H. A. Nordström,
1818.

b Det bruna ämnet, ſom vid ammoniak-ſalternas ſönderdelning afſkiljt ſig, blef nu ett föremål för underſökningen, och befanns, genom de förſök, ſom i det följande ſkola beſkrifvas, vara en egen, hittills okänd, brännbar mineralkropp, hvilken jag, för att utmärka deſs ſlägtſkap i egenſkaper med tellurium, kallat Selenium, af Σελήνη, måna.

Fig. 1.1 The first publication on selenium. The figure shows the (**a**) front page and (**b**) an excerpt of the text on p. 49 from the original reference where Berzelius first described his discovery of selenium and named the element [8]; a scanned copy of this book is at present freely available on internet through a search in Google Books. An English translation of the text given in (**b**) reads as follows: "The brown substance, which the decomposition of the ammonium salts yielded, now became an object of investigation, and was found, through the experiments, which in the following will be described, to be a separate, hitherto unknown, combustible mineral, which I, to mark its akin properties with tellurium, have named Selenium, from Σεληνη, moon (goddess)"

topic-skewed factors, and the fact that truly groundbreaking research is seldom reflected in citation numbers or other bibliometric readouts, at least not until years or decades after the initial discoveries. For one reason or the other, some very important findings never become acknowledged by an adequately high number of citations, such as with the first discoveries of selenium essentiality in bacteria by Pinsent [6] or in mammals by Schwarz [7]; two examples of quite rarely cited papers in relation to their evident importance in the history of selenium research. Units such as "impact factor," "h-index," or "crown index" are seldom more than, at best, distant reflections of scientific progress, when used in assessment of specific constellations of researchers, departments, or universities. However, bibliometry can certainly tell much about the development, trends, and flavors of whole research fields over time. For that particular purpose, we shall here use this tool as a method for the analysis of the history of selenium research. All the data as presented in this chapter were obtained in December 2010, using literature searches in the ISI Web of Science (http://isiknowledge.com/wos) or Scopus (http://www.scopus.com) databases, with the keywords "selenium," "selenocysteine," or "selenoprotein." Because these databases have little or no information about publications published before 1945, we shall here take a leap from the article of Berzelius published in 1818 until the year 1945 when continuing our analysis of the history of selenium research.

1.3.1 The First Papers from the Selenium Research Field as Found in the ISI Web of Science Database

In the ISI Web of Science database, eight articles can be found from the first year covered by the database (1945) using the keyword "selenium." These articles covered subjects of selenium toxicity (three articles), selenium levels in soil, plants, or animals (two articles), or the photodynamic properties of selenium, its spectral properties, or the oxidizing capacity of selenium dioxide (one article each). Indeed, selenium as solely a toxic compound for man and animals was the main view on selenium for many decades, before its important role as a natural constituent of selenocysteine and selenoproteins was discovered, as described in detail by Flohé in his recent review [4]. The subjects of those eight papers from 1945 that focused on agricultural, physical, or chemical properties of selenium are in principle the very same subjects that have made "selenium" a much more studied topic in research than the more specific "selenocysteine" or "selenoprotein" topics. Although some articles on genuine selenoproteins or related areas may be missed using just the keywords, "selenoprotein" and "selenocysteine," in database searches (thereby not necessarily finding articles on "Se-protein," "selenite," "selenate," "selenoenzyme," etc.), it is still of historic interest to list the first published papers found in the ISI database using only these two keywords. The very first articles found with the keyword "selenoprotein" were published in 1973 by Thressa Stadtman, where she reported that glycine reductase was a *selenoprotein*, i.e., a protein that in its native state contained selenium. The very first article on "selenocysteine" present in the

ISI database was published in 1976 and described the chromatographic behavior of carboxymethylated forms of cysteine and selenocysteine. In the same year (1976), Thressa Stadtman also published an article identifying the selenium-containing entity in glycine reductase as indeed being selenocysteine. After these reports, only few articles were published on "selenocysteine" or "selenoprotein" within the following years, and the ten first papers on these subjects stretch over the years 1973–1980 (for "selenoprotein") or 1976–1978 (for "selenocysteine"). See Table 1.1 for a complete list of these references. It should be noted, however, that during this period, i.e., for the years from 1945 to 1980, a total of 5,672 articles can be found in the ISI database on the topic of "selenium," with 248 articles published in the year

Table 1.1 The first papers on "selenium," "selenocysteine," and "selenoprotein" found in the ISI Web of Science database

Eight articles on "selenium" from 1945	First ten articles on "selenocysteine"	First ten articles on "selenoprotein"
Moxon, AL; Paynter, CR; Halverson, AW, *Effect of route of administration on detoxication of selenium by arsenic (1945) J. Pharm. Exp. Therap.*, 84 (2): 115–119	Rinaldi, A; Cossu, P; Demarco, C *Ion-exchange chromatography of s-(carboxymethyl)cysteine and se-(carboxymethyl) selenocysteine (1976)* J. of Chromatogr., 120 (1): 221–223	Stadtman, TC *Participation of a selenoprotein in anaerobic electron-transport reaction catalyzed by glycine reductase (1973)* Fed. Proc., 32 (3): 478
Robinson, WO *Determination of total selenium and arsenic in soils (1945) Soil science*, 59 (1): 93–95	Cone, JE; Martindelrio, R; Davis, JN; Stadtman, TC *Chemical characterization of selenoprotein component of clostridial glycine reductase - identification of selenocysteine as organoselenium moiety (1976)* PNAS, 73 (8): 2659–2663	Turner, DC; Stadtman, TC *Purification of protein components of clostridial glycine reductase system and characterization of protein A as a selenoprotein (1973)* Arch. Biochem. Biophys., 154 (1): 366–381
Trelease, SF *Selenium in soils, plants, and animals (1945)* Soil Science, 60 (2): 125–131	Demarco, C; Rinaldi, A; Dessi, MR; Dernini, S *Oxidation of se-carboxymethyl-selenocysteine by l-aminoacid oxidase and by d-aspartate oxidase (1976)* Mol. Cell. Biochem., 12 (2): 89–92	Stadtman, TC *Composition and some properties of selenoprotein of glycine reductase (1974)* Feder. Proc., 33 (5): 1291
Taboury, MMF **Toxicologie DU selenium et de ses composes chez les animaux et les vegetaux (1945)* Bulletin de la Societe de Chimie Biologique, 27 (4–6): 157–163	Shrift, A; Bechard, D; Harcup, C; Fowden, L *Utilization of seleno-cysteine by a cysteinyl-transfer-RNA synthetase from phaseolus-aureus (1976)* Plant Physiol., 58 (3): 248–252	Herrman, JL; Mcconnell, KP *Isolation and purification of a rat serum selenoprotein (1975)* Feder. Proc., 34 (3): 925

(continued)

Table 1.1 (continued)

Eight articles on "selenium" from 1945	First ten articles on "selenocysteine"	First ten articles on "selenoprotein"
Bacharach, AL *Selenium poisoning (1945)* British Medical J., 1 (4390): 276	Portanova, JP; Shrift, A *Usefulness of N-ethylmaleimide in identification of se-75-labeled selenocysteine (1977)* J. of Chromatogr., 139 (2): 391–394	Herrman, JL; Mcconnell, KP *Some properties of a rat serum selenoprotein (1976)* Feder. Proc., 35 (7): 1608
Preston, JS; Smith, GWG *The internal resistance of the selenium rectifier photocell, with special reference to the sputtered metal film (1945)* Proc. Phys. Soc. London, 57 (319): 1–11	Lyons, DE; Hawkes, CW; Forstrom, JW; Zakowski, JJ; Dillard, CJ; Litov, RE; Tappel, AL *Selenium-glutathione peroxidase - incorporation of selenium, selenocysteine as catalytic site, and effect on in vivo lipid peroxidation (1978)* Feder. Proc., 37 (6): 1339	Cone, JE; Martindelrio, R; Davis, JN; Stadtman, TC *Chemical characterization of selenoprotein compo nent of clostridial glycine reductase - identification of selenocysteine as organoselenium moiety (1976)* PNAS, 73 (8): 2659–2663
Waitkins, GR; Clark, CW *Selenium dioxide - preparation, properties, and use as oxidizing agent (1945)* Chemical Reviews, 36 (3): 235–289	Forstrom, JW; Zakowski, JJ; Tappel, AL *Identification of catalytic site of rat-liver glutathione peroxidase as selenocysteine (1978)* Biochem., 17 (13): 2639–2644	Cone, JE; Rio, RMD; Stadtman, TC *Characterization of selenoprotein of clostridial glycine reductase complex (1977)* Feder. Proc., 36 (3): 876–876
Feldman, C *The Spectrographic detection of selenium in the dc arc flame (1945)* J. Optical Soc. Amer., 35 (2): 180–184	Cini, C; Demarco, C *Carboxymethyl-selenopyruvic acid as product of oxidative deamination of carboxym-ethyl-selenocysteine (1978)* Italian J. of Biochem., 27 (2): 104–110	Cone, JE; Delrio, RM; Stadtman, TC *Clostridial glycine reductase complex - purification and characterization of selenoprotein component (1977)* JBC, 252 (15): 5337–5344
	Zakowski, JJ; Forstrom, JW; Condell, RA; Tappel, AL *Attachment of seleno-cysteine in catalytic site of glutathione peroxidase (1978)* BBRC, 84 (1): 248–253	Mcconnell, KP; Burton, RM; Kute, T; Higgins, PJ *Selenoprotein from rat testes cytosol (1978)* Feder. Proc., 37 (6): 1813–1813
	Ng, BH; Anderson, JW *Synthesis of selenocysteine by cysteine synthases from selenium accumulator and non-accumulator plants (1978)* Phytochem., 17 (12): 2069–2074	Hartmanis, M *A new selenoprotein from clostridium-kluyveri that copurifies with thiolase (1980)* Feder. Proc., 39 (6): 1772–1772

1970 and 434 articles in 1980, which illustrates how the field of selenium research had clearly caught on, even if the "selenoprotein" or "selenocysteine" research fields were yet only in their initial phases.

1.3.2 Size and Development of the Selenium Research Field

Among the articles covered by the ISI Web of Science database, it is quite striking how the publications found using the keyword "selenium" are so many more than those found using the keywords "selenocysteine" or "selenoprotein," also when covering articles published in recent years. Even more publications are found on "selenium" when using the Scopus database, which in addition covers a number of other documents including patents, conference proceedings, etc. In total, close to 111,000 publications on "selenium" could be found in the Scopus database by mid-December 2010, while those retrieved on the topic of "selenocysteine" were about 2,050, and when using the keyword "selenoprotein," about 1,750 papers could be found. The corresponding numbers for searches in the ISI database were 39,157 papers on "selenium," 7,761 papers on "selenocysteine," and 1,608 articles on "selenoprotein." The noticeable divergence in these numbers between the Scopus and ISI databases is yet another illustration of the many levels of bias embedded in bibliometric analyses, clearly governed by aspects such as the rules of a database for inclusion of publications, the database curator's choices of keywords, classifications into topics, and more. Still, the results of analyses such as those made here should still reflect the overall trends in the history and development of selenium research.

A graph of the number of papers over time as found in the ISI database vividly displays how the "selenium" research field has grown rapidly over the decades, while the articles found using the more specific keywords "selenocysteine" or "selenoprotein" are significantly less in number, although these are also growing steadily during the last 2 decades (Fig. 1.2). For the last couple of years, the publication frequency is about 2,000 papers per year published on the topic of "selenium" (as found in the ISI database) while those on "selenocysteine" or "selenoprotein" are typically between 150 and 200 papers published per year. Some of these latter articles are found regardless of the choice of "selenoprotein" or "selenocysteine" as keyword, but surprisingly many articles are found in the database searches using only one of these two keywords.

1.3.3 Subject Areas in Selenium Research

With industrial usage of selenium in glass, ceramics, photocopiers, rectifiers, solar cells, and more, and because of its properties as a catalyst in nonorganic chemistry, a large number of research publications on selenium are not at all related to biology or biochemistry. This, naturally, is a fact that differs from the topics of articles

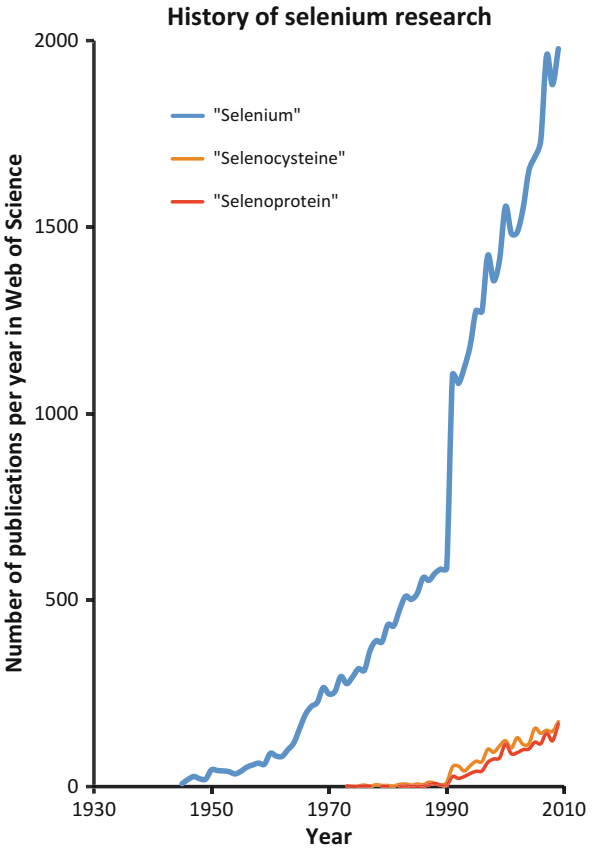

Fig. 1.2 Selenium research publications over time. The figure displays the number of publications for each year between 1945 and 2010 as found in the ISI Web of Science database using the key words "selenium," "selenocysteine," or "selenoprotein"

studying selenocysteine or selenoproteins, where the vast majority of such papers are classified by ISI as being focused on topics such as "Biochemistry & Molecular Biology," "Cell Biology," or "Endocrinology & Metabolism." When it comes to the papers on "selenium," biologically related subject areas amount to about half of the articles, with the remaining half mainly focused on topics relating to different subject areas of chemistry and physics (Fig. 1.3).

1.3.4 The Most Cited Publications on "Selenium," "Selenoproteins," and "Selenocysteine" Until Present Day

One method of analyzing what subjects of selenium research that have raised most interest is to analyze citation patterns. This type of analysis will, naturally, result in a

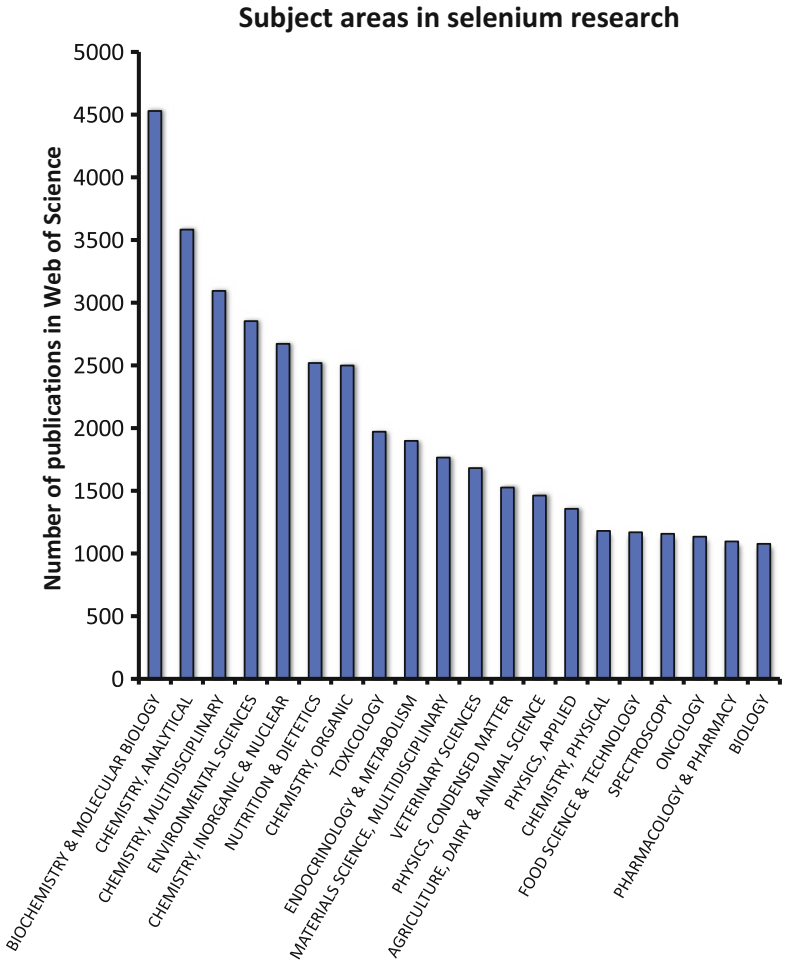

Fig. 1.3 Subject areas in selenium research. The *bar graph* shows subject areas within selenium research as listed in the ISI Web of Science database, displaying all subject areas that encompass at least 1,000 publications each

bias toward review articles, which are typically cited more than original reports, older articles that have had time to attract more citations, and articles that have a contextual impact on research topics involving many different research fields. However, in spite of such shortcomings, much can still be learned about the accumulated trends in the history of selenium research when performing these types of citation analyses. Using Scopus to find the 25 most cited articles on "selenium" until today reveals that the three most cited articles dealt with the use of selenium in electrophysical chemistry, the fourth paper related to the use of selenium to help solving the phase problem in crystallography, and the fifth and sixth papers were reviews on oxidative stress or antioxidant enzymes (Table 1.2). The profiles and "ranks" of these papers in this citation analysis are likely to be representative of the accumulated historic trends in

Table 1.2 The 25 most cited publications on "selenium" as listed in the Scopus database

	Keyword "selenium" gives 109,990 documents in total in Scopus					
	Citations					
	Year	<2008	2008	2009	2010	Total
	Total citations	43,026	7,557	7,840	6,922	65,345
1	Bruchez Jr., M., Moronne, M., Gin, P., Weiss, S., Alivisatos, A.P. Semiconductor nanocrystals as fluorescent biological labels (1998) *Science*, 281 (5385), pp. 2013–2016.	2,134	505	533	449	3,621
2	Xia, Y., Yang, P., Sun, Y., Wu, Y., Mayers, B., Gates, B., Yin, Y., Yan, H. One-dimensional nanostructures: Synthesis, characterization, and applications (2003) *Advanced Materials*, 15 (5), pp. 353–389.	1,764	623	607	553	3,547
3	Chan, W.C.W., Nie, S. Quantum dot bioconjugates for ultrasensitive nonisotopic detection (1998) *Science*, 281 (5385), pp. 2016–2018.	1,916	466	502	414	3,298
4	Terwilliger, T.C., Berendzen, J. Automated MAD and MIR structure solution (1999) *Acta Crystallographica Section D: Biological Crystallography*, 55 (4), pp. 849–861.	2,092	231	195	158	2,676
5	Finkel, T., Holbrook, N.J. Oxidants, oxidative stress and the biology of ageing (2000) *Nature*, 408 (6809), pp. 239–247.	1,302	270	267	293	2,132
6	Hayes, J.D., Pulford, D.J. The glutathione S-transferase supergene family: Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance (1995) *Critical Reviews in Biochemistry and Molecular Biology*, 30 (6), pp. 445–600.	1,673	110	121	95	1,999
7	Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G., Hoekstra, W.G. Selenium: Biochemical role as a component of glutathione peroxidase (1973) *Science*, 179 (4073), pp. 588–590.	1,268	174	203	184	1,829
8	Grundy, S.M., McBride, P., McKenney, J.M., Pasternak, R.C., Stone, N.J., Van Horn, L., Becker, D., Luepker, R.V. Detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III) (2002) *Circulation*, 106 (25), pp. 3143–3421.	781	325	333	309	1,748

(continued)

Table 1.2 (continued)

	Keyword "selenium" gives 109,990 documents in total in Scopus					
	Citations					
	Year	<2008	2008	2009	2010	Total
9	Vurgaftman, I., Meyer, J.R., Ram-Mohan, L.R. Band parameters for III–V compound semiconductors and their alloys (2001) *J. of Applied Physics*, 89 (11 I), pp. 5815–5875.	980	241	254	218	1,693
10	Gardner, M.J., Bowman, S., Paulsen, I.T., James, K., Eisen, J.A., Rutherford, K., Salzberg, S.L., Nelson, K.E. Genome sequence of the human malaria parasite Plasmodium falciparum (2002) *Nature*, 419 (6906), pp. 498–511.	1,049	164	164	160	1,537
11	Clark, L.C., Gross, E.G., Krongrad, A., Lesher Jr., J.L., Park, H.K., Sanders Jr., B.B., Smith, C.L., Graham, G.F. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin: A randomized controlled trial (1996) *J. of the American Medical Association*, 276 (24), pp. 1957–1963.	1,179	116	121	106	1,522
12	Murray, C.B., Kagan, C.R., Bawendi, M.G. Synthesis and characterization of monodisperse nanocrystals and close-packed nanocrystal assemblies (2000) *Annual Review of Materials Science*, 30, pp. 545–610.	847	205	220	219	1,491
13	Hennekens, C.H., Ridker, P.M., Willett, W., Peto, R., Buring, J.E., Manson, J.E., Stampfer, M., Gaziano, J.M. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease (1996) *NEJM*, 334 (18), pp. 1145–1149.	1,271	73	69	34	1,447
14	Stephens, N.G., Parsons, A., Schofield, P.M., Kelly, F., Cheeseman, K., Mitchinson, M.J., Brown, M.J. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS) (1996) *Lancet*, 347 (9004), pp. 781–786.	1,336	44	33	29	1,442
15	Puntes, V.F., Krishnan, K.M., Alivisatos, A.P. Colloidal nanocrystal shape and size control: The case of cobalt (2001) *Science*, 291 (5511), pp. 2115–2117.	858	166	151	134	1,309

(continued)

Table 1.2 (continued)

	Keyword "selenium" gives 109,990 documents in total in Scopus					
	Citations					
	Year	<2008	2008	2009	2010	Total
16	Smedley, P.L., Kinniburgh, D.G. A review of the source, behaviour and distribution of arsenic in natural waters (2002) *Applied Geochemistry*, 17 (5), pp. 517–568.	572	189	246	201	1,208
17	Pyykkö, P. Strong closed-shell interactions in inorganic chemistry (1997) *Chemical Reviews*, 97 (3), pp. 597–636.	866	108	110	103	1,187
18	Flederling, R., Kelm, M., Reuscher, G., Ossau, W., Schmidt, G., Waag, A., Molenkamp, L.W. Injection and detection of a spin-polarized current in a light-emitting diode (1999) *Nature*, 402 (6763), pp. 787–790.	968	86	69	62	1,185
19	Yu, B.P. Cellular defenses against damage from reactive oxygen species (1994) *Physiological Reviews*, 74 (1), pp. 139–162.	925	85	96	73	1,179
20	Kroemer, G., Zamzami, N., Susin, S.A. Mitochondrial control of apoptosis (1997) *Immunology Today*, 18 (1), pp. 44–51.	1050	36	28	25	1,139
21	Ames, B.N. Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases (1983) *Science*, 221 (4617), pp. 1256–1263.	940	61	59	49	1,109
22	Lawrence, R.A., Burk, R.F. Glutathione peroxidase activity in selenium deficient rat liver (1976) *Biochemical and Biophysical Research Communications*, 71 (4), pp. 952–958.	818	80	107	104	1,109
23	Rayman, M.P. The importance of selenium to human health (2000) *Lancet*, 356 (9225), pp. 233–241.	677	148	140	134	1,099
24	Brown, B.G., Alaupovic, P., Frohlich, J., Serafini, L., Huss-Frechette, E., Wang, S., DeAngelis, D., Bolson, E.L. Simvastatin and niacin, antioxidant vitamins, or the combination for the prevention of coronary disease (2001) *New England J. of Medicine*, 345 (22), pp. 1583–1592.	768	130	106	92	1,096
25	Steinmetz, K.A., Potter, J.D. Vegetables, fruit, and cancer prevention: A review (1996) *J. of the American Dietetic Association*, 96 (10), pp. 1027–1039.	844	96	73	59	1,072

Date of creation: 16 December 2010

selenium research. The seventh paper in that list is an original study from 1973 by Hoekstra and coworkers identifying the presence of selenium in glutathione peroxidase, while the topics of the remaining papers on the list reflect a wide variety of subjects that either directly or indirectly relate to the role of selenium in chemistry, physics, or biology (Table 1.2). In a similar analysis of the most cited papers found using the keyword "selenocysteine," the top article describes a bioinformatic method to find tRNA genes in genomic sequences, the second and third papers are two general reviews on the thioredoxin system, the fourth paper is the first description of the complete set-up of selenoproteins in mammals, while the fifth paper is the already mentioned review of Thressa Stadtman on selenocysteine from 1996 (Table 1.3). The whole list of the 25 most cited papers on selenocysteine probably describes rather well the major trends in research on this amino acid. Clearly, this research field is focused on either of two main subjects, namely the catalytic role of the amino acid in

Table 1.3 The 25 most cited publications on "selenocysteine" as listed in the Scopus database

	Keyword "selenocysteine" gives 2,055 documents in total in Scopus					
	Citations					
	Year	<2008	2008	2009	2010	Total
	Total citations	10,305	1,248	1,294	1,046	13,893
1	Lowe, T.M., Eddy, S.R. tRNAscan-SE: A program for improved detection of transfer RNA genes in genomic sequence (1997) *Nucleic Acids Research*, 25 (5), pp. 955–964.	830	173	199	196	1,398
2	Arnér, E.S.J., Holmgren, A. Physiological functions of thioredoxin and thioredoxin reductase (2000) *European J. of Biochemistry*, 267 (20), pp. 6102–6109.	514	85	96	77	772
3	Nordberg, J., Arnér, E.S.J. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system (2001) *Free Radical Biology and Medicine*, 31 (11), pp. 1287–1312.	388	80	83	84	635
4	Kryukov, G.V., Castellano, S., Novoselov, S.V., Lobanov, A.V., Zehtab, O., Guigó, R., Gladyshev, V.N. Characterization of mammalian selenoproteomes (2003) *Science*, 300 (5624), pp. 1439–1443.	266	88	86	62	502
5	Stadtman, T.C. Selenocysteine (1996) *Annual Review of Biochemistry*, 65, pp. 83–100.	402	36	32	21	491
6	Berry, M.J., Banu, L., Larsen, P.R. Type I iodothyronine deiodinase is a selenocysteine-containing enzyme (1991) *Nature*, 349 (6308), pp. 438–440.	377	18	9	14	418
7	Combs Jr., G.F., Gray, W.P. Chemopreventive agents: Selenium (1998) *Pharmacology and Therapeutics*, 79 (3), pp. 179–192.	266	39	32	21	358

(continued)

Table 1.3 (continued)

	Keyword "selenocysteine" gives 2,055 documents in total in Scopus					
	Citations					
	Year	<2008	2008	2009	2010	Total
8	Ganther, H.E. Selenium metabolism, selenoproteins and mechanisms of cancer prevention: Complexities with thioredoxin reductase (1999) *Carcinogenesis*, 20 (9), pp. 1657–1666.	261	36	32	24	353
9	Mustacich, D., Powis, G. Thioredoxin reductase (2000) *Biochemical Journal*, 346 (1), pp. 1–8.	228	28	38	26	320
10	Tamura, T., Stadtman, T.C. A new selenoprotein from human lung adenocarcinoma cells: Purification, properties, and thioredoxin reductase activity (1996) *PNAS*, 93 (3), pp. 1006–1011.	253	11	14	10	288
11	Low, S.C., Berry, M.J. Knowing when not to stop: Selenocysteine incorporation in eukaryotes (1996) *Trends in Biochemical Sciences*, 21 (6), pp. 203–208.	251	12	12	5	280
12	Gladyshev, V.N., Jeang, K.-T., Stadtman, T.C. Selenocysteine, identified as the penultimate C-terminal residue in human T-cell thioredoxin reductase, corresponds to TGA in the human placental gene (1996) *PNAS*, 93 (12), pp. 6146–6151.	228	16	19	13	276
13	Sies, H., Sharov, V.S., Klotz, L.-O., Briviba, K. Glutathione peroxidase protects against peroxynitrite-mediated oxidations: A new function for selenoproteins as peroxynitrite reductase (1997) *J. of Biological Chemistry*, 272 (44), pp. 27812–27817.	230	11	21	10	272
14	Berry, M.J., Banu, L., Chen, Y., Mandel, S.J., Kiefer, J.D., Harney, J.W., Larsen, P.R. Recognition of UGA as a selenocysteine codon in Type I deiodinase requires sequences in the 3′ untranslated region (1991) *Nature*, 353 (6341), pp. 273–276.	224	17	15	14	270
15	Hatfield, D.L., Gladyshev, V.N. How selenium has altered our understanding of the genetic code (2002) *Molecular and Cellular Biology*, 22 (11), pp. 3565–3576.	172	39	29	24	264
16	Boyington, J.C., Gladyshev, V.N., Khangulov, S.V., Stadtman, T.C., Sun, P.D. Crystal structure of formate dehydrogenase H: Catalysis involving Mo, molybdopterin, selenocysteine, and an Fe4S4 cluster (1997) *Science*, 275 (5304), pp. 1305–1308.	231	17	12	3	263

(continued)

Table 1.3 (continued)

	Keyword "selenocysteine" gives 2,055 documents in total in Scopus					
	Citations					
	Year	<2008	2008	2009	2010	Total
17	Kang, S.W., Baines, I.C., Rhee, S.G. Characterization of a mammalian peroxiredoxin that contains one conserved cysteine (1998) *J. of Biological Chemistry*, 273 (11), pp. 6303–6311.	217	15	19	9	260
18	Osawa, S., Jukes, T.H., Watanabe, K., Muto, A. Recent evidence for evolution of the genetic code (1992) *Microbiological Reviews*, 56 (1), pp. 229–264.	212	12	13	17	254
19	Blum, G., Ott, M., Lischewski, A., Ritter, A., Imrich, H., Tschape, H., Hacker, J. Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an Escherichia coli wild-type pathogen (1994) *Infection and Immunity*, 62 (2), pp. 606–614.	233	7	5	4	249
20	Alexander, F.W., Sandmeier, E., Mehta, P.K., Christen, P. Evolutionary relationships among pyridoxal-5′-phosphate-dependent enzymes. Regio-specific alpha, beta and gamma families (1994) *European J. of Biochemistry*, 219 (3), pp. 953–960.	221	10	9	7	247
21	Christianson, D.W. Structural biology of zinc (1991) *Advances in Protein Chemistry*, 42, pp. 281–355.	208	13	18	7	246
22	Böck, A., Forchhammer, K., Heider, J., Leinfelder, W., Sawers, G., Veprek, B., Zinoni, F. Selenocysteine: The 21st amino acid (1991) *Molecular Microbiology*, 5 (3), pp. 515–520.	186	19	20	11	236
23	Combs Jr., G.F. Selenium in global food systems (2001) *British J. of Nutrition*, 85 (5), pp. 517–547.	114	38	40	32	224
24	Chambers, I., Frampton, J., Goldfarb, P., Affara, N., McBain, W., Harrison, P.R. The structure of the mouse glutathione peroxidase gene: the selenocysteine in the active site is encoded by the "termination" codon, TGA. (1986) *The EMBO journal*, 5 (6), pp. 1221–1227.	185	10	17	10	222
25	Gesteland, R.F., Atkins, J.F. Recoding: Dynamic reprogramming of translation (1996) *Annual Review of Biochemistry*, 65, pp. 741–768.	196	5	7	10	218

Date of creation: 16 December 2010

Table 1.4 The 25 most cited publications on "selenoprotein" as listed in the Scopus database

	Keyword "selenoprotein" gives 1,746 documents in total in Scopus					
	Citations					
	Year	<2008	2008	2009	2010	Total
	Total	8,683	1,466	1,444	1,166	1,2759
1	Rayman, M.P. The importance of selenium to human health (2000) *Lancet*, 356 (9225), pp. 233–241.	677	148	140	134	1,099
2	Bianco, A.C., Salvatore, D., Gereben, B., Berry, M.J., Larsen, P.R. Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases (2002) *Endocrine Reviews*, 23 (1), pp. 38–89.	377	90	69	58	594
3	Hakak, Y., Walker, J.R., Li, C., Wong, W.H., Davis, K.L., Buxbaum, J.D., Haroutunian, V., Fienberg, A.A. Genome-wide expression analysis reveals dysregulation of myelination-related genes in chronic schizophrenia (2001) *PNAS*, 98 (8), pp. 4746–4751.	386	68	62	41	557
4	Kryukov, G.V., Castellano, S., Novoselov, S.V., Lobanov, A.V., Zehtab, O., Guigó, R., Gladyshev, V.N. Characterization of mammalian selenoproteomes (2003) *Science*, 300 (5624), pp. 1439–1443.	266	88	86	62	502
5	Maquat, L.E. Nonsense-mediated mRNA decay: Splicing, translation and mRNP dynamics (2004) *Nature Reviews Molecular Cell Biology*, 5 (2), pp. 89–99.	292	87	67	49	495
6	Stadtman, T.C. Selenocysteine (1996) *Annual Review of Biochemistry*, 65, pp. 83–100.	402	36	32	21	491
7	Brigelius-Flohé, R. Tissue-specific functions of individual glutathione peroxidases (1999) *Free Radical Biology and Medicine*, 27 (9–10), pp. 951–965.	310	56	46	36	448
8	Lu, T., Pan, Y., Kao, S.-Y., Li, C., Kohane, I., Chan, J., Yankner, B.A. Gene regulation and DNA damage in the ageing human brain (2004) *Nature*, 429 (6994), pp. 883–891.	233	62	67	69	431
9	Squadrito, G.L., Pryor, W.A. Oxidative chemistry of nitric oxide: The roles of superoxide, peroxynitrite, and carbon dioxide (1998) *FRBM*, 25 (4–5), pp. 392–403.	335	31	30	24	420

(continued)

Table 1.4 (continued)

	Keyword "selenoprotein" gives 1,746 documents in total in Scopus					
	Citations					
	Year	<2008	2008	2009	2010	Total
10	Ganther, H.E. Selenium metabolism, selenoproteins and mechanisms of cancer prevention: Complexities with thioredoxin reductase (1999) *Carcinogenesis*, 20 (9), pp. 1657–1666.	261	36	32	24	353
11	Ursini, F., Heim, S., Kiess, M., Maiorino, M., Roveri, A., Wissing, J., Flohé, L. Dual function of the selenoprotein PHGPx during sperm maturation (1999) *Science*, 285 (5432), pp. 1393–1396.	248	20	30	21	319
12	Tamura, T., Stadtman, T.C. A new selenoprotein from human lung adenocarcinoma cells: Purification, properties, and thioredoxin reductase activity (1996) *PNAS*, 93 (3), pp. 1006–1011.	253	11	14	10	288
13	Emery, A.E.H. The muscular dystrophies (2002) *Lancet*, 359 (9307), pp. 687–695.	145	35	38	54	272
14	Halliwell, B. Antioxidant defence mechanisms: From the beginning to the end (of the beginning) (1999) *Free Radical Research*, 31 (4), pp. 261–272.	185	27	37	22	271
15	Berry, M.J., Banu, L., Chen, Y., Mandel, S.J., Kiefer, J.D., Harney, J.W., Larsen, P.R. Recognition of UGA as a selenocysteine codon in Type I deiodinase requires sequences in the 3′ untranslated region (1991) *Nature*, 353 (6341), pp. 273–276.	224	17	15	14	270
16	Hatfield, D.L., Gladyshev, V.N. How selenium has altered our understanding of the genetic code (2002) *Molecular and Cellular Biology*, 22 (11), pp. 3565–3576.	172	39	29	24	264
17	Arthur, J.R. The glutathione peroxidases (2000) *CMLS*, 57 (13–14), pp. 1825–1835.	152	29	28	38	247
18	Croteau, W., Davey, J.C., Galton, V.A., St. Germain, D.L. Cloning of the mammalian type II iodothyronine deiodinase. A selenoprotein differentially expressed and regulated in human and rat brain and other tissues (1996) *J. of Clinical Investigation*, 98 (2), pp. 405–417.	210	10	7	6	233

(continued)

Table 1.4 (continued)

	Keyword "selenoprotein" gives 1,746 documents in total in Scopus					
	Citations					
	Year	<2008	2008	2009	2010	Total
19	Berry, M.J., Banu, L., Harney, J.W., Larsen, P.R. Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons (1993) *EMBO Journal*, 12 (8), pp. 3315–3322.	193	7	10	7	217
20	Behne, D., Hilmert, H., Scheid, S., Gessner, H., Elger, W. Evidence for specific selenium target tissues and new biologically important selenoproteins (1988) *BBA - General Subjects*, 966 (1), pp. 12–21.	165	9	16	13	203
21	Whanger, P.D. Selenium and its relationship to cancer: An update (2004) *British J. of Nutrition*, 91 (1), pp. 11–28.	102	44	26	24	196
22	Gromer, S., Arscott, L.D., Williams Jr., C.H., Schirmeri, R.H., Becker, K. Human placenta thioredoxin reductase. Isolation of the selenoenzyme, steady state kinetics, and inhibition by therapeutic gold compounds (1998) *J. of Biological Chemistry*, 273 (32), pp. 20096–20101.	133	20	26	15	194
23	Behne, D., Kyriakopoulos, A. Mammalian selenium-containing proteins (2001) *Annual Review of Nutrition*, 21, pp. 453–473.	133	26	21	13	193
24	Behne, D., Kyriakopoulos, A., Meinhold, H., Kohrle, J. Identification of type I iodothyronine 5′-deiodinase as a selenoenzyme (1990) *BBRC*, 173 (3), pp. 1143–1149.	161	10	11	7	189
25	Arteel, G.E., Briviba, K., Sies, H. Protection against peroxynitrite (1999) *FEBS Letters*, 445 (2–3), pp. 226–230.	167	10	5	5	187

Date of creation: 16 December 2010

the active site of selenoproteins, or the unique UGA-directed translational insertion machinery for selenocysteine (Table 1.3). The analogous list using "selenoprotein" as keyword instead of "selenocysteine" gives, interestingly, a somewhat different list (although several papers are found in both lists), with a similar profile in subject areas but perhaps slightly more medically oriented (Table 1.4). When taken together, the articles listed in Tables 1.2–1.4 should well represent the most studied aspects in the

history of selenium research, or at least the major trends in that research as reflected through the high number of citations related to these papers.

1.4 The Future of Selenium Research?

It is rather straightforward to look back and discuss how the history of selenium research has developed. As was briefly done in this chapter, a simple bibliometric analysis could illustrate that development. What results will a similar analysis give when performed in 10 years from now, or in 50 or 200 years? Naturally, we cannot know how the future of selenium research will unfold, but we can trust that it shall be exciting. With this field of research at present being under rapid development (see Fig. 1.1), it is clear that the potential of new selenium-related discoveries of major importance waits around the corner. Such findings may possibly involve discoveries of selenoproteins with hitherto unknown features or links to health and disease, the development of novel chemical methods or technological advancements based on selenium usage, new methods for selenium soil remediation or nutritional status assessment, or some other selenium discoveries that may prove to have a major impact on the shape of this as well as other research fields. The accumulated contents of the many chapters found in this book should give good hints about the flavors of some of those discoveries that await to be unfolded in the near future.

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References

1. Böck A, Forchhammer K, Heider J et al (1991) Mol Microbiol 5:515
2. Stadtman TC (1996) Annu Rev Biochem 65:83
3. Hatfield DL, Gladyshev VN (2002) Mol Cell Biol 22:3565
4. Flohe L (2009) Biochim Biophys Acta 1790:1389
5. Arnér ESJ (2010) Exp Cell Res 316:1296
6. Pinsent J (1954) Biochem J 57:10
7. Schwarz K, Foltz CM (1957) J Am Chem Soc 79:3292
8. Berzelius JJ (1818) Undersökning af en ny Mineral-kropp, funnen i de orenare sorterna af det i Falun tillverkade svaflet. Afhandlingar i fysik, kemi och mineralogi 6:42

Part I
Selenocysteine Biosynthesis and Incorporation into Protein

Chapter 2
Selenocysteine Biosynthesis and the Replacement of Selenocysteine with Cysteine in the Pathway

Xue-Ming Xu, Anton A. Turanov, Bradley A. Carlson, Min-Hyuk Yoo, Vadim N. Gladyshev, and Dolph L. Hatfield

Abstract The biosynthetic pathway of selenocysteine (Sec), the 21st amino acid in the genetic code, has been established in eukaryotes and archaea using comparative genomic and experimental approaches. In addition, cysteine (Cys) was found to arise in place of selenocysteine in thioredoxin reductase (TR) in NIH 3T3 cells and in mice. An analysis of the selenocysteine biosynthetic pathway demonstrated that replacement of selenide with sulfide in generating the active cysteine donor, thiophosphate, resulted in cysteine being donated to the acceptor molecule, which is likely dehydroalanyl-tRNA$^{[Ser]Sec}$, yielding Cys-tRNA$^{[Ser]Sec}$. The identification of the pathways for biosynthesis of selenocysteine and cysteine in mammals is discussed in this chapter.

2.1 Introduction

Selenocysteine (Sec) is a naturally occurring protein amino acid in the three domains of life, eukaryotes, archaea, and eubacteria, and is the only known protein amino acid in eukaryotes whose biosynthesis occurs on its tRNA, designated Sec tRNA$^{[Ser]Sec}$ [1, 2]. Bock and collaborators established the biosynthesis of Sec in eubacteria in 1991 [3] but its biosynthesis in eukaryotes and archaea remained elusive until only in the last few years [1, 4]. In addition, cysteine (Cys) was reported to occur in place of Sec in naturally occurring selenoproteins, e.g., the thioredoxin reductases (TR) [5],

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but the mechanism of how Cys arose in place of Sec was not determined. We recently observed that Cys is also biosynthesized using the Sec biosynthetic machinery to generate Cys-tRNA$^{[Ser]Sec}$ and the resulting Cys is inserted into some, but apparently not all, selenoproteins [6]. The biosynthesis of Sec and the de novo synthesis of Cys using the Sec biosynthetic machinery and the insertion of Cys into protein in place of Sec are discussed herein.

2.2 Sec Biosynthesis

The biosynthesis of Sec, as established in mammals, is shown in Fig. 2.1. The background on how the biosynthesis was deciphered in eukaryotes and archaea is discussed below.

2.2.1 Background

In 1970, two separate studies involving the same tRNA that would subsequently provide the foundation for the biosynthesis of Sec were reported. Maenpaa and Bernfield [7] found that a minor seryl-tRNA within the total seryl-tRNA population formed phosphoseryl-tRNA in rooster liver and these investigators speculated that this tRNA might have a role in the biosynthesis of the phosphoseryl moieties in phosvitin, a protein containing over 50% phosphoserine residues. At the same time, a minor seryl-tRNA was found in bovine and chicken livers that decoded specifically the nonsense (stop) codon, UGA [8]. Since a topic of considerable interest in the late 1960s and early 1970s was whether nonsense suppressor tRNAs occurred in higher vertebrates, it was suggested that the UGA decoding tRNA was an opal suppressor tRNA. Phosphoseryl-tRNA and the UGA decoding seryl-tRNA were subsequently shown to be the same tRNA [9]. Further characterization of this tRNA, including identifying it as the tRNA that inserts Sec into protein (designated as Sec tRNA$^{[Ser]Sec}$), and that it exists in organs and tissues of higher vertebrates in two isoforms, which are selectively used in synthesizing different subclasses of selenoproteins, can be found elsewhere ([10] and reviewed in [11] and Chap. 44).

It should also be noted that in 1989, Sec was shown to be biosynthesized on tRNA$^{[Ser]Sec}$ in *Escherichia coli* [12] and mammalian cells [13] demonstrating unequivocally that Sec was the 21st amino acid in the genetic code. These observations ruled out the possibility that phosphoserine, which was known to be attached to tRNA$^{[Ser]Sec}$, was initially incorporated into selenoproteins and then modified posttranslationally to Sec that would of course had made phosphoserine the 21st amino acid.

In addition to Sec tRNA, the seryl-tRNA synthetase that attaches serine to Sec tRNA, and the kinase that phosphorylates seryl-tRNA in forming phosphoseryl-tRNA, there were other protein factors, reported by several investigators, whose role in selenium metabolism had not been identified. These included a 48 kD protein in

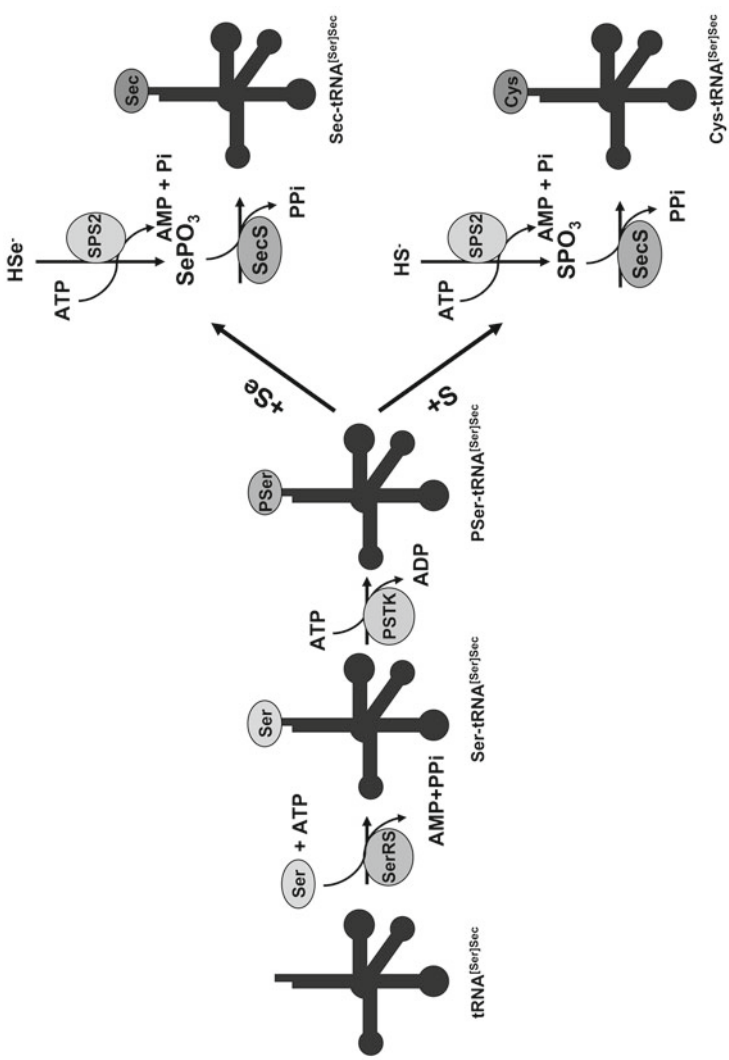

Fig. 2.1 Biosynthesis of Sec and de novo synthesis of Cys. The pathway of Sec biosynthesis in eukaryotes and archaea, wherein the synthesis is complete by the generation of selenophosphate from selenide and ATP in the presence of SecS and the donation of selenium to the active selenium acceptor to form Sec-tRNA$^{[Ser]Sec}$ is shown (see *upper portion* of the figure for the final steps in Sec biosynthesis). The de novo synthesis of Cys on Sec tRNA$^{[Ser]Sec}$, wherein sulfide replaces selenide in generating thiophosphate and its donation to the active selenium acceptor to yield Cys-tRNA$^{[Ser]Sec}$ is also shown (see *lower portion* of the figure for the final steps in Cys biosynthesis)

patients with an autoimmune chronic hepatitis that co-precipitated with Sec $tRNA^{[Ser]Sec}$ in cell extracts from such patients [13]. This protein was designated the soluble liver antigen (SLA) [14] and was found to form a complex with other proteins involved in the Sec insertion machinery [15]. SLA was subsequently reported to occur as a separate family within a large superfamily of diverse pyridoxal phosphate-dependent transferases [16] and was proposed to be selenocysteine synthase (SecS) in mammals [11, 16–18].

In addition, two genes were identified in mammals as having homology to the enzyme in *E. coli* that synthesized the active selenium donor, selenophosphate synthetase (SPS) [19]. SPS was initially designated as SelD in *E. coli* [20] and the mammalian genes coding for SPS as *sps1* [21, 22] and *sps2* [23]. The product of *sps2* is a selenoprotein (SPS2) suggesting that it might be an autoregulatory protein in selenoprotein biosynthesis [23, 24]. Initial experimental studies with SPS1 and SPS2 had shown that: (1) mutation of Sec to Cys in SPS2 had enzyme activity [23–25] and the Cys mutant form could complement *SelD*$^{-}$ cells following transfection of the *SelD*$^{-}$ cells with the Sec → Cys mutant *sps2* [25]; and (2) transformation of *E. coli* cells with *sps1* or *sps2* suggested that SPS2 had a role in the synthesis of selenophosphate and that SPS1 was involved in Sec recycling via a selenium salvage pathway [26].

The biosynthesis of Sec in eukaryotes and archaea was solved using the components in the above studies as described below.

2.2.2 Seryl-tRNA Synthetase (SerRS)

The biosynthesis of Sec begins with the aminoacylation of $tRNA^{[Ser]Sec}$ by seryl-tRNA synthetase (SerRS) in the presence of serine, ATP, and Mg^{++} as follows:

$$tRNA^{[Ser]Sec} + \text{serine} + ATP \xrightarrow{SerRS + Mg^{2+}} \text{seryl - } tRNA^{[Ser]Sec} + AMP + PPi$$

2.2.3 Phosphoseryl-tRNA Kinase (PSTK)

Identification of the kinase that is responsible for phosphorylating the serine moiety on the UGA decoding seryl-tRNA [7, 8] remained elusive for many years. This kinase, which was designated phosphoseryl-tRNA kinase (PSTK), was finally identified initially using a comparative genomic approach that searched completely sequenced genomes of archaea for a kinase-like protein that was present in those organisms that utilized the selenoprotein synthesizing machinery and was absent in those that did not [27]. Two candidates were detected, and their homology was next searched in eukaryotic genomes that did and did not use a functional Sec insertion machinery. Orthologs of one of these candidate kinases were present in eukaryotes

synthesizing selenoproteins but absent in eukaryotes lacking these proteins. The gene for this protein was cloned from mouse genomic DNA, the protein product expressed, isolated and its biochemical properties examined that unequivocally identified it as PSTK [27]. PSTK was shown to carry out the following reaction:

$$\text{Seryl - tRNA}^{[\text{Ser}]\text{Sec}} + \text{ATP} \xrightarrow{\text{PSTK} + \text{Mg}^{2+}} O\text{ - phosphoseryl - tRNA}^{[\text{Ser}]\text{Sec}} + \text{ADP}$$

2.2.4 Selenophosphate Synthetases (SPS1 and SPS2)

The protein products of *sps1* and *sps2* were generated by cloning the corresponding mouse genes into expression vectors, expressing and isolating the proteins for further study [1, 28]. A mutation was initially introduced into *sps2* to change Sec to Cys in SPS2. Similarly, *Caenorhabditis elegans sps2* that normally contains Cys in place of Sec and *SelD* was cloned and the products expressed and isolated. Mouse SPS1 and SPS2 (mSPS1 and mSPS2(Cys)), *C. elegans* SPS (cSPS2) and *E. coli* SPS (SelD) were all examined for their ability to synthesize the active donor, monoselenophosphate [1]. Selenide and ATP were incubated individually with each protein to assess whether they could synthesize the active selenium donor. mSPS2(Cys), cSPS2, and SelD generated selenophosphate but SPS1 did not, demonstrating that eukaryotic SPS2 is responsible for making the active selenium donor, and that SPS1 likely has another metabolic role [1]. SPS2 acts on selenide and ATP in yielding selenophosphate by the following reaction:

$$\text{Selenide} + \text{ATP} \xrightarrow{\text{SPS2}} \text{selenophosphate} + \text{AMP} + \text{Pi}$$

2.2.5 Selenophosphate Synthase (SecS)

As no homologous sequences to bacterial SecS, previously designated by Böck and collaborators as SelA [29], could be found in eukaryotes that encode a functional Sec insertion machinery in their genomes, we applied a computational and comparative genomic strategy, similar to that used to identify *pstk*, in searching for a *SecS* gene in eukaryotes [1]. The search was confined to eukaryotes and archaea whose genomes had been sequenced and the organisms synthesize selenoproteins, and the genomes of those organisms that did not synthesize selenoproteins were used as controls. In addition to identifying genes that are involved in the Sec insertion machinery, another gene was detected that might be SecS in mammals [1]. A homologous sequence was found in all eukaryotes and archaea encoding the functional selenoprotein insertion machinery but not in sequenced genomes of organisms not making selenoproteins.

The sequence of the purported mammalian SecS matched that of SLA [1], the 48 kD protein in patients with an autoimmune chronic hepatitis that co-precipitated

with $tRNA^{[Ser]Sec}$ (see above). The mouse *SLA* gene was cloned, expressed, and the gene product characterized [1]. The purified protein bound tightly with phosphoseryl-$tRNA^{[Ser]Sec}$, less well with $tRNA^{[Ser]Sec}$, and poorly or not at all with seryl-$tRNA^{[Ser]Sec}$, seryl-$tRNA^{Ser}$ or $tRNA^{Ser}$, strongly suggesting that the substrate for the *SLA* gene product was phosphoseryl-tRNA. Furthermore, this protein rapidly hydrolyzed the phosphate from the substrate leaving, most likely, dehydroalanyl-$tRNA^{[Ser]Sec}$ bound to the enzyme [1]. This complex was then shown to accept the active selenium donor, selenophosphate, that resulted in selenocysteyl-$tRNA^{[Ser]Sec}$ (see Fig. 2.1) demonstrating unequivocally that SLA is indeed mammalian SecS.

2.3 De novo Biosynthesis of Cys

Cys is considered to be an essential amino acid in mammals in that it was not thought to be synthesized de novo and must be obtained from the diet or synthesized from methionine. However, we recently reported that Cys can be biosynthesized de novo in mammals by using the Sec biosynthetic machinery [6]. Although this pathway synthesizes Cys de novo, the Cys synthesized does not substitute for the essential requirement of Cys insertion into protein in response to the UGU/UGC codons. The key steps in the replacement of Sec with Cys are: (1) sulfide can substitute for selenide in the SPS2 catalyzed reaction yielding thiophosphate; and (2) thiophosphate can react with the *O*-phosphoseryl-$tRNA^{[Ser]Sec}$ intermediate which is most likely dehydroalanine that was generated by the interaction of SecS to yield Sec $tRNA^{[Ser]Sec}$ (Fig. 2.1). The details of Cys replacement of Sec on $tRNA^{[Ser]Sec}$ and the significance of Cys insertion into protein in mammalian cells in culture or in mouse liver as a consequence of selenium status are discussed below.

2.3.1 Cys/Sec Replacement in vitro

The precise means of how Cys replaces Sec in the biosynthetic pathway was determined by using the enzymes and other components used in establishing how the latter amino acid was synthesized [6]. The only differences were that (1) sulfide was used in place of selenide in the presence of SPS2 and ATP to generate thiophosphate, and (2) thiophosphate was used in place of selenophosphate as the active sulfur donor in the presence of SecS and phosphoseryl-$tRNA^{[Ser]Sec}$. Thiophosphate did indeed replace selenophosphate that served as the active sulfur donor to the active acceptor molecule generated by SecS that in turn yielded Cys-$tRNA^{[Ser]Sec}$ (Fig. 2.1).

2.3.2 Cys/Sec Replacement in NIH 3T3 Cells

To elucidate the intracellular relevance of the replacement of Sec by Cys in the Sec biosynthetic pathway, we initially examined the effect of adding thiophosphate to

Table 2.1 C-Terminal peptide sequences of mouse TR1 and TR3 from NIH 3T3 cells and liver[a]

Experiment	Source of TRs	Protein	Peptide sequences	Sec (%)	Cys (%)
I	NIH 3T3, control	TR1	R.SGGDILQSGCUG	90	
		TR1	R.SGGDILQSGCCG		10
	NIH 3T3, SPO_3 treated	TR1	R.SGGDILQSGCUG	4	
		TR1	R.SGGDILQSGCCG		96
		TR1	KRSGGDILQSGCCG		
IIa	Liver, 0 ppm Se	TR1	R.SGGDILQSGCUG	49	
		TR1	R.SGGDILQSGCCG		51
		TR3	K.RSGLEPTVTGCCG		
IIb	Liver, 0.1 ppm Se	TR1	R.SGGDILQSGCUG	91	
		TR1	R.SGGDILQSGCCG		9
		TR1	K.RSGGDILQSGCUG		
		TR1	K.RSGGDILQSGCCG		
		TR3	R.SGLEPTVTGCUG		
		TR3	R.SGLEPTVTGCCG		
IIIc	Liver, 2 ppm se	TR1	R.SGGDILQSGCUG	100	ND[b]
		TR1	K.RSGGDILQSGCUG		
		TR3	R.SGLEPTVTGCUG		
		TR3	K.RSGLEPTVTGCUG		

[a]TR1 and TR3 were affinity isolated from NIH 3T3 cells, either treated or untreated with thiophosphate (Experiment I), or from livers of mice fed selenium deficient, selenium sufficient or selenium supplemented diets (Experiments IIa–c). The percent of Sec/Cys in the resulting C-terminal selenoprotein peptides was analyzed by MS/MS as described previously [6]
[b]ND not detectable

NIH 3T3 cells in culture [6]. The resulting TR1 was isolated, purified, and the ratio of Sec to Cys encoded by UGA determined by mass-spectrometry analysis (Table 2.1, Experiment I). In untreated cells, wherein the cells were grown in the presence of adequate amounts of selenium, the Sec to Cys ratio was 9:1 demonstrating that Cys was incorporated into TR1 at the UGA-encoded site even in the presence of adequate amounts of selenium in the medium. On the other hand, more than 90% Cys was present at the UGA-Sec position in TR1 within cells treated with thiophosphate.

2.3.3 Cys/Sec Replacement in Mice

We next assessed the ratio of Cys/Sec in liver TR1 and TR3 of mice maintained on identical diets, except for varying amounts of selenium that included deficient, adequate, and supplemented levels (Table 2.1, Experiments IIa–c, respectively) [6]. The ratio of Sec to Cys in liver TR1 and TR3 was ~1:1 in mice fed a selenium-deficient diet and ~9:1 in mice fed a selenium-adequate diet. Mice maintained on a selenium-supplemented diet had undetectable levels of Cys in TR1 and TR3 at the UGA

encoded site. Interestingly, the degree of replacement of Cys with Sec intracellularly appeared to be a reflection of the level of selenium in the medium (Table 2.1, Experiment I) or diet (Table 2.1, Experiment II).

2.4 Conclusions

Sec is the 21st amino acid in the genetic code, the only known protein amino acid in eukaryotes whose biosynthesis occurs on its tRNA, and the last known protein amino acid in eukaryotes whose biosynthesis was finally resolved [1]. The enzymes and other factors involved in Sec biosynthesis were initially found and subsequently identified to be involved in Sec biosynthesis in a variety of ways. For example, Sec $tRNA^{[Ser]Sec}$ was first reported to be a minor seryl-tRNA that decoded specifically the stop codon, UGA, in bovine and chicken livers [8] and subsequently was shown to be Sec $tRNA^{[Ser]Sec}$ [13]. PSTK was described as a kinase that phosphorylated the serine moiety on a minor seryl-tRNA in rooster liver to form phosphoseryl-tRNA [7] and was also subsequently identified many years later by computational and comparative genomic and experimental approaches to be the kinase involved in synthesizing one of the intermediates in the Sec pathway in eukaryotes and archaea [27]. SecS was initially described as a protein factor that bound Sec $tRNA^{[Ser]Sec}$ in human liver of patients with an autoimmune disease [14] and was then identified by computational and comparative genomic and experimental approaches as SecS [1]. SPS1 and SPS2 were originally found in the mammalian genome by their homology to the eubacterial SelD [21–23], but their experimental characterization demonstrated that only SPS2 was the enzyme responsible for making the active selenium donor [1]. Each of these components was cloned, expressed, isolated, and characterized to establish the biosynthesis of Sec [1].

In addition, Cys was found to be synthesized de novo by using SPS2 and replacing selenide with sulfide-yielding thiophosphate that donated sulfur to the active acceptor, most likely, dehydroalanine, that was attached to SecS yielding Cys-$tRNA^{[Ser]Sec}$ [6]. This pathway was shown to be operative in mammalian cells and in mice ingesting normal amounts of selenium and to insert Cys in some, but likely not all, selenoproteins [6]. In mice on a selenium-deficient diet, the levels of Sec and Cys in TR1 and TR3 were approximately equal suggesting a possible physiological significance of such a pathway. It was suggested that the replacement of Sec by Cys in some selenoproteins "may provide possibilities for regulating the expression of (specific) selenoproteins and their functions as well as elucidating the biological roles of selenium" [6]. Clearly, the finding that Cys can replace Sec in certain selenoproteins opens the door to many additional and fascinating studies further elucidating the significance of this novel pathway involving Cys and selenoproteins.

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Chapter 3
Molecular Mechanism of Eukaryotic Selenocysteine Incorporation

Michael T. Howard, Jonathan N. Gonzales-Flores, and Paul R. Copeland

Abstract Although substantial progress has been made in determining which factors are required for eukaryotic Sec incorporation, the mechanism by which the factors are able to alter the coding potential of an mRNA at specific UGA codons is still not known. What is clear is that a complex interplay between *cis*- and *trans*-acting factors regulates the selenocysteine (Sec) incorporation event both at the basal level and in determining the efficiency of the process. In this chapter, we dissect the current state of knowledge regarding this interplay and delve into the increasingly important role that in vitro systems will play in determining the precise mechanism by which Sec is incorporated into selenoproteins.

3.1 Introduction

The a priori assumption upon discovery of the genetic code, over 4 decades ago, was that it would be universal in nature [1]. Any change in the code would result in global changes in the proteome and likely be cataclysmic to the fitness of the organism; consequently, the code once established would be immutable and fixed. Subsequent discoveries of organisms and organelles that reassign codons on a genome-wide level, as well as a growing number of examples of *cis*- and *trans*-acting signals that alter decoding of select codons in specific mRNAs, and the addition of selenocysteine (Sec) and pyrrolysine to the list of 20 cotranslationally

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DOI 10.1007/978-1-4614-1025-6_3,

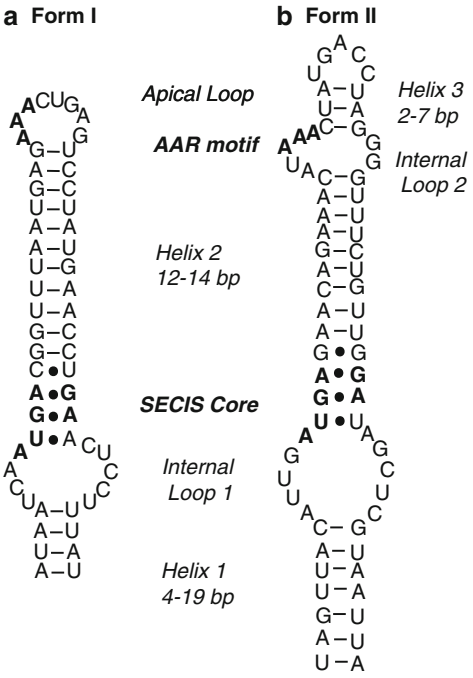

Fig. 3.1 SECIS element diagram. The two types of SECIS elements are exemplified here by the first (**a**) and second (**b**) SECIS elements in the selenoprotein P 3′ UTR. The Form II SECIS element is characterized by an additional short helix (helix 3) above the conserved AAR motif

inserted amino-acids dispelled the notion of a fixed universal genetic code (reviewed in [2–4]). The genetic code is evolving. The standard rules of decoding in most organisms dictate that the ribosome will terminate translation upon encountering any one of the three stop codons, UAA, UAG, or UGA. It may be no coincidence that gene-specific redefinition of codons occurs most often with stop codons. Termination codons occur only once per gene; consequently, assigning a dual meaning to these codons in an organism would minimize the impact on existing coding sequences and the resulting proteome. Further, impact on global protein expression is reduced by the evolution of *cis*-acting sequence elements and *trans*-acting factors that direct stop codon redefinition to select mRNAs in an organism. To date there are two *trans*-acting factors and one *cis*-acting RNA sequence that are required for converting specific UGA codons from signaling termination to one that signals Sec incorporation. In 1991 it was reported that type 1 iodothyronine deiodinase (DIO1) 3′ UTR contained a sparsely conserved sequence, termed the Sec insertion sequence (SECIS) element, that was required for the translation of full-length DIO1 both in vitro and in injected *Xenopus* oocytes [5]. Subsequent studies clarified that the SECIS element consisted of three small regions of conservation surrounded by an overall similar topology as shown in Fig. 3.1 [6–10]. In 1997 a protein was shown to specifically bind to the conserved AUGA motif, and in 2000 this protein was identified as a novel factor that was required for the Sec incorporation reaction in vitro. Because a previous study had reported a nonspecific SECIS-binding activity that they termed SECIS-binding protein (SBP) [11], the SBP

identified in 2000 was termed SBP2. That same year saw the identification of the Sec-specific elongation factor, eEFSec [12, 13], and there has been no further identification of factors that are required for Sec incorporation. Layered over the core of required components are other factors, both *cis* and *trans*-acting, which have been reported to modulate the Sec incorporation reaction. These include the novel SBPs discussed in Chap. 4 of this book as well as mRNA sequences in the coding regions of some selenoprotein genes that have dramatic effects on the efficiency of Sec incorporation both in vitro and in vivo, which are discussed in detail below.

In this chapter, we review current developments in our understanding of the Sec incorporation mechanism with an emphasis on the known roles of each of the required factors, the *cis*-acting elements that facilitate Sec incorporation, and describe potential in vitro systems that could prove to be essential in further deciphering the molecular mechanism(s) controlling UGA redefinition and Sec incorporation.

3.2 The Core Factors

3.2.1 SECIS Elements

The basic structure and function of SECIS elements has been extensively reviewed in the previous editions of this book [14, 15] and elsewhere [16–18]. The importance of SECIS function in vivo was underscored recently when it was found that a mutation in the Selenoprotein N gene (*SEPN1*) that caused SEPN1-related myopathy was mapped to the SECIS element. The AUGA → ACGA mutation was severe enough to completely eliminate detectable SEPN1 expression as determined by immunoblot of patient samples [19]. Recent investigations into SECIS function have shown dramatic variability in the efficiency with which different SECIS elements function in the context of a reporter gene where the difference in Sec insertion efficiency between high- and low-efficiency SECIS elements spanned more than three orders of magnitude [20]. This effect was shown to be primarily dictated by the sequence of the SECIS core, helix 2, and the apical loop (see Fig. 3.1) Although there was a generally similar trend across SECIS elements, the cell type in which the experiment was done, or the use of a cell free in vitro translation system (rabbit reticulocyte lysate), had a significant effect on which SECIS elements were strongest and the total difference between high- and low-efficiency sequences. Interestingly, the differences in SECIS efficiency could not be attributed to differential SBP2 binding. Together these findings strongly suggest the existence of other cell-specific factors that regulate the efficiency of Sec incorporation in vivo. One caveat to this study, however, is that these SECIS elements were taken out of their natural contexts, so any stabilizing or modulating effects mediated by the surrounding 3′ UTR sequence or more distant *cis*-acting elements were not considered. Indeed, the idea that a so-called “efficiency factor” could be a *cis*-acting RNA sequence is a possibility discussed in detail below.

The SECIS element has also recently been shown to be a platform for complex formation as it was found to be required for the association of two independently expressed SBP2 domains (see below) and for recruitment of eEFSec to form a stable SBP2/SECIS/eEFSec complex [21]. This finding provides valuable mechanistic insight into the process of Sec incorporation and implicates SBP2 and the SECIS in driving an eEFSec conformation that can be recognized by the ribosome. Future work designed to decipher the conformational changes that accompany complex formation will be required to build a molecular model of the events required for Sec incorporation.

In the 20 years that have passed since the discovery of the SECIS element, one of its features remains a mystery. The conserved sequence in the apical loop, the AAR motif, is absolutely required for Sec incorporation, but its function remains elusive. To date, no AAR sequence-specific binding proteins have been identified, and this is not for a lack of attempts (P.R. Copeland, unpublished observation). The fact that two SECIS elements (SelM and SelO) have C residues in place of the AAR motif reduces the likelihood that a sequence-specific RNA-binding protein is interacting with the terminal loop [22, 23]. This gives support to an as-yet unsubstantiated model where the terminal loop may be playing a role directly on the ribosome, likely assisting with eEFSec binding to the ribosomal A site and/or with Sec-tRNA^{Sec} accommodation.

3.2.2 SECIS-Binding Protein 2

SBP2 possesses three biochemically distinct domains (Fig. 3.2), the C-terminal half of the protein comprised of a Sec incorporation domain (SID), which is evolutionarily unique, and an RNA-binding domain (RBD), which is a member of the L7Ae RNA-binding family of proteins that interact with a variety of RNAs, specifically at kink turn motifs such as those found in rRNA, snRNA, and SECIS elements. These two domains, the SID and RBD, are sufficient for all three of the known functions of SBP2: SECIS element binding, ribosome binding, and Sec incorporation. The N-terminal half of the protein is also evolutionarily unique and has no known function, but presumably serves a regulatory role since SBP2 in many organisms (e.g., insects, protists, and worms) lacks the N-terminal domain entirely [24]. Although much is known about the RNA-binding properties of SBP2, little progress has been made in determining how it works to promote Sec incorporation. One model proposes that SBP2 stably binds ribosomes and upon SECIS element binding promotes a conformational change in the ribosome that allows eEFSec binding at the expense of the translation termination factor eRF1 [25]. Two findings have recently posed significant challenges to this model. First, it was found that the SID and RBD domains, when expressed as separate proteins, are fully active in Sec incorporation in vitro, but they do not stably interact with ribosomes, providing fairly clear evidence that the stable ribosome-binding activity is not required for Sec incorporation [21]. Second, it was found that SBP2 forms a stable SECIS-dependent, Sec-tRNA^{Sec}-independent complex

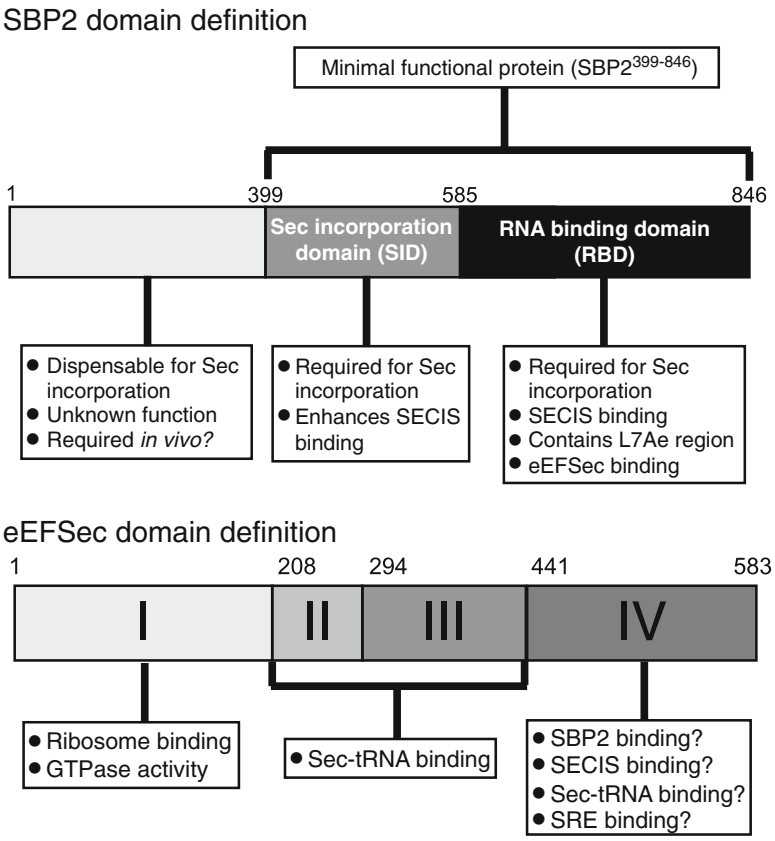

Fig. 3.2 SBP2 and eEFSec domain structures. Known or putative functions for each domain are indicated

with eEFSec, suggesting that SBP2 may act directly on eEFSec conformation upstream of the actual incorporation event, perhaps prior to ribosome binding [21].

Progress has been made in determining how the SID in SBP2 may function with respect to the RBD. Using the SID and RBD domains as separate proteins, it was shown that they form a SECIS-dependent complex and that the SID is involved in enhancing the SECIS-binding activity of the RBD. Since the SID does not exhibit a stable binding activity toward the SECIS element, it follows that the SID and RBD make direct contacts that are conformationally driven by SECIS binding. Interestingly when the conserved SID residues IILKE$^{526–530}$ (rat numbering -NP_076492.1) are mutated to alanine, the enhancement of RBD SECIS-binding activity is preserved, but the stable interaction between the SID and RBD is lost [21]. Surprisingly, the IILKE$^{526–530}$ mutant protein that contains both the SID and RBD domains completely lacks SECIS-binding activity, indicating that the presence of the mutated sequence is "blocking" access to the SECIS element. This finding aligns nicely with a separate study showing that the IILKE$^{526–530}$ residues are a determinant for SECIS specificity.

In that case it was found that the corresponding residues in the *Drosophila* version of SBP2 (SVRVY), which are not highly conserved, are necessary for binding to the form 2 SECIS elements found in *Drosophila* [26]. Based on these findings, this region of the SBP2 SID has clearly been identified as an indirect regulator of SECIS element affinity, making it a likely focus for determining the molecular basis for selective SECIS binding.

Beyond the SECIS and ribosome-binding activities of SBP2, two regions have been identified as being critical for an as-yet unidentified function that is likely proximal to the Sec incorporation event (i.e., driving a conformational change in eEFSec and/or the ribosome). One of these lies just upstream of the IILKE$^{526–530}$ sequence and was identified when the PLMKK$^{504–508}$ sequence was mutated to alanines. This version of SBP2 has neither SECIS nor ribosome-binding defects and yet is completely unable to support Sec incorporation. An identical phenotype was found when a sequence at the N-terminus of the RBD (FQ$^{648–649}$) was mutated, suggesting that these two regions form a single functional interface or that they perform two separate but essential functions. The latter scenario would fit with a model where SBP2 is required to promote conformational changes in both the ribosome and eEFSec either simultaneously or even sequentially.

3.2.3 Sec-Specific Elongation Factor

The Sec-specific elongation factor (eEFSec) in eukaryotes is a GTPase that is the exclusive carrier of the Sec-tRNA^{Sec} [12, 13]. The binding affinity of eEFSec to GTP is approximately three times higher than to GDP, thus it may not require a guanine nucleotide exchange factor (GEF). As for its tRNA-binding properties, eEFSec can only interact with Sec-tRNA^{Sec}, but not the serylated-tRNA^{Sec} precursor or the canonical aminoacyl-tRNAs [12, 13].

eEFSec consists of four domains (Fig. 3.2). Leibundgut et al. reported the complete crystal structure of archaeal EFSec [27]. This revealed a “chalice-like” structure consisting of Domains I, II, and III forming the cup of the chalice, whereas Domain IV is separated from the first three domains and forms the base of the chalice. The function of each eEFSec domain remains untested, but based on sequence conservation, the first three domains in eEFSec may have similar properties to the eukaryotic translation elongation factor, eEF1A. The elongation factor eEF1A is the main protein carrier that delivers all canonical aminoacyl-tRNAs to the ribosomal A-site during protein synthesis and is composed of three domains [28]. Domain I is required for GTPase activity and ribosomal factor-binding site interaction. Domain II is mainly involved in aminoacyl-tRNA binding, and Domain III is proposed to be involved in interactions with the T arm of aminoacyl-tRNAs (reviewed in [29]). Recently, it was shown that Sec-tRNA^{Sec} contains an anti-determinant for eEF1A binding at the base of the T arm, thus providing evidence of Domain III importance in tRNA recognition [30]. Domain IV in eEFSec, which is not present in eEF1A,

was proposed to be involved in interactions with SBP2 [31] and the "extra arm" of the Sec-tRNA^{Sec} [32].

eEFSec and SBP2 were first demonstrated to interact by a co-immunoprecipitation experiment in mammalian cells, forming a complex that was RNase sensitive [13]. Further studies in mammalian cells showed that eEFSec and SBP2 interactions were further enhanced by overexpression of the tRNA^{Sec} gene [31]. However, it was later demonstrated in a pure component system that SBP2 could not form a complex with eEFSec/GTP/Sec-tRNA^{Sec} and instead it caused Sec-tRNA^{Sec} release from eEFSec, although, the addition of a SECIS element was not tested [33]. Recently, Donovan et al. demonstrated in a native gel shift assay that eEFSec can form a complex with SBP2 in the presence of a SECIS element without the requirement of Sec-tRNA^{Sec} and/or GTP nucleotide [21]. This report also showed that the RBD of SBP2 and the SECIS element are sufficient for eEFSec recruitment. This suggests that the RBD of SBP2 and the SECIS element could form together a binding interface that is favorable for eEFSec interaction. Thus, taking together the in vivo and in vitro data so far, complex formation between eEFSec and SBP2 is driven by the SECIS element and possibly stabilized in the presence of Sec-tRNA^{Sec}. Putting together what is known about the function of SBP2, eEFSec, and the SECIS element, it seems likely that they form a stable and "active" Sec-tRNA^{Sec} delivery complex that can bind the ribosomal factor-binding site. Some elements in the complex (e.g., the SECIS loop) may play roles downstream, e.g., in Sec-tRNA^{Sec} accommodation into the A-site. This, however, is likely only half the story as the ribosome may also need to be primed to accept this active complex as discussed in Chap. 5.

eEFSec must be denied general access to the ribosomal A-site to prevent the Sec-tRNA^{Sec} from acting as a suppressor tRNA. Unlike eEFSec, eEF1A can obtain ribosomal A-site access without the requirement of additional factors. Near the ribosomal A-site is found the elongation factor-binding site that is composed of two elements: (1) the GTPase associating center (GAC) and (2) the sarcin-ricin loop (SRL). The GAC and SRL main function is to activate GTP hydrolysis in elongation factors, such as eEF1A and eEF2 [34]. Hüttenhofer et al. reported that the bacterial version of eEFSec, SelB, obtains ribosome-dependent GTP hydrolysis only when a bacterial SECIS element is added [35]. Their conclusion was that the bacterial SECIS element, which resides immediately downstream of the UGA-Sec codon rather than in the 3′ UTR, induces a conformational change within SelB to promote functional interactions with the ribosome. Indeed, eEFSec could be using a similar mechanism where SBP2 and the SECIS element act in concert to directly modify the eEFSec/GTP/Sec-tRNA^{Sec} ternary complex. In addition to the basal activity of the core complex and by analogy to the bacterial system, *cis*-acting RNA elements located immediately downstream of eukaryotic UGA-Sec codons could be interacting with the ternary complex to induce conformational changes that enhance the accommodation of Sec-tRNA^{Sec} (see Sect. 3.3). Further investigations are required to clarify the activating mechanism(s) that promotes functional interactions between eEFSec and the ribosome to allow Sec incorporation into nascent peptides during decoding of in-frame UGA-Sec codons.

3.3 *Cis*-Acting Elements Affecting Sec Incorporation

Although the efficiency of Sec incorporation is not known in vivo, the observation that termination appears to be the predominant event for selenoprotein genes with a single Sec-encoding UGA codon [5, 13, 36–39] has widely been interpreted as evidence for competition between termination of translation by eRF1/3 and Sec-tRNA^{Sec} decoding of the stop codon. Although the SECIS element itself can have a large impact on Sec incorporation efficiency (as discussed above in Sect. 3.2.1 and [40]), UGA-Sec sequence contexts that either favor eEFSec delivery of the Sec-tRNA^{Sec} to the ribosome and Sec incorporation or antagonize release factor catalyzed termination of translation are expected to increase redefinition efficiency.

3.3.1 Sequence Context Effects on Termination Efficiency

One factor known to effect termination efficiency is the identity of the stop codon, where in mammals the order is: [UAA > UAG > UGA]. The differences likely stem from the nature of direct contacts between the eukaryotic termination factor (eRF1) and the stop codon that induce conformational changes in the ribosome complex required to trigger peptide hydrolysis [41, 42]. In addition, multiple studies have highlighted the importance of local stop codon sequence context, especially the two codons preceding and the base following the stop codon, in determining termination efficiency [43–46]. However, these *cis*-acting sequences alone are not sufficient to predict termination efficiency [47], indicating that a larger sequence context is involved. The nature of this effect is likely to be complex and may include RNA secondary structure as well as the primary sequence, and even the composition of the nascent peptide chain in the exit tunnel of the ribosome.

3.3.2 Sequence Context Effects on Sec Incorporation

In contrast to stop codon readthrough due to near-cognate tRNA decoding, redefinition of UGA-Sec codons to encode Sec requires recruiting the eEFSec ternary complex for cognate decoding by Sec-tRNA^{Sec}. Evidence for an effect of UGA-sequence context on the efficiency of Sec insertion efficiency initially came from studies where nucleotides adjacent to the UGA-Sec codons for the iodothyronine deiodinase [48, 49] and PHGPx [39] were varied. In most cases, readthrough efficiency was found to be increased in contexts that resulted in inefficient termination. A thorough analysis of readthrough efficiency of the 10 UGA-Sec codons encoded by the rat Selenoprotein P (*SEPP1*) gene (with each UGA-Sec codon containing the surrounding native 24 nucleotide sequence context) in rabbit reticulocyte lysate revealed a lack of correlation between Sec incorporation efficiency and the nucleotide immediately following the UGA-Sec codon [50]. This study illustrated that

the sequence context effect is complex and supports a model in which a larger *cis*-acting sequence context determines Sec insertion efficiency. Here, it was proposed that this larger context may work together with Sec incorporation factors to determine readthrough efficiency.

Further evidence for an extended context effect on Sec incorporation efficiency comes from phylogenetic analysis demonstrating the potential for stable and conserved RNA structures located downstream of the UGA-Sec codons in a subset of selenoprotein genes [51]. In support of the importance of these elements, the same RNA secondary structures were independently identified in two selenoprotein genes, *SEPN1* and *SELT*, in a genome-wide search for deeply conserved functional RNA structures [52]. Detailed experimental analysis of the larger sequence context surrounding the *SEPN1* UGA-Sec codon demonstrated an effect on Sec incorporation efficiency in vitro [51, 53] and in vivo [54] (also see Chap. 22). This *cis*-acting element, designated the Sec codon redefinition element (SRE), consists of upstream sequences and a highly conserved stem-loop structure that starts six nucleotides downstream of the UGA-Sec codon. Using reporter assays in cultured mammalian cells, the *SEPN1* SRE is sufficient to cause high level (4–6%) readthrough of UGA and UAG codons in mammalian cells, which was not dependent on the presence of the 3′ UTR SECIS element [51, 53]. When the *SEPN1* SECIS element was included in the 3′ UTR, the SRE was not required for readthrough but had a significant stimulatory effect. Experiments in rabbit reticulocyte lysates provided direct evidence that the SRE stimulates Sec incorporation, rather than near-cognate tRNA decoding of the UGA codon [53] suggesting it may play a direct role in recruiting or ribosomal accommodation of the Sec-tRNA^{Sec}. In contrast to the cell-based model originally used, SRE stimulation of readthrough was specific for UGA codons and required both SBP2 supplementation and the 3′ UTR SECIS element. Importantly, by supplementing the rabbit reticulocyte lysate with 75-Se labeled Sec-tRNA^{Sec}, it was also shown that the SRE increased incorporation of Sec into the full-length product.

3.3.3 Sequence Context Effects from a Distance

Evidence that distant (non-SECIS) *cis*-acting elements can alter UGA redefinition was recently discovered in selenoprotein mRNAs from the ciliate *Euplotes crassus* [55]. Several genes were identified that contain multiple UGA codons. *E. crassus* has the requisite Sec incorporation machinery as well as tRNAs capable of decoding UGA codons as cysteine. The *Euplotes* thioredoxin reductase 1 (eTxnrd1) gene contains seven UGA codons. Transfection experiments in HEK293 cells and mass spectrometry analysis of the native protein purified from *E. crassus* revealed that the first six UGA codons are decoded as cysteine and only the final UGA codon in the penultimate codon position was decoded as Sec. Replacing the eTxnrd1 3′ UTR with the 3′ UTR of *SELT* from *Toxoplasma* relaxed the positional requirement allowing insertion of Sec at upstream UGA codons. The authors propose a model in which the 3′ UTR from eTxnrd1 contains a *cis*-acting RNA structure that prevents

the SECIS element from accessing the ribosome during decoding of upstream UGA codons. The contribution of the local sequence context at each UGA codon was not examined in this study.

Collectively, these results clearly demonstrate that an extended UGA-Sec sequence context and even distant *cis*-acting elements can affect both termination and Sec incorporation efficiency. It is unclear if these *cis*-acting elements share common mechanisms with *cis*-acting elements stimulating near-cognate tRNA decoding of stop codons as the mechanism(s) by which these elements act remains uncertain. The intriguing possibility that the *cis*-acting elements in selenoprotein mRNAs interact directly with components of the Sec insertion machinery or the ribosome to facilitate decoding by Sec-tRNA^{Sec} is suggested by several lines of evidence but requires further study.

3.3.4 Mechanism of Sec Incorporation in Transcripts with Multiple UGA Codons

Several selenoprotein genes have now been identified with the potential to encode more than one Sec residue. These include an alternatively spliced isoform of *SEPN1* (alternate transcript 1) [56], selenoprotein L [57], and *SEPP1*. Surprisingly, the total number of UGA-Sec codons in *SEPP1* ranges among species from 10 in humans to as many as 28 in sea urchin [58]. As demonstrated in rabbit reticulocyte lysate, the efficiency of Sec incorporation at each UGA-Sec codon when examined alone and in its native context varies between ~5 and 25% [50]. Yet, purification of selenoprotein P from plasma reveals the majority of protein to be full-length with several prematurely UGA-terminated species having been identified [59, 60]. In contrast to the model whereby Sec incorporation competes inefficiently with termination, the production of full-length protein from these messages would seem to demand highly efficient Sec incorporation due to the compounding effect of termination at each UGA-Sec codon.

It has been suggested that *SEPP1* may utilize a special mechanism for Sec incorporation due to the exceptional number of UGA-Sec codons and the observation that *SEPP1* mRNAs are unique in having two conserved 3′ UTR SECIS elements. In one model [61], it is proposed that each of the two SECIS elements in the *SEPP1* RNA has different functions with the distal SECIS element serving to incorporate Sec at the first UGA inefficiently, acting as a checkpoint for Sec incorporation factors, and the proximal SECIS dedicated to redefinition of the remaining UGA codons with high efficiency. It was concluded that the *SEPP1* gene has evolved unique properties to accommodate the incorporation of multiple Secs into one polypeptide.

A recent study examining Sec incorporation efficiency in messages containing a subset of the *SEPP1* UGA-Sec codons suggests that the ability to incorporate Sec with high efficiency may not be a unique feature of the *SEPP1* mRNA but rather an intrinsic property of Sec incorporation [62]. In this study, it was found that

incorporation of Sec was inefficient at a first UGA-Sec codon but increased by roughly one order of magnitude at downstream UGA-Sec codons. The observed increase in Sec incorporation "processivity" was not unique to *SEPP1* SECIS, as replacing the two *SEPP1* SECIS elements with single SECIS elements derived from other selenoprotein genes revealed similar results. A modified model was proposed in which the SECIS complex is loaded onto the ribosome prior to, or during, decoding of the first UGA-Sec codon. Once assembled, the ribosome proceeds to the next UGA-Sec codon reprogrammed for highly efficient Sec incorporation. A key observation in this study was that the efficiency of Sec incorporation at each UGA-Sec codon in the message was dependent on the identity and relative strength of the SECIS in the 3′ UTR. This was interpreted as evidence for a continued interaction of the SECIS element with the ribosome at each UGA-Sec codon. Consequently, it was proposed that the SECIS element and associated factors track with the ribosome following Sec insertion at the first UGA-Sec codon. The high level of termination at the first UGA-Sec codon could then be explained by the preceding ribosomes encountering the first UGA-Sec codon without having access to the SECIS element and the *trans*-acting factors associated with the ribosome engaged in decoding the remainder of the open reading frame. Under this model, ribosomes that have recruited the Sec incorporation machinery decode UGA-Sec codons as Sec with high efficiency and termination at UGA codons is inefficient.

3.4 Putting It All Together: In Vitro Reconstitution

In this chapter, we have provided a "bottom up" perspective on Sec incorporation, describing the factors, *cis*-sequences, and events most proximal to the actual Sec-tRNA^{Sec} delivery event (see Fig. 3.3). One of the major hurdles in definitively determining the core mechanism of Sec incorporation is creating a system in which Sec incorporation can be reconstituted from purified components. The use of rabbit reticulocyte lysates has been a valuable intermediate in this endeavor as they are naturally devoid of endogenous SBP2. Two recent studies highlight the utility of rabbit reticulocyte lysates in not only helping to decipher the core mechanism but also reproducing results obtained in living cells [20, 63]. Our efforts to find or create a similar lysate that is devoid of both SBP2 and eEFSec have thus far been unsuccessful, but current efforts to make home-made lysates from eEFSec-null *Drosophila* embryos will likely yield favorable results and provide an alternative intermediate system that will allow significant progress to be made with regard to eEFSec function (P.R. Copeland, unpublished results). The major hurdle in building a completely reconstituted system is that in vitro reconstitution of eukaryotic translation initiation is inefficient and technically challenging. One potential way to circumvent this is to use a translation elongation system that bypasses the initiation phase by the use of an internal ribosome entry site (IRES) from the cricket paralysis virus [64]. This *cis*-acting sequence allows for a complete bypass of translation initiation as it

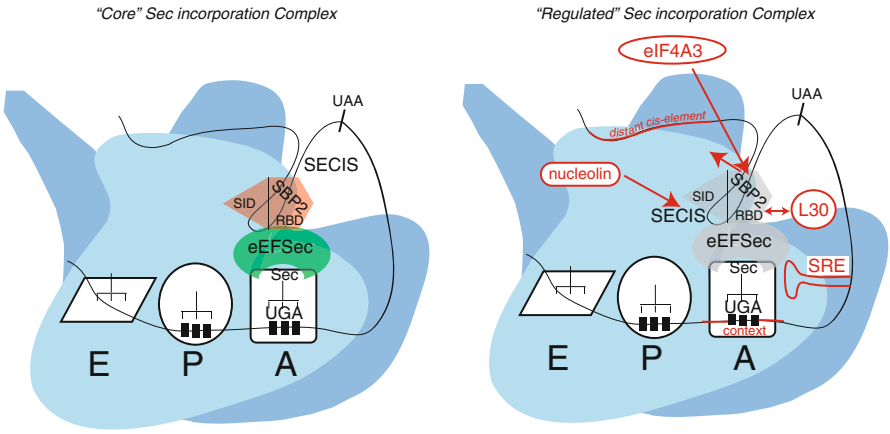

Fig. 3.3 Models of Sec incorporation. The *left panel* shows a Sec incorporation event mediated by the factors known to be required, while the *right panel* shows the factors (*red*) that may impact the efficiency and/or processivity of the Sec incorporation reaction. These factors include the *cis*-elements discussed in the text such as the SRE, codon context and distant 3′ UTR-based elements as well as *trans*-factors discussed in Chap. 4 such as nucleolin, ribosomal protein L30, and eukaryotic initiation factor A3 (eIF4A3)

directly recruits 80 S ribosomes. One potential caveat of such a system is that there may be a role for translation initiation factors in supporting Sec incorporation. A recent study has addressed this by determining that CrPV IRES-driven Sec incorporation is possible in vitro, albeit at a slight but consistently lower efficiency [65]. Ultimately, such a system will allow detailed molecular interaction studies based on fluorescence resonance energy transfer (FRET) as well as the ability to create intermediate Sec incorporation complexes that will reveal the steps required for the Sec incorporation event and the role that newly identified *cis*- and *trans*-acting factors play in modulating this process. One clear example of how this system may be utilized is in carefully dissecting the interplay between translation termination and Sec incorporation. By altering the ratios of Sec incorporation factors and termination factors and assessing the ability of an active Sec incorporation complex to form on purified ribosomes in the presence or absence of eRF1, one could gain clear insight into how these two competing processes work in concert to provide regulated synthesis of selenoproteins. This is just one of countless experiments that will reveal the inner workings of the Sec incorporation machinery, ultimately shedding light on how one might regulate this process in vivo.

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Chapter 4
SECIS-Binding Proteins Regulate the Expression of the Selenoproteome

Donna M. Driscoll and Jodi L. Bubenik

Abstract The incorporation of selenocysteine into selenoproteins during translation is dictated by a UGA codon in the mRNA. The recoding of UGA as selenocysteine instead of stop depends on a stem-loop structure in the 3′ untranslated region of the mRNA. This element acts as a platform for RNA-binding proteins, including components of the basal selenocysteine incorporation machinery (SECIS-Binding Protein 2 and ribosomal protein L30) and two newly discovered regulatory proteins that selectively modulate selenoprotein expression (eukaryotic initiation factor 4a3 and nucleolin). Thus, multiple RNA-binding proteins may act in a combinatorial manner to regulate the expression of the selenoproteome.

4.1 Introduction

The health benefits of selenium in humans are largely attributed to its presence as selenocysteine (Sec), which is found in a small but important subset of proteins, called selenoproteins. Sec is considered the 21st amino acid because it is encoded by the UGA stop codon and is incorporated into selenoproteins during translation.

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The recoding of UGA as Sec depends on the Sec Insertion Sequence (SECIS) element, a stable stem-loop in the 3′ untranslated region (UTR) of the selenoprotein mRNA [1]. All eukaryotic SECIS elements share a similar structure composed of two stems separated by an internal loop. The apical region of SECIS contains either an apical loop (Type 1 SECIS) or apical bulge (Type 2 SECIS) [2, 3]. Two conserved motifs are required for Sec incorporation: the SECIS core and the AAR motif [4]. Over the last 10 years, significant advances have been made in defining the role of the SECIS element in the Sec incorporation mechanism. A number of proteins have been shown to bind to the SECIS element in vitro. This chapter will focus on four SECIS-binding proteins whose functions in selenoprotein synthesis have been established. SECIS-binding protein 2 (SBP2) [5] and ribosomal protein L30 (L30) [6] are components of the basal Sec incorporation machinery in eukaryotes, whereas eukaryotic initiation factor 4a3 (eIF4a3) [7] and nucleolin [8] are regulatory proteins that play opposing roles in modulating the expression of a subset of selenoproteins. SBP2, a low abundance protein, appears to be dedicated solely to selenoprotein synthesis. By contrast, L30, eIF4a3, and nucleolin are abundant multifunctional RNA-binding proteins that have been co-opted into the Sec incorporation pathway. These four proteins will be considered in the context of their SECIS-binding activities and distinct roles in dictating the expression of the selenoproteome.

4.2 SBP2

SBP2 is the best-characterized *trans*-acting protein in the selenoprotein biosynthetic pathway. SBP2 was initially detected in rat testis extracts as a 120 kDa protein that bound to SECIS elements in UV crosslinking assays [9]. This binding activity was purified by RNA affinity chromatography using a SECIS element as the ligand [10]. The purified protein was analyzed by mass spectrometry and the peptide sequence information was used to isolate a cDNA clone encoding the binding activity [5]. This powerful strategy of biochemical purification coupled with mass spectrometry was also successfully used to identify L30, nucleolin, and eIF4a3 as SECIS-binding proteins.

The full-length rat SBP2 cDNA encodes a novel protein of 846 amino acids [5]. Interestingly, the protein is not highly conserved across species, although there are local regions of high sequence conservation. SBP2 is a limiting and likely an essential factor for selenoprotein synthesis in mammalian cells [11]. The N-terminal half of the protein is not required for Sec incorporation and does not contain any known motifs [5]. Elucidating the function of this region has proved difficult in the absence of testable hypotheses. The C-terminal half of SBP2 performs several critical functions during Sec incorporation, including binding to the SECIS element, recruiting the Sec-specific elongation factor, EFSec, and interacting with the ribosome [12–14]. The importance of the last two activities to the Sec incorporation mechanism is discussed in Chaps. 3 and 5.

4.2.1 Expression of SBP2

Given the pivotal role of SBP2 in dictating the expression of the selenoproteome, it is important to elucidate the expression and regulation of this protein. SBP2 protein and activity levels vary between tissues and cell types [10, 15]. This variation is not explained by differences in SBP2 mRNA levels [5, 15], suggesting that the expression of SBP2 may be regulated at the translational level. Indeed the 3′ UTR of the SBP2 mRNA contains several highly conserved regions that interact with members of the Turnover and Translation Regulatory RNA-binding Protein family [15]. To add to this complexity, SBP2 has been shown to be a redox-sensitive protein. Oxidative stress resulted in the accumulation of SBP2 in the nucleus and a decrease in selenoprotein synthesis in cells [16]. Understanding how the expression and activity of SBP2 is regulated is an important direction for future research.

SBP2 protein is expressed at low levels in somatic tissues, which has implications for models of Sec incorporation. It has been proposed that SBP2 remains stably bound to SECIS elements in selenoprotein mRNAs through multiple rounds of translation [11]. Based on an analysis of relative transcript levels, the ratio of the SBP2 mRNA to the total pool of selenoprotein mRNAs ranges from 1:200 to greater than 1:1,000 in different mouse tissues [17]. Presumably, SBP2 is substoichiometric to the number of potential mRNA targets in a cell. Given the stoichiometry of the players involved, the proposed requirement for a stable SBP2:SECIS interaction seems unlikely, unless most selenoprotein mRNAs are not competent for translation. Thus, alternative models in which SBP2 acts transiently at SECIS should be considered.

4.2.2 SECIS-Binding Activity of SBP2

Based on mutagenesis studies, the binding of SBP2 to the SECIS element requires an intact SECIS core [5]. RNA footprinting studies revealed that SBP2 binds to both strands of the SECIS core and along the 5′ side of the internal loop and helix 1 [18]. A recent study identified additional determinants for SBP2 binding in helix 2 and showed that SBP2 preferentially binds to RNAs that contain a large internal loop [19]. Subtle differences in these determinants outside of the SECIS core may explain how SBP2 can distinguish among SECIS elements from different selenoprotein mRNAs.

The SECIS core contains two sheared tandem G-A pairs [20], which have been shown to undergo a kink-turn folding in other mRNAs [21, 22]. Several lines of indirect evidence suggest that the SECIS element can also undergo an open-to-kinked transition [6]. This hypothesis needs to be validated using biophysical approaches. Interestingly, SBP2 appears to bind to the open form of the SECIS element but not the kinked form [6]. The implications of this differential binding for the mechanism of Sec incorporation are discussed in Sect. 4.3.1.

4.2.3 RNA-Binding Domain of SBP2

SBP2 and L30 are both members of the L7Ae family of RNA-binding proteins. This family includes the archaeal ribosomal protein L7Ae, other eukaryotic ribosomal proteins, and proteins involved in RNA processing, ribonucleoprotein assembly, and termination of protein synthesis [23]. The family members share a similar RNA-binding domain, called the L7Ae motif, and they often bind to kink-turn motifs in RNA [24]. The L7Ae motif in SBP2 most closely resembles the domain found in the spliceosomal 15.5 kDa protein. Using this homology as a guide, the Krol laboratory identified amino acids in the SBP2 L7Ae motif that are critical for interacting with the SECIS core [25]. However, the L7Ae motif alone was not sufficient to mediate SECIS binding. Based on sequence conservation, mutagenesis studies, and secondary structure predictions, Bubenik and Driscoll [26] proposed that the SBP2 RNA-binding domain is bipartite. Both the L7Ae motif and a second noncontiguous region that is located N-terminal to this motif are required for the SECIS-binding activity of SBP2 [26]. Two subsequent studies also demonstrated the importance of this region, which has also been termed SID or K-rich [12, 27]. Whether this region directly contacts the RNA or induces a conformational change in the L7Ae motif to enhance SECIS binding is controversial.

4.2.4 SBP2 as an Essential Factor for Sec Incorporation

SBP2 has been shown to be an essential factor for Sec incorporation in a rabbit reticulocyte lysate in vitro translation system [5]. siRNA-mediated knockdown of SBP2 in mammalian cells led to a reduction in the expression of endogenous selenoproteins [16]. Whether SBP2 is required for selenoprotein synthesis in vivo remains to be established but the sentiment in the field is that an SBP2 knockout is likely to be lethal. Importantly, reducing SBP2 levels in cultured cells has biological consequences. The knockdown of SBP2 in mesothelioma and neuroblastoma cell lines led to a reduction in telomere length, possibly due to lesions induced by oxidative damage [28]. Papp et al. [29] showed that depletion of SBP2 caused an increase in reactive oxygen species, leading to oxidative stress and apoptosis. The effects of the SBP2 knockdown in these two studies were presumably due to the loss of selenoprotein production. However, the possibility that SBP2 plays other roles in the cell in addition to Sec incorporation cannot be excluded.

4.2.5 SBP2 as the Master Regulator of Selenoprotein Synthesis

An early study from the Berry lab showed that SBP2 is the limiting factor for Sec incorporation when selenoprotein mRNAs are overexpressed in transfected cells [11].

The overexpression of SBP2 also had a differential effect on the Sec incorporation activity of various SECIS elements. These results led the authors to propose that SBP2 dictates the efficiency of Sec incorporation and ultimately, the expression of the selenoproteome. A subsequent study used the converse approach of siRNA knockdown to show that reducing SBP2 levels had various effects on the levels of individual selenoprotein mRNAs [30]. Based on a co-immunoprecipitation approach, SBP2 was preferentially bound to certain endogenous selenoprotein mRNAs compared to others in cells [30]. The conclusion of this study was that SBP2 determines the expression of the selenoproteome by differentially regulating selenoprotein mRNA stability and/or translational efficiency.

The finding that SBP2 plays a critical role in selectively regulating the expression of individual selenoproteins raises the question as to how this selectivity is determined. The simplest model is that individual selenoprotein mRNAs contain SECIS elements that differ in their affinity for SBP2. The protein has been shown to have a selective SECIS-binding activity when it is expressed in transfected cells and in vitro translation assays but other cellular factors are present in these systems [26, 30]. Indeed, when purified recombinant SBP2 was tested in gel shift assays, the protein bound to different SECIS elements with similar affinity [18]. There are several possible explanations for the lack of selectivity observed in these experiments. First, the bacterially expressed SBP2 may have been improperly folded or lacking a critical posttranslational modification. Second, the SECIS-binding activity of the recombinant protein was tested using minimal SECIS elements [18]. Additional sequences in the 3′ UTR may influence the affinity of the SBP2:SECIS interaction. Finally there may be other proteins in the cell that modulate the affinity or selectivity of SBP2. This hypothesis is supported by the recent finding that eIF4a3 prevents the binding of SBP2 to a subset of selenoprotein mRNAs, as discussed in Sect. 4.4.

4.2.6 Importance of the SBP2:SECIS Interaction in Human Disease

An exciting area of research is the newly discovered link between mutations in the SBP2 gene and human disease. Several SBP2 mutations and their physiological consequences are summarized in a recent review [31]. The effects of these mutations specifically on thyroid hormone metabolism are discussed in more detail in Chap. 29. In the first report of a genetic defect in SBP2, mutations in the SBP2 gene in two families were linked to decreased iodothyronine deiodinase expression and thyroid hormone dysfunction [32]. This was the first demonstration of an inherited defect in deiodinase activity in humans. Despite normal mRNA levels, the activities of type 2 iodothyronine deiodinase (Dio2) and glutathione peroxidase 1 (GPx1) were reduced in these individuals. The expression of other selenoproteins appeared to be unaffected since the patients were otherwise healthy, suggesting a selective defect in SBP2 activity [32]. One family had a missense mutation (R→Q) in the first region of the bipartite SBP2 RNA-binding domain. This single amino acid change

altered the RNA-binding affinity of SBP2, so that the mutant protein did not stably interact with SECIS elements from certain selenoprotein mRNAs, including Dio2 and GPx1 [26]. This selective SECIS-binding defect would explain the mild phenotype of the patients since the essential selenoproteins would still be expressed, while others like Dio2 and GPx1 would be lost.

Since this initial report, the search for additional SBP2 mutations in the human population has intensified. Such mutations appear to be rare and result only in partial SBP2 deficiency, presumably because complete loss of SBP2 function would be lethal. Recent studies have identified several patients who are compound heterozygotes for unique SBP2 mutations that result in variable phenotypes, ranging from mild to severe [33–35]. An emerging theme in the field is that the extent of the defect of an individual mutant SBP2 protein dictates which subset of selenoproteins show impaired synthesis, thus determining the complexity of the phenotype.

4.3 Ribosomal Protein L30

The second SECIS-binding protein to be characterized in depth is ribosomal protein L30, which is a component of the large ribosomal subunit in eukaryotes. Unlike SBP2, L30 is an abundant protein that is ubiquitously expressed in mammalian tissues. Although L30 is primarily associated with the ribosome, a small fraction exists in other cellular compartments. L30 is found in the nucleolus where it is involved in rRNA processing, in the nucleus where it binds to the L30 pre-mRNA to inhibit splicing, and in the cytoplasm where it binds to the mature L30 mRNA to inhibit translation [36–38]. The existence of this autoregulatory feedback loop suggests that the expression levels of L30 need to be tightly controlled in mammalian cells.

4.3.1 SECIS-Binding Activity of L30

L30 has been shown to bind to SECIS elements both in vitro and in cells [6]. This interaction is specific as mutations that disrupted the SECIS core abrogated L30 binding. Based on mutagenesis studies, L30 and SBP2 have similar nucleotide requirements for binding to the SECIS element [6]. A major question that remains to be answered is whether L30 and SBP2 bind to identical or overlapping sites on SECIS elements. As discussed above, the SECIS element may undergo an open-to-kinked conformational transition. In vitro binding studies suggested that SBP2 only binds to the open form of SECIS elements. By contrast, L30 can interact with either the open or kinked conformer [6]. Interestingly, the affinity of L30 is much higher for an SBP2:SECIS complex than for the free SECIS alone, which suggests that SBP2 may remodel SECIS elements so that they become high-affinity targets for L30. As binding of L30 induces a kink in the L30 pre-mRNA [39], L30 may also induce the SECIS core to undergo a kink-turn folding. SBP2 may not be sufficiently flexible to do this, given that this protein has a much larger RNA-binding domain

than L30. These studies suggest a model in which the SECIS element acts as a molecular switch that undergoes conformational changes upon protein binding as discussed in [6].

4.3.2 Role of L30 in Sec Incorporation

In addition to the SECIS-binding activity of L30, there is also functional evidence that this protein plays a role in Sec incorporation. The overexpression of L30 in rat hepatoma cells enhanced the UGA recoding activity of a co-transfected reporter construct that detects translational readthrough in transfected cells [6]. Thus, L30 was limiting in this system in the presence of overexpressed reporter RNAs. However, definitive evidence that L30 is essential for endogenous selenoprotein synthesis is still needed. As L30 is an essential gene in yeast [40], targeted disruption of the L30 gene in mice is likely to be lethal. The alternative strategy of siRNA knockdown is likely to be a more fruitful line of investigation.

The identification of L30 as a component of the eukaryotic UGA recoding machinery led to new ideas about the mechanism of Sec incorporation. We proposed a specific order of events in which SBP2 binds to the SECIS element in an early targeting event, and L30 acts at the ribosome during Sec incorporation [6]. This model takes into consideration the observations that SBP2 is expressed at very low levels in most somatic tissues and binds SECIS elements with high specificity and affinity. By contrast, L30 is an abundant, ubiquitous protein that binds to the SBP2:SECIS complex with higher affinity than to the free SECIS element alone. The rationale and supporting evidence for this model are presented in [6]. An alternative and equally viable model in which SBP2 acts at the ribosome is discussed in [13].

A number of critical questions about the mechanism of action of L30 remain. Does L30 tether the SECIS element to the ribosome or does the protein leave the ribosome to bind to the SECIS element? There are several examples of other nonessential ribosomal proteins that perform noncanonical functions unrelated to polypeptide synthesis, including L13a, which leaves the ribosome to participate in the translational silencing of interferon-induced mRNAs in macrophages [41]. Does ribosome-associated L30 or the extra-ribosomal pool of L30 participate in the Sec incorporation mechanism? Finally, is the SECIS-binding activity of L30 sufficient to promote UGA recoding? It is intriguing to speculate that L30 may perform additional functions during this process, such as tethering the SECIS element to a specific site on the ribosome or suppressing termination at the UGA/Sec codon.

4.4 eIF4a3

When selenium becomes limiting, cells need to prioritize the utilization of this important micronutrient in order to ensure the proper expression of the selenoproteome. During selenium deficiency, there is a hierarchy of expression in which the synthesis

of essential selenoproteins is maintained while other less important selenoproteins are poorly expressed [42–45]. While SBP2 may contribute to the preferential translation of certain selenoprotein mRNAs, this protein is not regulated by selenium. A major advance in the field was the recent discovery that eIF4a3 serves as a link between selenium status and differential selenoprotein expression.

eIF4a3 belongs to the DEAD box family of RNA-dependent ATPases [46]. The protein, which is ubiquitously expressed in mammalian cells, is predominantly nuclear [47]. Despite sharing homology with other initiation factors, eIF4a3 has no known role in protein synthesis. The canonical function of eIF4a3 is to bind to spliced mRNAs during the formation of the exon junction complex [47, 48]. As part of the exon junction complex, eIF4a3 plays a role in nonsense-mediated decay, a surveillance pathway that eliminates mRNAs that contain premature stop codons [49, 50]. By contrast, eIF4a3 binds selectively to a subset of SECIS elements and regulates selenoprotein expression at the level of mRNA translation [7].

4.4.1 SECIS-Binding Activity of eIF4a3

The stable interaction of eIF4a3 with spliced mRNAs depends on other proteins in the exon junction complex [51]. However, the binding of eIF4a3 to the SECIS element is specific and selective. Purified eIF4a3 bound with high affinity to SECIS elements from GPx1 and Selenoprotein R (SelR), which are nonessential selenoproteins. By contrast, eIF4a3 bound with low affinity to the SECIS elements from two mRNAs that encode essential selenoproteins [7]. Thus, the selective eIF4a3:SECIS interaction must rely on a different mechanism than the sequence-independent binding of eIF4a3 to spliced transcripts.

Based on mutagenesis studies, the internal loop of the GPx1 SECIS is required for binding of eIF4a3. When this region was replaced with a different internal loop, eIF4a3 was still able to bind, suggesting that the protein recognizes additional determinants. Of the four SECIS elements tested, eIF4a3 interacted with two Type 1 elements that contained an apical loop, but not with two Type 2 elements that contained an apical bulge [7]. These results suggest that the apical loop may be part of the signature motif for eIF4a3. The identification of the nucleotide sequences and/or structures in SECIS elements that are required for binding of eIF4a3 will provide critical insight into how this protein discriminates among selenoprotein mRNAs.

4.4.2 eIF4a3 Is a Negative Regulator of Sec Incorporation

The binding of eIF4a3 to a SECIS element has functional consequences. eIF4a3 inhibited UGA recoding directed by the GPx1 and SelR SECIS elements in an in vitro translation system [7]. This effect was specific, as eIF4a3 had no effect when the UGA codon was replaced with UGU/Cys. Likewise the recoding activities

of SECIS elements that did not bind eIF4a3 were not inhibited when the protein was added to the assay. How does eIF4a3 inhibit UGA recoding? eIF4a3 has been shown to have a helicase activity in vitro. The simplest model is that eIF4a3 unwinds the SECIS element, thus eliminating the stem-loop structure. However, neither the helicase activity nor the ATPase activity of eIF4a3 was required for the protein to inhibit Sec incorporation. Instead, eIF4a3 competes with SBP2 for binding to the SECIS element because the two proteins share overlapping binding sites [7]. Furthermore, once eIF4a3 is bound to the SECIS element, it cannot be displaced by SBP2. Since eIF4a3 is predominantly nuclear in cells, the protein likely binds to the SECIS element before the GPx1 mRNA is exported to the cytoplasm for translation. The interaction of eIF4a3 with the SECIS element would mask the SBP2-binding site and consequently prevent Sec incorporation.

4.4.3 eIF4a3 Is Regulated by Selenium

Based on in vitro studies, eIF4a3 acts as a translational repressor for GPx1 and SelR. One might expect that the synthesis of these two selenoproteins would be compromised in normal cells given that eIF4a3 is widely expressed. Insight into this conundrum came from the discovery that eIF4a3 is regulated by selenium status [7]. It appears that there is sufficient eIF4a3 in selenium-adequate cells to carry out its canonical function of binding to spliced mRNAs in the exon junction complex. However, the amount of eIF4a3 is limiting with respect to binding to selenoprotein mRNAs. In response to selenium deficiency, eIF4a3 protein levels are upregulated several-fold. This increase in eIF4a3 is required for selective translational repression because siRNA knockdown of eIF4a3 rescued GPx1 expression in selenium-deficient cells [7]. Furthermore, the overexpression of eIF4a3 in selenium-adequate cells reduced GPx1 protein levels with no effect on mRNA levels [7]. Thus, eIF4a3 is necessary and sufficient to repress the synthesis of GPx1. eIF4a3-mediated translational repression is an attractive model for nutrient regulation of gene expression where rapid and transient changes in protein synthesis might be desired in response to dietary fluxes. These studies provided the first mechanistic insight into how the translation of nonessential selenoprotein mRNAs is selectively inhibited to ensure the synthesis of essential selenoproteins when selenium is limiting.

4.4.4 Additional Roles for eIF4a3 in Regulating Selenoprotein Synthesis?

In addition to inhibiting GPx1 synthesis, does eIF4a3 direct a translational regulon in which the synthesis of a cohort of selenoproteins is repressed in selenium-deficient cells? What happens when selenium status is restored? One interesting idea is that eIF4a3 may dissociate from the GPx1 SECIS element in response to an

event such as a selenium-dependent posttranslational modification. The mRNA would then be available for interaction with SBP2, rapidly restoring GPx1 synthesis when selenium becomes available. Finally, is there a role for the SECIS-bound eIF4a3 in mediating the degradation of the GPx1 mRNA? As discussed above, eIF4a3 and the exon junction complex participate in the nonsense-mediated decay pathway. It is intriguing to speculate that the binding of eIF4a3 to the SECIS element could lead to the formation of a pseudo-exon junction complex in an ectopic location, resulting in mRNA degradation.

4.5 Nucleolin

Nucleolin is best known for its classical role in facilitating ribosome biogenesis in the nucleolus. Over the last few years, it has become clear that the protein also performs a variety of unexpected functions in other cellular compartments [52]. Nucleolin regulates the expression of several cellular and viral transcripts at the posttranscriptional level by altering mRNA stability or translation in the cytoplasm, as discussed in [8]. A recent study established that nucleolin acts as a positive regulator for the translation of selenoprotein mRNAs that encode essential functions [8].

4.5.1 SECIS-Binding Activity of Nucleolin

Nucleolin was initially identified as a putative SECIS-binding protein by screening a bacterial expression library with a radiolabeled GPx1 SECIS probe [53]. This study did not determine whether nucleolin bound to other SECIS elements or whether the nucleolin:SECIS interaction was functionally important. A subsequent study showed that nucleolin is a selective SECIS-binding protein. Nucleolin bound with higher affinity to SECIS elements from selenoproteins that are preserved in selenium deficiency and/or exhibit a severe phenotype when deleted in mice [8]. Interestingly, nucleolin had a very low affinity for the SECIS element from the GPx1 mRNA, which encodes a nonessential selenoprotein. Thus, it is not clear why nucleolin was identified as a GPx1 SECIS-binding protein in the earlier ligand screening experiments.

Mutational analysis of SECIS elements revealed that the upper part of the basal stem is required for nucleolin binding [8]. This region may directly interact with nucleolin or it may be required to stabilize the SECIS structure so that nucleolin can bind elsewhere on the molecule. How does nucleolin discriminate among SECIS elements? A number of different nucleolin-binding sites have been identified in preribosomal RNAs and in the 5′ or 3′ UTRs of several cellular mRNAs [54–57]. To date, a consensus binding site common to the SECIS elements that are bound by nucleolin with high affinity has not been identified.

Defining the domains in nucleolin that are required for SECIS binding is another important area for investigation. The central region of nucleolin contains four non-identical RNA recognition motifs (RRM), which are found in many proteins that are involved in RNA processing and metabolism [58]. Some of these RNA-binding proteins contain multiple RRMs. In such a protein, the individual motifs or combinations of RRMs often have different binding specificities, allowing the protein to interact with more than one target mRNA sequence. In the case of nucleolin, studies on pre-ribosomal RNA found that binding of this protein to the nucleolin recognition element (NRE) requires only RRMs 1 and 2, whereas all four RRM domains are essential for binding to another sequence, the Evolutionary Conserved Motif [59, 60]. It will be of interest to identify the RRMs in nucleolin that mediate SECIS binding.

4.5.2 Role of Nucleolin in Selenoprotein Synthesis

Unlike eIF4a3, the expression and activity of nucleolin is not regulated by selenium. Even in selenium-adequate cells, SBP2 and L30 are both limiting factors for Sec incorporation. Thus, the limiting UGA recoding machinery may need to be preferentially recruited to a subset of transcripts, which encode selenoproteins that perform critical functions. The role of nucleolin in regulating selenoprotein expression was investigated using an siRNA strategy [8]. siRNA knockdown of nucleolin inhibited the synthesis of essential selenoproteins, with no effect on the expression of nonessential selenoproteins. Furthermore, the levels and the nuclear/cytoplasmic localization of selenoprotein mRNAs were not altered in the nucleolin-deficient cells. These results support the hypothesis that nucleolin is required for the optimal expression of a subset of selenoproteins, which encode essential functions.

There are a number of mechanisms by which nucleolin could enhance selenoprotein mRNA translation. The simplest model is that nucleolin converts a SECIS element into a more effective competitor for the UGA recoding machinery, which is limiting in cells. Nucleolin may bind to the SECIS element and recruit SBP2 or other factors in the Sec incorporation pathway through protein:protein interactions. Alternatively, nucleolin may stabilize the structure of the SECIS element or modify its conformation so that high affinity interactions with SBP2 can occur. Finally, it will be important to validate the physiological significance of these studies in an in vivo setting. Of particular interest is whether nucleolin plays a role in preserving the synthesis of essential selenoproteins in animal models of selenium deficiency.

4.6 Other SECIS-Binding Proteins

In addition to the four proteins discussed above, several other putative SECIS-binding proteins have been observed. Two groups reported discrepant results as to whether nuclease sensitive element-binding protein 1 (NSEP1), also known as

DNA-binding protein B, binds to the GPx1 SECIS element [61, 62]. A later study showed that the siRNA knockdown of NSEP1 led to a twofold decrease in GPx1 levels but the expression of other selenoproteins or control proteins other than actin was not analyzed [63]. Whether the reduction in GPx1 expression was mediated at the level of mRNA stability or translation was not investigated in this study. Proteins of 47.5 and 60–65 kDa in cell extracts have also been reported to bind to the GPx1 SECIS element [64, 65]. However, the identity of these proteins and the functional significance of these interactions have not been determined. Of note, the unknown 47.5 kDa SECIS-binding protein may have been eIF4a3, which has a similar molecular weight.

4.7 Conclusions

An exciting development in the field of selenoprotein biology is that the SECIS element interacts with a number of *trans*-acting factors. These SECIS-binding proteins have been shown to be involved either in the Sec incorporation mechanism or in the regulation of this pathway. Although SBP2 plays an important role in dictating the expression of the selenoproteome, eIF4a3 and nucleolin perform critical functions in determining which selenoproteins are synthesized. These recent discoveries shift the current paradigm from a simplified SBP2-centric view to a more complex model in which multiple SECIS-binding proteins combinatorially regulate the expression of individual selenoproteins or subsets of selenoproteins. In the future, we hope to identify polymorphisms or mutations in the L30, nucleolin, or eIF4a3 genes that impact selenoprotein expression and consequently human health.

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Chapter 5
A Ribosomal Perspective on the Mechanism of Selenocysteine Incorporation

Kelvin Caban and Paul R. Copeland

Abstract Selenocysteine (Sec) is cotranslationally inserted into polypeptides during the elongation phase of protein synthesis in response to specific UGA codons. As UGA normally signals translation termination, the Sec incorporation complex is required to modify the canonical translation machinery. Thus, a thorough understanding of the Sec incorporation mechanism necessitates careful consideration of the intricacies of general translation, specifically during the elongation phase. Here, we consider the current body of evidence that supports a key role for the ribosome in regulating the process of Sec incorporation.

5.1 Introduction

Selenocysteine (Sec) incorporation is accomplished by the action of at least two *trans*-acting factors: SECIS-binding protein-2 (SBP2) and the Sec-specific elongation factor (eEFSec; see Chap. 3). These two factors convert a translation termination reaction into an elongation reaction by changing the coding potential of UGA codons found upstream of SECIS elements. Interestingly, SBP2 is known to bind to the ribosome both in cells and in vitro [1–4], suggesting that it is providing a signal to the ribosomes that Sec codons should be bound by the eEFSec ternary complex (eEFSec/Sec-tRNA^{Sec}/GTP) rather than the translation termination complex. Although Sec incorporation is in direct competition with translation termination, this chapter focuses entirely on the elongation phase of translation. This is because Sec codons are fully competent for translation termination even in the presence of a full complement of Sec incorporation factors [1]. Thus, termination appears to occur as a default reaction when Sec incorporation is not possible or occurs at a reduced efficiency.

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Ribosomes contain three tRNA-binding sites formed by both the large and small subunit, referred to as the A, P, and E sites. The A site binds aminoacyl-tRNA (aa-tRNA), the P site binds peptidyl-tRNA (and a unique initiator tRNA), and the E (Exit)-site binds deacylated tRNA prior to its departure from the ribosome. In addition, protein synthesis requires numerous *cis*-elements and *trans*-acting factors that work in concert with the ribosome and tRNA molecules to efficiently and faithfully decode the mRNA in three phases: initiation, elongation, and termination.

The bulk of protein synthesis takes place during the elongation phase. Two elongation factors sequentially bind the ribosome and utilize the energy of GTP hydrolysis to catalyze two major reactions: (1) delivery of the aa-tRNA to the ribosome by elongation factor Tu (EF-Tu; eEF1A in eukaryotes), and (2) translocation of the mRNA–tRNA complex by elongation factor G (EF-G; eEF2 in eukaryotes). The latter brings the next codon to the ribosomal A site allowing the cycle to repeat until a termination signal is encountered. Here, we dissect the molecular events in the elongation phase and put them into the context of the requirements for successful incorporation of selenocysteine. Since most mechanistic studies have been performed in bacteria, we refer to the bacterial elongation factors, EF-Tu (the tRNA carrier protein whose eukaryotic analogue is eEF1A or eEFSec for Sec), EF-Ts (the guanine nucleotide exchange factor [GEF] that is required for maintaining the GTP-bound state of EF-Tu), and EF-G (the translocase whose eukaryotic analogue is eEF2).

5.2 Initial Binding

The elongation cycle begins following translation initiation with the initiator tRNA in the P site, and an empty A site. The aa-tRNA is delivered to the ribosome as a ternary complex with EF-Tu and GTP. The very first event that is thought to occur is referred to as initial binding, which is a rapid and transient interaction between the ternary complex and the ribosome. This interaction is codon-independent and may function to recruit the ternary complex to the ribosomal A site [5, 6]. Even though this event is codon-independent, it is likely that eEFSec is normally excluded from initial binding as this would likely inhibit normal ternary complex binding. Thus, this step may represent the first barrier that must be overcome when switching from canonical elongation to one that is likely mediated by the SBP2/SECIS interaction. Since SBP2 has also been shown to form a stable SBP2/SECIS/eEFSec complex, it is possible that the function of this complex is distinct from one that may regulate ribosome conformation as discussed below.

Initial binding is believed to involve a protein–protein interaction between EF-Tu and one of the L7/L12 ribosomal stalk proteins (hereafter L12). Mutagenic studies coupled with kinetic analysis of this initial binding event suggests that it involves an interaction between helix D in the G-domain of EF-Tu (Domain I) and helices 4/5 of the C-terminal domain of L12 [7]. Interestingly, EF-Tu also uses Helix D to interact with the N-terminal domain of its GEF EF-Ts, and it has been proposed that the EF-Tu/L12 interaction resembles that of the EF-Tu/EF-Ts complex [7]. Interestingly,

the Sec-specific elongation factors, eEFSec and SelB, have several deletions that correspond to regions in EF-Tu that are involved in its interaction with EF-Ts. Not only does this suggest the lack of a GEF for eEFSec and SelB, but it also raises the intriguing possibility that these deletions interfere with initial binding by disrupting the interaction between the Sec-specific elongation factors and the ribosomal stalk proteins. A potential function for SBP2 on the ribosome then would be to alter the conformation of the L12 stalk to allow recruitment of the eEFSec ternary complex to the ribosomal A site, or alternatively to alter the conformation of eEFSec so it can interact with the L12 stalk. This may, in fact, be more likely because in this way SBP2 won't interfere with eEF1A TC binding.

In eukaryotes, the L12 stalk is replaced by an analogous complex that consists of the phosphoproteins (P-proteins) P1, P2 (L12), and P0 (L10) [8]. Although the P-proteins do not share sequence homology with L12, the eukaryotic factors are functionally equivalent. The functional significance of the P-proteins in specifying the recruitment of the eukaryotic elongation factors, eEF1A and eEF2, was demonstrated by exchanging the bacterial L12 stalk proteins for the eukaryotic counterparts. This replacement conferred a functional interaction between prokaryotic ribosomes and eukaryotic elongation factors demonstrating the importance of the stalk proteins for achieving specificity across these two domains [9]. This study suggests that initial binding is conserved, but a codon-independent interaction has not been reported in eukaryotes. In addition, it should be noted that the molecular basis for the interaction between eEF1A and the eukaryotic ribosome has not been characterized; hence, there is no experimental evidence to suggest that the interaction between eEF1A and the eukaryotic stalk proteins is mediated through helix D in the G-domain of eEF1A as has been proposed for EF-Tu.

L12 is also important for GTPase activation of EF-Tu following codon recognition. Along with L11, which binds H43–44 at the base of the L12 stalk, this region is referred to as the GTPase-associated center (GAC) [10]. Indeed, ribosomes lacking the L12 stalk proteins display a ~1,000-fold decrease in the rates of ribosome-stimulated GTP hydrolysis of EF-Tu [11]. In bacteria, the SECIS element is required to stimulate the ribosome-dependent GTPase activity of SelB, supporting the idea that it is in a conformation that is unable to functionally interact with L12 in the absence of the SECIS [12]. It would be interesting to see if SBP2 could similarly stimulate the latent GTPase activity in eEFSec through its interaction with the ribosome.

5.3 Codon Recognition: Kinetic Proofreading and Induced Fit

Initial binding is followed by codon recognition, which occurs in two discriminatory steps and is driven by two distinct mechanisms: kinetic proofreading and induced fit [13]. During kinetic proofreading, the aa-tRNAs are selected on the basis of anticodon–codon complementarity as dictated by the rules of Watson–Crick base pairing. As such, cognate tRNA will bind the A site with the highest affinity, while

non-cognate tRNAs on the other side of the spectrum cannot bind and are rapidly rejected. Near-cognate tRNAs can associate with the A site as well as cognate tRNAs, but exhibit a substantial increase in their dissociation rates [14]. Codon recognition occurs in an initial selection step following initial binding of the ternary complex, and then again following GTP hydrolysis and dissociation of the aa-tRNA from EF-Tu in a separate proofreading step [15].

Aside from this thermodynamic discrimination, ribosomes also actively participate in the selection process via a defined set of conformational changes that lead to the acceleration of two rate-limiting steps in the tRNA selection pathway: (1) GTPase activation followed by rapid GTP hydrolysis, and (2) accommodation of the tRNA into the peptidyl transferase center (PTC) followed rapidly by peptide bond formation. These conformational changes on the ribosome reflect an induced fit mechanism triggered in the presence of cognate tRNA. The crystal structure of the small ribosomal subunit programmed with cognate or near-cognate tRNA fragments called anticodon stem loops (ASLs) confirmed these earlier observations that suggested an induced fit mechanism in response to cognate tRNA [16–18]. In the presence of a cognate ASL, residues A1492, A1493 in the decoding center of the ribosome were completely flipped such that they could directly engage the codon–anticodon duplex and monitor its geometry. The small subunit was also observed to undergo a global conformational change referred to as domain closure. During domain closure, the shoulder and head domains of the small subunit rotate toward the decoding center. This reconfiguration allows nucleotide G530 in helix 18 of the shoulder domain to rotate from a *syn*- to an *anti*-conformation such that it can also interact with and monitor the codon–anticodon mini-helix. While these X-ray crystal structures were performed using ASLs and the small ribosomal subunit in isolation, the most recent crystal structure of the 70S ribosome complexed with EF-Tu ternary complex has corroborated these initial findings [19]. Since most eukaryotic ribosomes evolved to support termination factor accommodation at UGA codons, the question arises whether the ribosomal conformation changes that occur during canonical codon recognition also occur during Sec incorporation. The fact that codon/anticodon pairing is not sufficient to fully explain translational fidelity suggests that there may be unique conformational changes required to change the identity of a stop codon that may not be intrinsic to the ribosome and thus relegated to the functions of SBP2 or eEFSec or even through the Sec-tRNA^{Sec} itself as discussed below.

5.4 Communication Between Functional Centers

As mentioned above, cognate codon–anticodon interactions in the decoding center lead to an increase in the rate of GTP hydrolysis. This indicates that the information in the decoding center has to be reported to the GAC of the ribosome to activate the elongation factor's latent GTPase activity. The global domain closure induced upon binding of the cognate tRNA suggested that information in the decoding center was being transmitted to the GAC through the intersubunit bridges [17]. At odds with

this hypothesis, however, was an earlier study showing that two tRNA fragments corresponding to the ASL/D-arm and acceptor end/T-arm were incapable of stimulating GTP hydrolysis even in the presence of paromomycin [20], despite the fact that paromomycin was shown to induce the domain closure of the small subunit in the presence of both cognate and near-cognate ASL fragments [17] and to stimulate GTP hydrolysis for cognate and near-cognate tRNA [21]. The requirement for an intact tRNA suggests that the signal from the decoding center is propagated through the tRNA body instead of the intersubunit bridges.

In 1971, a $tRNA^{Trp}$ mutant with a G24A substitution in the D-arm was identified and named the Hirsh suppressor [22]. The Hirsh suppressor is near-cognate with respect to the UGA codon, but is somehow capable of evading rejection during codon recognition and thus functions as a UGA suppressor. Direct evidence in favor of signal propagation through the tRNA was revealed by kinetic studies demonstrating that the Hirsh suppressor was capable of accelerating the rate of GTP hydrolysis and peptide bond formation even when ribosomes were programmed with a near-cognate codon [23]. This indicates that the Hirsh suppressor tRNA can adopt the conformation normally induced by cognate tRNA binding and stabilized by domain closure. However, in the absence of structural data, we cannot rule out that the Hirsh suppressor is somehow inducing base flipping and domain closure on its own without the need for a cognate codon–anticodon interaction. If this is true, it would suggest that you need both the conformational changes (base flipping and domain closure) that may be contributing to this signal propagation through the intersubunit bridges as well as, an intact tRNA. In support of this, it was recently demonstrated that the Hirsh suppressor was unable to enhance GTP hydrolysis and peptide bond formation when the residues in the decoding center (A1492, A1493, and G530) were mutated indicating that this suppressor has not completely bypassed the molecular events that canonically lead to transmission of this signal [24]. These studies open up the distinct possibility that the Sec-$tRNA^{Sec}$ plays an active role in determining the efficiency of the Sec incorporation reaction, perhaps ultimately providing the molecular explanation for differential utilization of the Sec-$tRNA^{Sec}$ isoforms (see Chap. 44).

5.5 Proofreading

Following GTP hydrolysis, domain rearrangements within EF-Tu result in release of the aa-tRNA from the ternary complex, thereby freeing the 3′ $^{74}CCA^{76}$ acceptor end (CCA-end) containing the amino acid [25, 26]. Upon dissociation from EF-Tu, the codon–anticodon base pair in the decoding center is interrogated once again in a second discriminatory step called proofreading [15]. Herein, near-cognate tRNAs that get past initial selection are rapidly rejected while cognate tRNAs become fully accommodated in the ribosomal A site and make stabilizing contacts with the PTC. Accommodation is followed rapidly by peptide bond formation [27]. Cognate tRNAs accelerate the rate of accommodation, and thus proofreading, like initial selection, is also believed to operate through an induced fit mechanism.

5.6 tRNA Accommodation and Peptide Bond Formation

Peptide bond formation takes place in the PTC, the catalytic active site of the ribosome [28]. The PTC is located in a cleft below the central protuberance of the ribosome where it spans across the large subunit portion of the A and P site. This region is composed almost entirely of RNA from the central loop (C-loop) of domain V and the helices that protrude from it. The boundaries of the PTC are formed by two distinct regions referred to as the A and P-loop [29]. Directly below the PTC is the entrance to the ribosomal exit tunnel where the nascent polypeptide passes as the elongating ribosome translates the mRNA. The peptidyl tRNA is stabilized by interactions with the P-loop, which include a Watson–Crick base pair between C74 and G2251, and C75 and G2252 (*Escherichia coli* numbering used throughout unless indicated). In addition, the terminal A76 forms a stacking interaction with the ribose of A2451, and hydrogen bonds with the 2′ OH of A2450. When the A site is empty, the PTC is in an "un-induced state"; in this state both sides of the peptidyl ester group are sequestered by nucleotides U2585, C2063, and A2451, thus protecting it from nucleophilic attack [30]. Binding of aa-tRNA to the PTC results in conformational changes that are required to properly align the tRNA substrates to allow the peptidyl transferase reaction. This substrate-induced fit mechanism involves a shift of U2506 to prevent a steric clash with the amino acid moiety of the aa-tRNA, and the movement of nucleotides A2602, G2583, U2584, and U2585 which swings 90° away from the P site and exposes the peptidyl ester [31].

The CCA-end of the aa-tRNA is stabilized by several interactions with the A-loop [28]. These include stacking interactions between C74 and U2555, a Watson–Crick base pair between C75 and G2553, and a type I A-minor interaction between the terminal A76 and G2583. In addition to the protections resulting from direct contacts between the tRNA and the PTC, chemical probing studies have also revealed nucleotides whose reactivity changes due to allosteric effects [32]. Interestingly, when comparing aa-tRNA to deacylated tRNA bound to the ribosomal A site, three nucleotides (A2451, A2439, and A2602) in domain V showed altered chemical reactivities. These results indicate that the amino acid moiety can affect the confirmation of the PTC. Interestingly, while C74 is critical for the orientation of the tRNA substrates and for inducing the aforementioned conformational changes that expose the peptidyl ester to nucleophilic attack, the amino acid moiety is also thought to play an important role in shifting the equilibrium toward the induced state [30].

As stated above, the inability of the eEFSec ternary complex to decode the UGA codon in the absence of SBP2 suggests that it does not have direct access to the ribosomal A site. Another putative function for SBP2 on the ribosome may involve conformational changes in the PTC so that binding of the Sec-tRNA^{Sec} is enhanced, thus allowing this unique aa-tRNA to accommodate and take part in peptide bond formation. Alternatively, SBP2 can modify the position of the peptidyl tRNA in the P site relative to the Sec-tRNA^{Sec} in the A site.

5.7 The Role of the Esterified Amino Acid in tRNA Selection

tRNAs vary with respect to their nucleotide sequence, posttranscriptional modifications, and the amino acid they are esterified with. Each amino acid contains a particular functional group that imparts unique chemical properties to their cognate tRNA. Despite this molecular diversity, tRNAs are able to uniformly bind the ribosome suggesting that they are functionally equivalent substrates [33], raising the question of how the ribosome may deal with the unique chemistry of the Sec residue. During initial selection, the amino acid is masked by the elongation factor, but following GTP hydrolysis the amino acid becomes exposed to the ribosomal A site as the aa-tRNA becomes accommodated in the PTC. At this moment, the amino acid could affect tRNA binding and peptide bond formation. Moreover, as the polypeptide chain is extended, the amino acids gradually move through the nascent peptide exit tunnel. Molecular dynamic simulations of the ribosomal exit tunnel using different amino acid side chains reveal binding crevices and suggest that the tunnel is capable of interacting differently with various amino acids [34]. Indeed, a specific peptide motif in the secretory monitor protein, SecM, has been shown to stall the ribosome through interactions with rRNA residues in the exit tunnel [35].

Early studies comparing the binding affinities of deacylated and aa-tRNAs in the absence of elongation factor suggested that the amino acid was an important contributor in achieving uniform binding to the ribosome [36]. While certain tRNAs such as Gly-tRNA^{Gly} bound equally well whether it was amino-acylated or deacylated, other tRNAs varied by as much as two orders of magnitude. When in vitro transcribed tRNAs lacking their posttranscriptional modifications were compared to their native counterparts, they displayed substantially reduced binding to both the A and P sites of the ribosome. In addition, elements within the tRNA body were recently identified as being important in tuning the tRNA [37]. These findings suggest that the various tRNA molecules have evolved with unique features that function in concert to achieve uniformity of binding.

Given the importance of codon recognition in tRNA selection by accelerating the rate-limiting steps (GTPase activation and accommodation) through induced fit mechanisms, it has been informative to assay whether misacylated tRNAs affect GTP hydrolysis and the end point of peptide bond formation. Effraim et al. [38] used smFRET to follow the dynamics of misacylated tRNAs in real time through the various stages of the tRNA selection pathway. In addition, they measured dipeptide formation in the presence or absence of competitor tRNAs. Using a recently engineered tRNA aminoacylation ribozyme capable of accepting various aminoacyl and tRNA substrates, they engineered six tRNAs by mixing tRNA^{Phe}, tRNA^{Ala}, and tRNA^{Lys} (i.e., Ala-tRNA^{Phe}, Lys-tRNA^{Phe}, etc.). Surprisingly, misacylated tRNA resulted in dipeptide yields similar to that observed with the correctly acylated native tRNA substrates. However, when assayed under more stringent conditions (in the presence of competitor native tRNA substrate), misacylated tRNAs exhibited a 2–4 fold decrease in dipeptide formation, clearly demonstrating that misacylated tRNAs are indeed selected by the ribosome at lower efficiencies. smFRET studies showed

that the rate of codon recognition/GTP hydrolysis and accommodation/peptide bond formation for the misacylated tRNA was unaffected. Instead, the decrease observed in the competition assay was attributed to an increase in A site sampling events prior to codon recognition. This result is surprising because during A site sampling, the amino acid moiety is buried in a pocket on the elongation factor.

Future studies using a wider range of misacylated and native tRNA substrates will no doubt provide a greater understanding of the role that the amino acid plays during the tRNA selection process. This is a key area of research for the selenocysteine field as it seems highly likely that special accommodation of Sec is required for efficient and processive incorporation of this highly reactive amino acid. This will, of course, require the development of a completely reconstituted Sec incorporation system as described in Chap. 3.

5.8 Pretranslocation State Ribosomes Recruit EF-G

Following tRNA delivery and peptide bond formation, the ribosome undergoes a conformational transition from the posttranslocation state (POST) to the pretranslocation state (PRE). POST state ribosomes contain peptidyl tRNA in the P site and an empty A site, while PRE state ribosomes are characterized by occupation of the A site with peptidyl-tRNA and deacylated tRNA in the P site. This transition represents the beginning of the third major catalytic event that occurs during the translation elongation cycle – translocation of the tRNA–mRNA complex (3). Although this step may seem downstream of the Sec incorporation event, in fact the event does not end until the uniquely large $tRNA^{Sec}$ is released at the E site. Indeed, reduced rates of translocation during Sec incorporation may explain the observation that selenoprotein mRNAs are associated with lighter polysomes than control mRNAs of the same length [39, 40]. Translocation results in movement of the peptidyl tRNA from the A site to the P site, and the simultaneous movement of the deacylated tRNA from the P site to the E site. This movement of the tRNAs pulls the mRNA in the 5′ direction so that the next codon is positioned in the ribosomal A site thus allowing the cycle to repeat until a termination codon is reached. Translocation is catalyzed by a second translation elongation factor, the GTP-dependent ribosomal translocase, EF-G.

Both EF-G and EF-Tu bind to the elongation factor binding site composed of the SRL on H95, and the GAC on H43–44. A fundamental mechanistic question is how the ribosome distinguishes between these two elongation factors such that they do not interfere with each other during translation? Several structural and biochemical studies have provided insight on key differences between the PRE and POST translocation states of the ribosome that may allow for the sequential recruitment of these factors at the appropriate time.

One major difference between PRE and POST state ribosomes can be seen when comparing the cryo-EM structures of ribosomes trapped in these two functional states (reviewed in [41]). While the SRL appears to be relatively immobile, the GAC switches from an open to a closed conformation. In the PRE state, the GAC is

positioned toward Helix 89 (H89) near the SRL (closed), while in the POST state it shifts away from H89 (open). Consistent with these observations, the insertion of an additional base pair in the stem of H42 below the GAC predicted to constitutively mimic the POST state, reduced the binding, GTPase activity, and translocation activity of EF-G in vitro, whereas EF-Tu binding and function were unaffected [41]. Thus, the conformation of the GAC seems to be an important regulator of this selective binding event.

Yet another piece of the puzzle was uncovered by Zavialov and Ehrenberg when they demonstrated that EF-G binding and activity was controlled by the status of the tRNA in the P site [42]. PRE state ribosomes contain deacylated tRNA in the P site, while POST state ribosomes contain peptidyl-tRNA in the P site. Interestingly, treatment of POST state ribosomes with puromycin was sufficient to restore EF-G binding and ribosome-dependent GTPase activity. Puromycin is an aa-tRNA mimetic and thus functions as a substrate in peptide bond formation. However, unlike the situation in vivo where the peptide is transferred to the aa-tRNA and remains in the A site, when the peptide is transferred to puromycin it dissociates from the ribosome leaving a deacylated tRNA in the P site and an empty A site.

Cryo-EM analysis of these puromycin-treated POST state ribosomes showed that not only were these ribosomes competent for EF-G binding, but they also exhibited the same conformational flexibility exhibited by PRE state ribosomes [43]. Thus, the presence of peptidyl-tRNA in the P site locks the ribosome such that it is conformationally constrained. The mechanism that leads to this locked state at present remains unknown, but the enhanced stability of the POST state ribosome may be required to facilitate delivery of the aa-tRNA by EF-Tu. Despite the nonphysiological nature of this puromycin-treated POST state ribosome, these results imply that the removal of the peptide from the P site tRNA during peptide bond formation unlocks the ribosome, and this unlocking is an apparent prerequisite for stable EF-G binding and function. Thus the key question for Sec incorporation is whether the heretofore unexplored interplay between eEFSec and eEF2 is sufficient to promote the PRE/POST transition or does this require the function of an additional factor.

5.9 Intermediate States During Translocation

Removal of the peptide from the P site tRNA during peptidyl transfer is required to unlock the ribosome into a flexible conformation that confers EF-G binding. Early evidence for a conformational change on the ribosome following peptidyl transfer was reported by the Noller group [32]. Chemical probing of PRE state ribosomes in the absence of EF-G indicated that deacylated tRNA in the P site, and peptidyl-tRNA in the A site could spontaneously sample hybrid states. In the classical configuration, the tRNA remains completely bound to the P site (P/P site) or the A site (A/A site). In the hybrid state, the ASL of the P and A site tRNAs remains bound to the small subunit, while the CCA-ends shift and interact with the adjacent E and P sites, respectively. Single molecule studies using fluorescence-labeled tRNA molecules added to surface immobilized ribosomes support the idea that the tRNA molecules

are dynamic and fluctuate between a classical and a hybrid state [44]. Kinetic studies using mutant tRNAs and mutant rRNAs shown to destabilize the classic state, and therefore favor hybrid state formation, result in increased rates of EF-G stimulated translocation indicating that hybrid state formation is functionally relevant [45].

Cryo-EM analysis of PRE state ribosomal complexes using near physiological concentrations of magnesium (3.5 mM) suggests that PRE state ribosomes populate two macro states in the absence of EF-G: macro state I (MSI) and macro state II (MSII) [46]. In addition to the transition from the classical (A/A and P/P) to the hybrid (A/P, P/E) tRNA-binding states, MSII is also characterized by a counter-clockwise rotation (ratcheting) of the small subunit relative to the large subunit when visualized from the solvent side, and a conformational rearrangement of the dynamic L1 stalk.

The L1 stalk is located ~100Å from the PTC in *E. coli* ribosomes, and is composed of ribosomal protein L1 and its rRNA-binding site formed by H76–78 on the large subunit. Ribosomes devoid of L1 become trapped in the classic tRNA-binding state and exhibit a reduced rate of protein synthesis [47, 48]. During the MSI to MSII transition, the L1 stalk exchanges between an open position extended away from the subunit interface, to a closed position where it folds inward toward the E site and makes contacts with the deacylated tRNA in the hybrid P/E state. Following translocation, the L1 stalk maintains its contacts with the deacylated tRNA in the E/E site in what has been described as a half-open conformation [49, 50]. These results implicate the L1 stalk in the translocation mechanism and additionally in the removal of deacylated tRNA from the E site. The modulation of the L1 stalk induced upon peptide bond formation or unlocking of the ribosome exemplifies the capacity of the ribosome to communicate across large distances using allosteric networks. This fact is even more remarkable when considering that EF-G, which binds to the factor-binding site at the base of the L12 stalk nearly 170 Å away, can allosterically regulate the L1 stalk. In POST state ribosomes, the L1 stalk is in an open conformation making it accessible to *trans*-factors that can bind and potentially modulate the translation elongation cycle [51]. This example of allostery is an attractive model for the potential function of SBP2 on the ribosome. Although its binding site has not been determined, it is likely not to be involved in stable interactions at the factor-binding site or GAC as this would interfere with canonical translation. Thus in a fashion similar to the communication between the L1 stalk and EF-G, it is possible that SBP2 signals to the functional centers of the ribosome from a distant-binding site.

5.10 Conclusion

The molecular mechanisms that drive the Sec incorporation reaction remain undetermined. In order to fully understand how a unique set of Sec-specific factors are able to modify something as complex and efficient as the molecular machine responsible for protein synthesis, future experiments should be designed in the context of the tremendous body of work that has deciphered the molecular events leading to peptide bond formation during the elongation phase of translation.

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Chapter 6
Chemical Basis for the Use of Selenocysteine

Erik L. Ruggles, Gregg W. Snider, and Robert J. Hondal

Abstract Since the discovery of selenocysteine as the 21st amino acid in the genetic code, two streams of thought have dominated the question of why selenium is used to replace sulfur in enzyme active sites in the form of selenocysteine. These ideas are that selenocysteine is (i) a "relic of the anaerobic world" and (ii) "catalytically superior" to the use of sulfur as cysteine. This latter idea is due to the experimental finding that the replacement of selenocysteine with cysteine in enzyme active sites results in a large drop in catalytic activity, and has been interpreted to mean that selenocysteine is essential for catalyzing the formation of product from substrate. We and others have previously proposed that selenocysteine is not catalytically essential since cysteine homologs of selenocysteine enzymes exist and catalyze their enzymatic reactions with comparable efficiency. Here, and elsewhere, we discuss the idea that the use of selenocysteine confers an enzyme with the ability to resist irreversible inactivation by oxidation.

6.1 Introduction

Selenocysteine (Sec, U) is distinct from the other 20 common proteinogenic amino acids due to the complexity of its insertion into the polypeptide chain, which involves recoding of a stop codon as a sense codon, the use of a *cis*-acting factor in the mRNA, and multiple protein accessory factors [1]. The elaborate nature of the recoding process likely indicates that Se has a unique chemical function that the S-atom of cysteine (Cys) cannot fulfill.

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What is the special chemical function of Se that explains its use in enzymes? A popular idea is that Sec is "catalytically superior" to the use of Cys in enzymes and is necessary for the conversion of substrate to product. This idea likely originated from the fact that mutation of the active-site Sec residue in enzymes to Cys results in large drop in the catalytic rate constant (k_{cat}) [2, 3]. Another early experiment that lent support for this idea was the substitution of Sec into a naturally occurring Cys-enzyme. In the case of a Cys-containing phospholipid hydroperoxidase, this Sec for Cys substitution resulted in an enzyme with higher catalytic activity than the wild type enzyme [4]. However, it was later shown by Stadtman that the Sec-containing selenophosphate synthetase from *H. influenzae* did not have higher catalytic activity than the Cys-containing ortholog from *E. coli* [5]. This result led Stadtman to suggest "…a role of selenocysteine in *H. influenzae* that is not catalytic." This latter idea has not been widely championed in the field.

A specific catalytic role for Se in enzymes has been difficult to discern because most of the physico-chemical properties of Se and S are quite similar as has been noted in a recent review [6]. Many researchers have focused on the differences in nucleophilicity and acidity between Se and S as rationales for the use of Sec in enzymes [7]. As we have pointed out in a recent review article, when Se and S have equal ionization states, the ratio of Se nucleophilicity to S nucleophilicity is modest, in the range of 5–10 [8]. There are also multiple examples of Cys residues in enzymes with pK_a values lower than 5 [8]. Despite these high similarities between Se and S, my research group (and others) has tried to determine a specific catalytic role for Se in the enzyme thioredoxin reductase (TR). We initially focused on what we have termed as a "niche rationale" for the use of Se in TR by explaining its role in the catalytic cycle as a superior leaving group (due to a lower pK_a) and stabilizing a certain conformer of the enzyme due to longer C–Se and Se–S bond lengths [9]. However, as more experimental evidence accumulated, we began to question this model for the role of Se in TR (and other enzymes) and started to focus on a role for Se that is not catalytically essential as originally suggested by Stadtman as well as others [10].

While there is no question that Se plays an important mechano-chemical function in the enzymes where it occurs (we refer to the chemical property of Se that supports conversion of substrate to product as its mechano-enzymatic function), we, and others, argue that S can compensate for the absence of Se in enzyme active sites due to chemical tuning of the active-site microenvironment. We hypothesized that the mechano-enzymatic function of Se should be related to its non-catalytic, biological function in enzymes [8]. Review of our mechanistic experiments led us to posit that the electrophilicity of Se was the mechano-enzymatic function that allowed TR to convert substrate to product. At the same time, electrophilicity of the Se atom also allows it (and the enzyme) to resist irreversible inactivation by oxidation as discussed below.

One possible way in which a selenoenzyme can resist irreversible inactivation by oxidation is shown in Fig. 6.1. Both Cys- and Sec-enzymes require a reduced thiol or selenol in their respective active-sites to be in the active, functional state. Upon exposure to oxidant (such as H_2O_2), both enzymes can be oxidized to inactive forms as either the sulfenic (Enz-SOH) or selenenic acid (Enz-SeOH) oxidation states. Both the Enz-SOH and Enz-SeOH forms can be reduced back to the active state

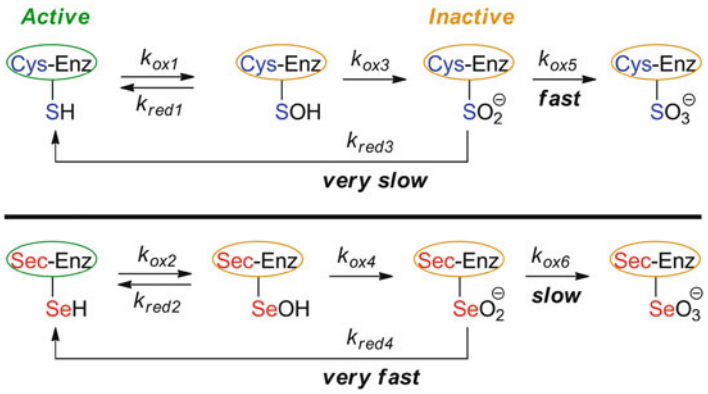


Fig. 6.1 Cys- and Sec-enzymes require a reduced thiol or selenol, respectively, to be in the active, functional state. Each enzyme can be oxidized by H_2O_2 to inactive Enz-SOH and Enz-SeOH forms, respectively, with $k_{ox2} > k_{ox1}$. Reduction by thiol restores both inactive forms back to the active state with $k_{red2} > k_{red1}$. Addition of a second equivalent of H_2O_2 to Enz-SOH and Enz-SeOH oxidation states leads to formation of Enz-SO_2^- and Enz-SeO_2^-, respectively. Presumably $k_{ox4} > k_{ox3}$, though this has not been experimentally determined. However, $k_{red4} >> k_{red3}$, with the sulfinic acid being extremely resistant to reduction. The Enz-SeO_2^- form resists further oxidation to Enz-SeO_3^-, while Enz-SO_2^- is oxidized to Enz-SO_3^- relatively easily. In this case, $k_{ox6} << k_{ox5}$

by the addition of exogenous thiol. A key chemical difference between Cys- and Sec-enzymes is revealed when the two enzymes are oxidized to the sulfinic acid (Enz-SO_2^-) and seleninic acid (Enz-SeO_2^-) forms. The sulfinic acid form of a Cys-enzyme cannot be chemically reduced back to Enz-SH by the addition of thiols such as glutathione because the S-atom of Enz-SO_2^- is not very electrophilic. In contrast, the Enz-SeO_2^- form of a selenoenzyme can be readily reduced back to Enz-SeH, as has been shown by the work of Hilvert and coworkers through their study of seleno-subtilisin [11]. The reason for the much faster reduction of $RSeO_2^-$ compared to RSO_2^- is because Se is much more electrophilic than S [12, 13], and it is this superior ability of Se to accept electrons relative to S that can explain both its mechano-enzymatic function and its chemico-biological (non-catalytic) function in enzymes.

A second chemical difference between Se and S that can help a selenoenzyme resist irreversible inactivation by oxidation is the fact "that while S(VI) is a stable oxidation state for sulfur relative to S(IV) exactly the reverse is true for Se(VI) vs. Se(IV)" [14]. As shown in Fig. 6.1, this means that it is *more difficult* to further oxidize the Enz-SeO_2^- form of a Sec-enzyme to the selenonic acid form (Enz-SeO_3^-) than is the same oxidation of the Enz-SO_2^- form of a Cys-enzyme to the sulfonic acid form (Enz-SO_3^-). The reason for the slower oxidation of Se(IV) to Se(VI) is also related to the electropositive character of Se. The lone pair of electrons on $RSeO_2^-$ is not readily available for bonding, because they are strongly attracted to the positive Se nucleus. In contrast, the lone pair of electrons on RSO_2^- is readily available for nucleophilic attack onto electrophiles as shown by the reaction of *p*-toluensulfinic acid with benzeneseleninic acid [14]. In this reaction, the Se atom acts as the electron acceptor and S acts as the electron donor, the opposite of what is commonly thought about the nucleophilic character of Se.

6.2 Chemical Models to Study the Oxidation States of S and Se

We wished to quantify the rates of oxidation and reduction of Enz-SO_2^- and Enz-SeO_2^- in enzyme active sites. In order to simplify the problem, we chose small molecule S- and Se-model compounds to determine these rates. These compounds are benzenesulfinic acid ($PhSO_2^-$) and benzeneseleninic acid ($PhSeO_2^-$), respectively. A simple qualitative difference between the two compounds was immediately obvious to us upon addition of excess β-mercaptoethanol (βME) to both compounds. In the case of $PhSeO_2^-$, an immediate yellow precipitate formed upon the addition of βME (Fig. 6.2). We determined by mass spectrometry that this yellow precipitate was PhSe–SePh. In contrast, there was no evidence of reaction upon addition of βME to $PhSO_2^-$.

These reactions were more carefully followed using ^{1}H-NMR. The reduction of both the sulfinic and seleninic acids were carried out under an Ar atmosphere in K_2HPO_4/KH_2PO_4 buffered D_2O (Ar sparged) that was 50 mM in substrate. After obtaining an initial ^{1}H-NMR spectrum, βME was added (1.0–5.0 Eq). The observation of precipitate in the case of the seleninic acid substrate was also apparent in the ^{1}H-NMR spectrum due to the lack of aromatic signals (Fig. 6.2c). In order to slow the rate of reduction, the reaction was carried out in deuterated methanol at −65°C (data not shown). However, even at −65°C the reduction of the seleninic acid to the selenosulfide was too fast to be observed by ^{1}H-NMR. In contrast, the reduction of the sulfinic acid with βME was so slow that no reaction was observed after 2 weeks at room temperature (Fig. 6.2f). In order to increase the rate of the reduction of $PhSO_2^-$, the reaction was carried out in deuterated methanol at 85°C (data not shown). Even at this elevated temperature, the reduction of sulfinic acid was not observed even after 2 weeks.

The *oxidation* of both the sulfinic and seleninic acids to their respective sulfonic and selenonic forms were carried out under an Ar atmosphere in K_2HPO_4/KH_2PO_4 buffered D_2O (Ar sparged) that was 50 mM in substrate. After obtaining an initial ^{1}H-NMR spectrum, H_2O_2 was added (1.0 Eq for $PhSO_2^-$, while 10.0 Eq for $PhSeO_2^-$). The oxidation was monitored by ^{1}H-NMR at specific time points depending on the rate of substrate oxidation (minutes for $PhSO_2^-$ substrate, while days for $PhSeO_2^-$ substrate). In both cases the rate of substrate oxidation was determined as $\text{rate} = k[\text{substrate}]^1[H_2O_2]^1$. For the $PhSO_2^-$ substrate, the second order rate constant was determined by a plot of $[PhSO_2^-]^{-1}$ vs. time (s). Since the concentration of both $PhSO_2^-$ substrate and H_2O_2 are the same, the slope of the line obtained is the second order rate constant (k). For the $PhSeO_2^-$ substrate, the second order rate constant was determined using pseudo-first order kinetics due to the excess H_2O_2 required to promote oxidation. A plot of $\ln[PhSeO_2^-]$ vs. time (s) provided the pseudo-first order constant (k'). Since $k' = k[H_2O_2]_0$, the second order rate constant (k) could then be calculated. The ^{1}H-NMR spectra of the oxidation of $PhSeO_2^-$ and $PhSO_2^-$ by H_2O_2 monitored over time are shown in Fig. 6.3 (top and bottom, respectively).

As can be seen in the time courses, the oxidation of $PhSeO_2^-$ is very slow (even with 10 Eq of H_2O_2) and the oxidation of $PhSO_2^-$ is relatively fast, especially at acidic pH. While the reduction of each compound proved either to be too fast

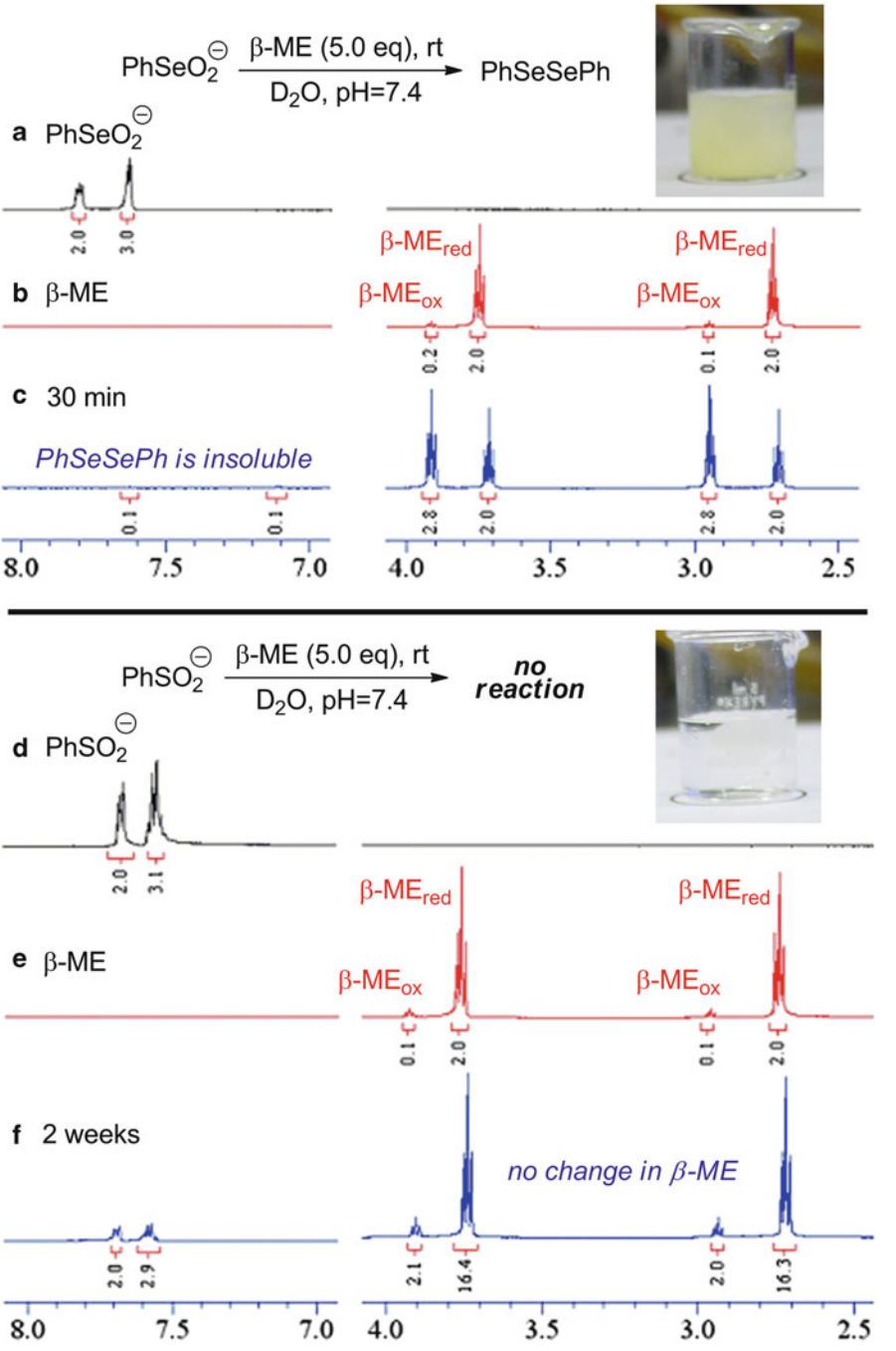


Fig. 6.2 The reduction of $PhSeO_2^-$ (**a–c**) and $PhSO_2^-$ (**d–f**) by βME monitored by 500 MHz ^{1}H-NMR. Upon addition of βME, PhSe-SePh is produced directly as a *yellow* precipitate (beaker in *upper right corner* of figure). This is not only visibly apparent in the reaction flask, but also in the ^{1}H-NMR spectra (**c**) due to the lack of aromatic signals. The reduction of $PhSO_2^-$ differs significantly as shown by the absence of reaction after 2 weeks (**f**). Compare the two reductions qualitatively by examining the reaction flasks

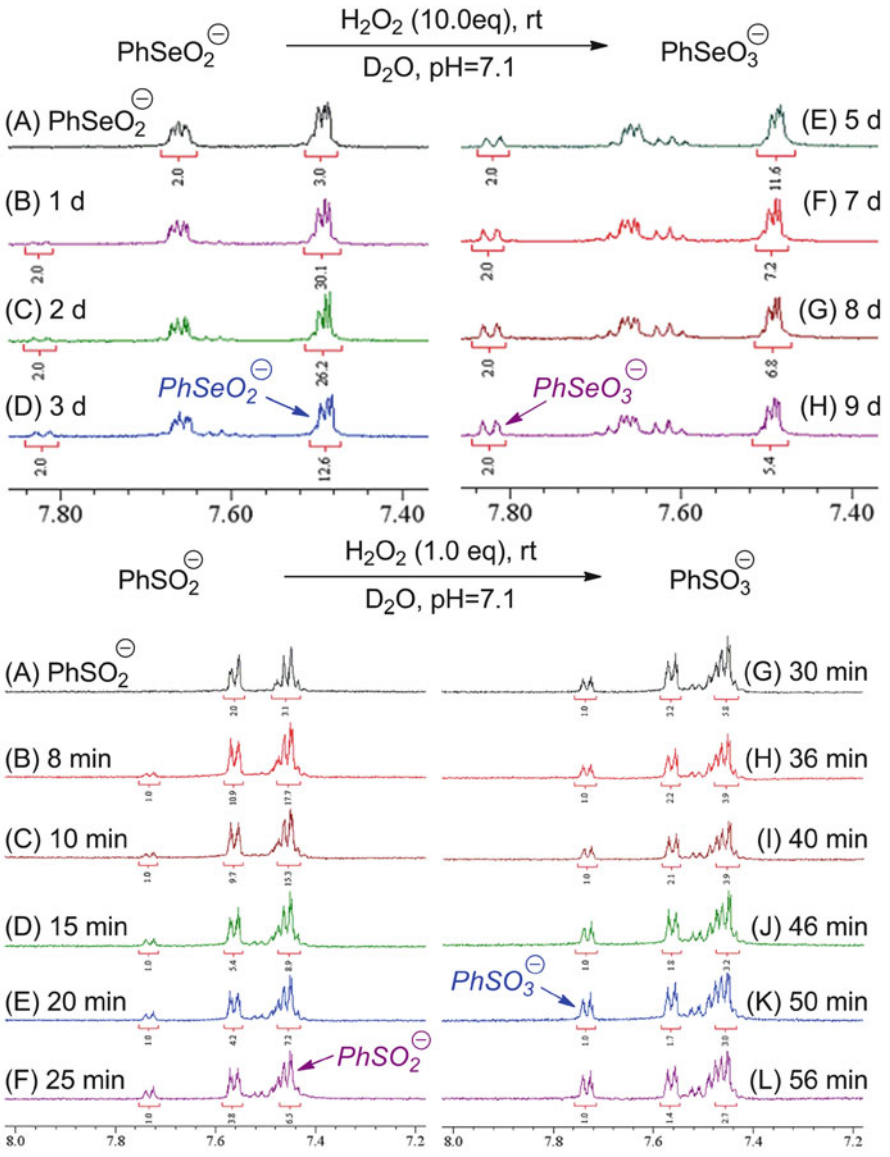
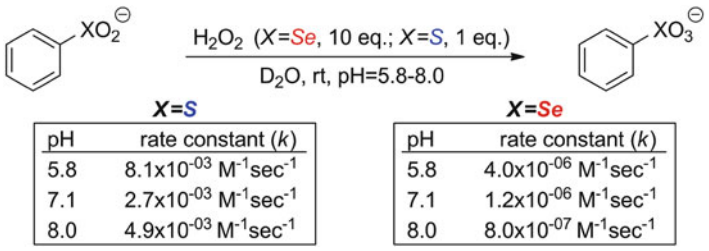


Fig. 6.3 The oxidation of $PhSeO_2^-$ (*top*) and $PhSO_2^-$ (*bottom*) by H_2O_2, monitored by a 500 MHz ^{1}H-NMR. For the oxidation of $PhSeO_2^-$, it takes a 10 Eq excess of H_2O_2 over 9 days to reach an approximately 50:50 mixture of $PhSeO_2^-$ to $PhSeO_3^-$. In contrast, the oxidation of the sulfur analogue requires only 1 Eq of H_2O_2 and 1 h reaction time to reach an approximately 50:50 mixture of $PhSO_2^-$ to $PhSO_3^-$



Scheme 6.1 Summary of experimentally determined rate constants at various pH values for the oxidation of $PhSeO_2^-$ and $PhSO_2^-$ to $PhSeO_3^-$ and $PhSO_3^-$, respectively. The oxidation of the S-compound was much faster than the Se-compound at all pH values, with the difference at acidic pH being the largest

($PhSeO_2^-$) or too slow ($PhSO_2^-$) to determine a rate constant using ^{1}H-NMR, the oxidations were on a time scale that allowed for determining oxidation rate constants and this data is summarized in Scheme 6.1. The data shows that the oxidation of $PhSO_2^-$ is ~2,200-fold faster than $PhSeO_2^-$ at pH 7.1 and ~2,000-fold faster at pH 5.8. We note that while we were not able to determine a rate constant for the reduction of $PhSeO_2^-$, Hilvert and coworkers were able to measure an observed rate constant for the reduction of a model seleninic acid compound ($RSeO_2^-$) using stopped-flow techniques. This rate constant was determined to be 3.3×10^6 M^{-1} min^{-1} [11]. Moreover, they showed that the pH optimum of this reduction was between 4 and 5. This increased rate of reduction at acidic pH parallels our own experiments using methaneseleninic acid as a substrate for a truncated TR missing the C-terminal Sec residue [15]. Thus, the data clearly shows very large differences in the chemistries of the oxides of Se and S; seleninic acid is reduced exceptionally fast by a thiol (especially at acidic pH) and sulfinic acid is reduced exceedingly slow. Based on our data, we estimate that the ratio of the rates of reduction of $PhSeO_2^-$ to $PhSO_2^-$ is $\geq 10^6$ (k_{red4}/k_{red3} in Fig. 6.1). Conversely, the oxidation of seleninic acid is relatively slow compared to the same oxidation of sulfinic acid (especially at acidic pH), and this is the basis for our assertions outlined in Fig. 6.1.

6.3 Sec-Containing Thioredoxin Reductase Resists Inactivation by Oxidation

We recently set out to test our hypothesis that Sec-enzymes resist irreversible inactivation by oxidation [15]. The results showed that mouse mitochondrial Sec-TR resisted inactivation from exposure to 50 mM H_2O_2 as shown in Fig. 6.4a. Our hypothesis predicts that a Sec-enzyme will be more resistant to inactivation by oxidation than a Cys-enzyme as outlined in Fig. 6.1. To test this prediction we tested

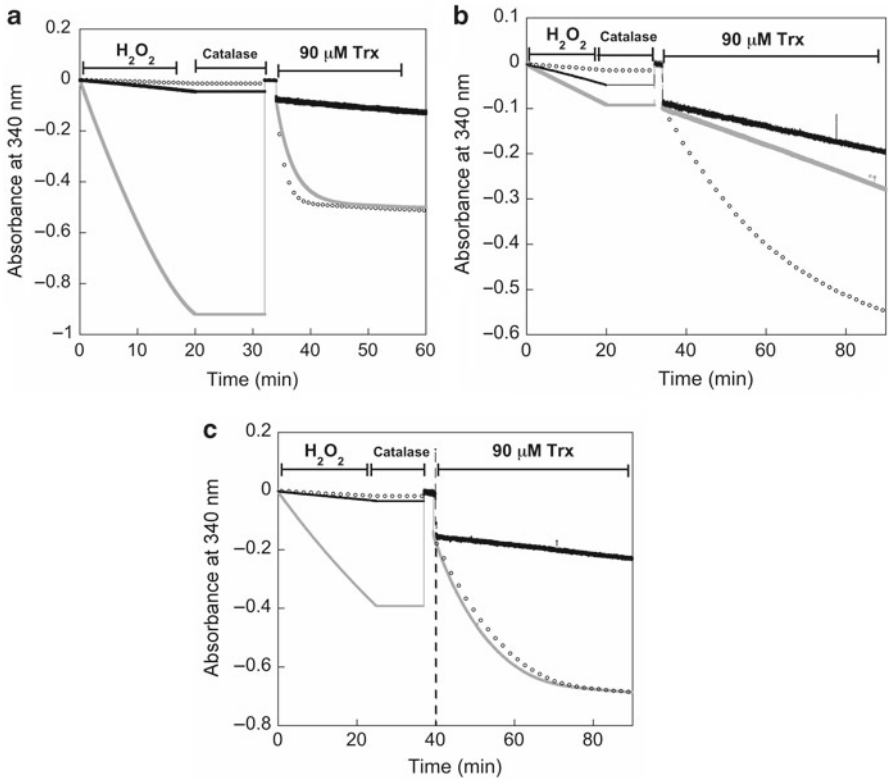


Fig. 6.4 Resistance of SecTR to inactivation by H_2O_2. (**a**) Exposure of mammalian SecTR-GCUG to 50 mM H_2O_2. For this experiment, the enzyme is incubated with (*grey line*) and without (*open circles*) 50 mM H_2O_2. The *black line* is the nonenzymatic control (no enzyme). The reaction progress is monitored by measuring the consumption of NADPH at 340 nm. After 20 min of incubation with 50 mM H_2O_2, catalase is added to consume the remaining H_2O_2 (12 min), after which 90 μM *E. coli* Trx is added to the reaction assay to assess if the Trx-reductase activity of the enzyme is affected [15]. The reaction progress curves of both peroxide treated and untreated are essentially the same. The same experiment (**b**) is repeated for wild type DmTR-SCCS (Cys-DmTR). The results show that the Trx-reductase activity of Cys-DmTR is greatly affected by exposure to 50 mM H_2O_2 (compare *open circles* to *grey line*). (**c**) When Sec is substituted for Cys in the DmTR-SCUG mutant (Sec "rescue"-TR), the enzyme becomes resistant to inactivation by peroxide (compare *open circles* to *grey line*)

the ability of a Cys-ortholog of mammalian TR to resist inactivation by exposure to H_2O_2. This ortholog is the Cys-containing TR from *D. melanogaster* (DmTR), which contains a C-terminal SC_1C_2S redox motif instead of the GC_1U_2G redox motif found in mammalian TR. The results of this experiment are shown in Fig. 6.4b [16], they show that the Cys-TR is significantly inactivated by exposure to 50 mM H_2O_2. Our hypothesis implies that replacement of the Cys residue in DmTR with a Sec residue should reverse this inactivation. We then constructed a mutant of DmTR in

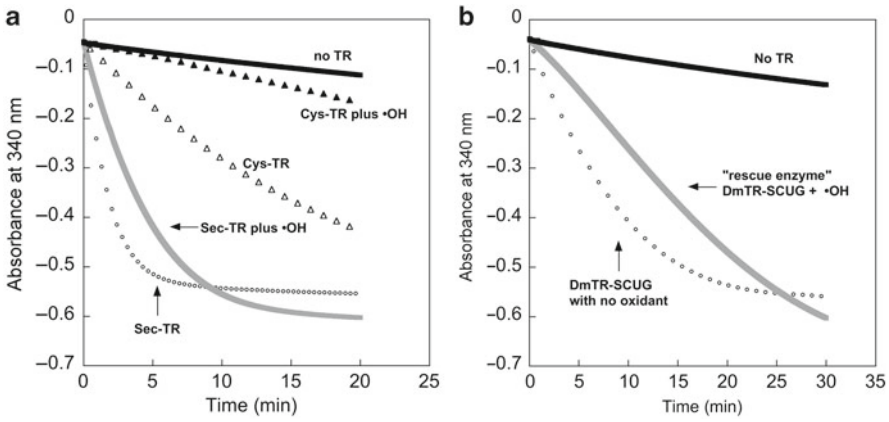


Fig. 6.5 Resistance of SecTR to inactivation by hydroxyl radical. (**a**) Exposure of mammalian SecTR-GCUG to •OH generated by Fe•EDTA/H_2O_2 in situ. Here, the enzyme is incubated with (*grey line*) and without (*open circles*) •OH followed by a quenching step. Trx is then added to the reaction mixture and activity is measured by loss of absorbance at 340 nm. The *black line* is the nonenzymatic control (no enzyme). While mammalian Sec-TR resists inactivation by •OH the Cys-ortholog (DmTR-SCCS) is largely inactivated as can be seen by comparing activity of the enzyme without •OH (*open triangles*) to the enzyme activity after •OH treatment (*closed triangles*). The presence of Se in the Sec "rescue"-TR (the DmTR-SCUG mutant) confers resistance to oxidation by •OH as shown by the plot depicted in (**b**). Compare DmTR-SCUG with •OH treatment (*grey line*) with DmTR-SCCS without •OH treatment (*open circles*)

which we replaced the active-site Cys_2 residue (the Cys residue in the second position of the dyad) with a Sec residue using protein semisynthesis [17]. Thus we have a pair of enzymes termed Cys-DmTR (with C-terminal sequence of SC_1C_2S) and Sec-DmTR (or also called Sec "rescue"-TR with C-terminal sequence of SC_1U_2G) that differ in sequence by only a single atom from ~6,000 atoms in each subunit. We then tested the ability of Sec-DmTR to resist inactivation by 50 mM H_2O_2. The results are shown in Fig. 6.4c. As can be seen from the data, the substitution of Sec for Cys confers the mutant enzyme with the ability to resist inactivation by oxidation from H_2O_2 as our hypothesis predicts.

Concomitant with the publication of our hypothesis that Sec-enzymes would resist irreversible inactivation by oxidation, Koppenol and coworkers also predicted that Sec-enzymes would resist inactivation by oxidation, with a specific prediction that Sec-enzymes would resist one-electron oxidations due to the high stability of a selanyl radical (RSe•) relative to a thiyl radical (RS•) ([18] and see also [19]). Indeed, they showed that the selanyl radical was more stable than the thiyl radical by a factor of 10^{10}. We tested this specific hypothesis with our Cys-DmTR and Sec-DmTR system mentioned above using the hydroxyl radical (•OH) as the one-electron oxidant. The hydroxyl radical was generated using Fenton chemistry with Fe•EDTA/H_2O_2 as the source of the radical. As shown in Fig. 6.5a, Cys-DmTR was largely

inactivated by •OH while the Sec-mitochondrial TR greatly resisted this inactivation. Similar to our results with H_2O_2, the Sec-"rescue" enzyme (DmTR-SCUG) gained the ability to resist inactivation by •OH as shown in Fig. 6.5b. The experimental results exactly match the prediction by Koppenol and coworkers.

The exact sequence of chemical events is not known that allows the Sec-TR to survive a one-electron oxidation reaction. Presumably, Se can donate a hydrogen atom (or an electron from the selenolate) to •OH allowing for the formation of H_2O. The resulting Enz-Se• radical could then react with a second molecule of •OH, forming Enz-SeOH. This selenenic acid form of the enzyme can then either be reduced back to Enz-SeH or further oxidized to Enz-SeO_2^-. In the case of TR, if the seleninic acid form is produced, it will quickly be reduced back to the selenol [15] and this seleninic form will strongly resist further oxidation as our experiments have shown. This property of Se allows the enzyme to survive catastrophic degradation of the peptide backbone [18]. In contrast, if S replaced Se, the formed thiyl radical would react by abstracting a H• radical from the peptide backbone, initiating a radical catalyzed degradation cascade of the peptide backbone with concomitant loss in enzyme activity [18]. One-electron oxidations of S can also result in the formation of RSO_2^- [20], or Enz-SO_2^-, which would irreversibly inactivate the enzyme.

6.4 Conclusions

As discussed here and elsewhere, we have hypothesized that the use of Sec in enzymes is due to factors other than for supporting efficient catalysis, e.g., that Sec is catalytically necessary to convert substrate to product. Previously [15], and as presented here, the data demonstrates that the presence of a Sec-residue in TR imbues the enzyme with the ability to resist irreversible inactivation by oxidation. This idea was originally proposed by Rocher and coworkers, though expressed in a different way [21]. Rocher proposed that the use of Se in glutathione peroxidase was to prevent "significant self-inactivation" due to reaction with hydroperoxides. Tolerance toward oxygen induced inactivation has also been proposed as a rationale for the use of Se in place of S in the NiFeSe cluster of a bacterial hydrogenase [22]. Resistance to irreversible oxidation comes in two forms: (i) the ability of Se-oxides to be recycled back to the parent selenol as shown in Fig. 6.1, and (ii) the ability of Se to resist inactivation by one-electron oxidation by one-electron oxidants. The former property would be advantageous in the "aerobic world," while the latter property would be advantageous in enzymes that might be exposed to one-electron oxidation events. One-electron oxidation events do not depend on the presence of oxygen and this could possibly explain the initial appearance of Sec in the "anaerobic world." Finally, we note that our hypothesis is not yet definitively accepted in the field and that multiple rationales may exist for the use of selenium in enzymes.

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Chapter 7
Evolutionary Basis for the Use of Selenocysteine

Louise White and Sergi Castellano

Abstract Evolutionary adaptations to dietary selenium may explain the use of selenocysteine in proteins. If so, adaptive signals should be present in the genomic regions of selenoprotein genes. It is, however, difficult to identify the signatures of adaptation left by natural selection in the genome of extant species (including humans). Furthermore, dietary adaptations to selenium may have happened in some species but not in others. For example, while dietary selenium does not seem to be a major selective force behind the evolution of selenocysteine use in vertebrate proteins, it may be an important factor in other lineages. Whether levels of selenium in the diet have driven the evolution of other functionally important amino acid changes in selenoproteins is not known. Dietary selenium may have also shaped the regulation of selenoprotein genes and of genes involved in the metabolism of selenium. Evolutionary genetics methods aimed at detecting signals of natural selection at the regulatory level are key to answering this question. Understanding the genetic basis of adaptations to levels of selenium in the diet would help shed light on the molecular mechanisms behind the metabolism of selenium.

7.1 Introduction

Selenocysteine (Sec) is a rare amino acid in present-day proteins. It is the rarity of Sec use in proteins that first suggests a unique and specialized role of selenium in protein function. Indeed, Sec residues provide unique catalytic properties to selenoenzymes (see Chap. 6 and references therein). It is then startling that the majority of selenoproteins in nature have homologs with cysteine (Cys) in place of Sec [1–4].

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This apparent puzzle about the exchangeability of Sec and Cys in protein function is amenable to evolutionary enquiry [5]. Evolutionary theory and methods to infer the exchangeability of Sec and Cys residues in proteins are examined in the first part of this chapter.

Evolutionary studies are also central to the study of other functionally important amino acid changes in selenoproteins. These changes are relevant to the role of selenium in protein activity. Amino acid changes in proteins involved in the metabolism of selenium may also result in adaptations to levels of selenium in the diet. In this regard, it is informative to first discuss the example of iron-related genes, as there is a rich body of evolutionary work on this essential trace element [6, 7]. Lessons from these studies are applicable to the evolutionary study of selenium-related genes. This is the focus of the middle sections of this chapter.

The essentiality of the trace element selenium may also play an important part in the evolution of selenium-dependent genes, as selenium levels vary greatly by geographical region [8–10]. Adaptations to major dietary shifts in humans and other animals [11] may also be important in the evolution of selenoproteins. The regulation of selenoprotein genes, in particular, may adapt to levels of selenium in the diet. Evolutionary adaptations in the regulation of genes involved in the metabolism of selenium are also possible. Understanding the genetic basis of these adaptations, and the diseases often associated with them, is a challenge in selenium biology. Detecting the genetic loci that have played a major role in dietary adaptations to selenium is the first step. Approaches for detecting signals of natural selection on selenium-related genes are discussed in the last part of this chapter.

7.2 Inferences of Natural Selection of Selenocysteine Residues

Selenocysteine is the defining feature of selenoproteins. It is thus of interest to understand the evolutionary forces shaping the use of Sec in proteins. The more so, since the strength and mode of natural selection acting on Sec sites should reflect the role of this amino acid in protein function [5]. The extent of exchangeability of Sec with Cys depends on the uniqueness of this role. Thus, a distinct contribution of Sec to protein function should be apparent in the rate and pattern of Sec/Cys exchanges between species and among populations [12].

In order to measure the action of natural selection on Sec sites in proteins, we need first to formalize the intuition above. Natural selection acts on changes with fitness consequences, that is, changes that affect the capacity of an organism to survive and reproduce. Selection acting upon deleterious mutations is known as negative (or purifying) selection. Similarly, selection acting upon advantageous mutations is known as positive selection. Variants that increase the fitness of an individual in its environment might increase rapidly in frequency as a result of positive selection. The identification of molecular changes that have been subject to positive selection provides the basis for understanding evolutionary adaptations at the molecular level. One of the main interests of evolutionary biology is to distinguish molecular variation

that is subject to natural selection, particularly positive selection, from neutral variation [13]. Neutral variants have no fitness consequences. Therefore, functionally equivalent amino acids are expected to evolve under neutrality. This is important because the neutral theory of evolution predicts the neutral rate of exchange of amino acids in proteins [14]. The extent of functional exchangeability between Sec and Cys can be learned from comparisons to this rate, as deviations from the neutral expectation are a sign of the action of natural selection.

We can use this idea to measure the exchangeability of Sec and Cys residues among selenoproteomes (the set of selenoproteins in a species). This provides an overall measure of exchangeability between Sec and Cys amino acids. Neutral patterns of selenoproteome divergence (between species) and diversity (among populations) indicate no fitness advantage or disadvantage of Sec over Cys (e.g., no distinct contribution of Sec to protein activity). Departures from neutrality are a signature of natural selection and can be interpreted as (1) selection against deleterious Sec/Cys mutations (purifying selection), which is consistent with low Sec/Cys exchangeability and denotes functional differences between Sec and Cys residues; or (2) selection favouring advantageous Sec/Cys mutations (positive selection), which can be interpreted as evidence for adaptive evolution and, in the case of selective pressures unrelated to protein function, high Sec/Cys functional exchangeability.

How do we test the nonneutral scenarios above for the evolution of Sec usage in proteins? In short, we need to compare them against a neutral scenario using a neutrality test. A neutrality test is a statistical test of a model in which all observed mutations are neutral. Under neutrality, the expected rate and pattern of variation in Sec and Cys sites can be inferred from population genetics theory or simulations of the evolutionary process (see [12] for details). This constitutes the null (undisturbed by selective forces) model of Sec usage in protein evolution. For example, neutral simulations of the evolution of the Sec residues present in the last common ancestor of all vertebrate species show that Sec/Cys exchanges, in the vertebrate phylogeny, are less common than expected under neutrality [5]. This is consistent with strong purifying selection and very low exchangeability between Sec and Cys residues in vertebrate proteins. Furthermore, Sec sites in the human genome, genotyped in a worldwide sample of human populations, show no variation [5]. The absence of polymorphism observed suggests that natural variation in these sites is rare, if at all present, in human populations. Because different human populations have different selenium nutritional histories, this result is consistent with a minor role for Se availability in shaping Sec use in human proteins.

It is important to acknowledge, however, that the neutrality tests above do not rule out that some of the identified Sec/Cys changes in the vertebrate lineage are adaptive. Similarly, adaptations to the use of Sec in human populations with extreme levels of selenium in their diets may exist. What is clear is that levels of selenium in the diet have not been a major selective force in the evolution of Sec usage throughout vertebrate evolution and recent human history. That is, selenium has not shaped the evolution of Sec in the majority of selenoproteins in vertebrate species and human populations. Whether this is the case in other lineages (including the lineage leading to vertebrates) is not known, as the evolutionary forces acting on Sec sites

could differ among lineages. In any case, the extent of constraint identified in vertebrate Sec sites suggests that functional differences are responsible for the low exchangeability between Sec and Cys residues. It is, however, possible that the higher catalytic activity usually attributed to Sec-containing enzymes only justifies a fraction of the extensive conservation in Sec and Cys sites during vertebrate evolution. Indeed, similar catalytic activity between homologous Sec- and Cys-containing enzymes, most likely due to additional compensatory substitutions in the active site of Cys-enzymes, has recently been reported [15–17]. Functional studies on present-day selenoproteins suggest that a broader range of substrates and pH in which selenoenzyme activity is possible [16], or other properties derived from the different catalytic mechanisms between Sec- and Cys-enzymes [17], may account for the constraint and the deleterious effect of Sec/Cys replacements in vertebrates. A more complex view of Sec in protein activity is emerging, and other biochemical and functional differences with fitness consequences may apply to the majority of uncharacterized selenoenzymes. The functional characterization of selenoproteins will be particularly relevant to evolutionary studies, in those lineages where a successful inference of natural positive selection can be made for a Sec/Cys substitution.

In conclusion, little evidence exists today for an adaptive role of Sec/Cys exchanges in proteins. While it is reasonable to expect the use of selenium to be adaptive in some selenoproteins, it is challenging to prove a direct role of natural positive selection in any single Sec residue. This is, however, a very exciting question in selenium biology and better data and evolutionary tests should provide an answer to this question.

7.3 Inferences of Natural Selection of Iron-Related Genes

Iron is a trace nutrient essential in humans as an enzyme cofactor in respiration, for DNA synthesis and for oxygen transport in the blood [18]. An imbalance of body iron can lead to pathological conditions. Iron deficiency is a major problem affecting 15–30% of the world's population and is prevalent in all geographical regions [7, 19]. Iron deficiency is known to be associated with central nervous dysfunction, impairment at work and in exercise, poor pregnancy outcomes, and an impaired immune response [20]. At the other extreme are iron overload disorders, which result in an excess of iron in the body (see below). It is therefore possible that excess or deficiency of dietary iron has shaped the evolution of genes involved in the metabolism of iron. Understanding the genetic basis of evolutionary adaptations to dietary iron is relevant to selenium, the deficiency or excess of which is also known to cause disease.

Heredity hemochromatosis is an iron overload disorder characterized by iron deposition in internal organs and a reduced life expectancy [21]. Heredity hemochromatosis is the most common autosomal recessive disorder in Europe [20] with most cases caused by mutations in the *HFE* gene, a regulator of iron absorption [21]. The most prevalent of these is a mutation that changes a cysteine residue at position 282 to tyrosine (C282Y), disrupting an intramolecular disulfide bridge and

leading to a nonfunctional protein [6]. While the exact mechanism of iron regulation by *HFE* is not well understood, it is clear that this mutation increases the intestinal absorption of iron from the diet [19]. In heterozygous individuals, the C282Y mutation increases iron serum and haemoglobin levels [20]. In homozygous individuals, excess iron is deposited in the liver and other organs [21].

The potential for levels of dietary iron to change in novel environments makes the *HFE* gene a possible target of local adaptation. It is reasonable to ask whether the *HFE* C282Y allele shows signatures of positive selection. The *HFE* C282Y allele is present in Europe at an overall frequency of 3.2%, with a north-south cline from 10% in Irish to 0% in Turkish populations, but is almost entirely absent from the rest of the world (where it exists this is likely the result of European genetic mixing with other populations) [22]. As a result of their higher iron absorption, a selective advantage for C282Y heterozygotes could be envisaged in environments or diets low in iron. How do we assess the role of natural selection on the *HFE* C282Y allele? Evolutionary theory tells us that under a neutral model of evolution, there is a direct relationship between the time since an allele arose and its frequency in a population. Thus an allele must be old to be frequent. If a young allele is frequent this may be taken as an indication that positive selection may have occurred. The origins of the C282Y allele have been estimated at between 1,725 and 3,150 years ago [6]. Under a neutral model, the C282Y mutation would have to have arisen about 56,900 years ago to reach the 3.2% frequency seen in Europe today (calculation after [23]). This discrepancy and the known functional effects of the allele suggest that positive selection may have played a role in shaping the distribution of *HFE* C282Y.

C282Y is not the only iron uptake enhancing mutation in the *HFE* gene. A separate mutation in the *HFE* gene that substitutes histidine for aspartate at residue 63 (H63D) can increase iron absorption [21]. This diversity of mutations producing similar phenotypes maintained within the population offers further suggestion of a selective advantage to increased iron absorption. The H63D mutation has a worldwide distribution but a significantly higher frequency in Europe than elsewhere [22, 24]. This concentration in Europe raises the possibility that there have been Europe-specific selective forces acting on *HFE* iron uptake enhancing mutations.

A second equally difficult question is: What selective pressures account for the inferences of natural positive selection in the *HFE* gene in Europeans? On the one hand, geographical variance in soil iron concentration has not been correlated with population level iron deficiency [10]. This suggests that the geology of Europe has not been a selective force acting upon *HFE* C282Y. This question, however, needs to be explored further as iron uptake and absorption is complex, both for plants from soils and for humans from food.

On the other hand, one speculative possibility is that an iron accumulation phenotype became important in Europe with the introduction of farming. Farming spread through Europe from the Middle East beginning around 9,000 years ago and led to a dietary shift from a meat rich high iron diet to a cereal based diet low in iron [25]. Additionally, domestication of animals led to the adoption of milk drinking. The high level of calcium in milk decreases iron absorption and the presence of lactoferrin further decreases iron availability [7, 26]. Interestingly, the areas of

Europe with the highest frequency of *HFE* C282Y are also the areas with the highest frequency of the *LCT* C13910T lactase persistence allele that allows the digestion of milk in adulthood [24, 27].

The inference of selection on the *HFE* gene is a good example of the role of population genetics in studying the genetic basis of recent dietary adaptations. It is also a good example of the uncertainties in identifying the selective factors behind natural positive selection. Whether the geography of selenium, dietary shifts, or both factors have played a role in the evolution of selenium-related genes is also unknown. If this is the case, amino acid changes in proteins involved in selenium metabolism may explain some of the adaptations to levels of selenium in the diet observed today.

7.4 Inferences of Natural Selection of Selenoprotein Genes

Very few statistical inferences of natural selection have identified selenocysteine genes as likely targets of positive selection. Indeed, only glutathione peroxidase 1 (GPx1) has been shown to carry putative signatures of natural selection [28]. Sequence variation in the coding and untranslated regions (including the SECIS element) of glutathione peroxidases 1–4, thioredoxin reductase 1 and selenoprotein P was explored in 102 individuals of four major ethnic groups in the United States. The studied selenoproteins have antioxidant properties and it is therefore possible that population differences in selenoprotein activity and expression influence risk for a range of complex diseases (e.g., cancer). Disease genes should be under negative selection when the disease phenotype leads to a reduction of fitness. Classic neutrality tests were carried out and the observed pattern of genetic variation was found to be consistent with neutrality for five genes. The *GPx1* gene, however, showed signatures of a possible selective sweep in the Asian population. Further confirmation of these signatures is needed. In any case, the causal interpretation of the inferred selective sweep in the *GPx1* gene is difficult to ascertain, and whether dietary selenium is responsible for this adaptive signature is not known. The nature of the evolutionary forces acting specifically on the Sec codon in these selenoprotein genes was, however, not pursued in this study.

Interestingly, a gene involved in selenium metabolism has been recently identified as a likely target of positive selection in ancestral modern humans [29]. Comparisons of the Neanderthal genome to present-day human genomes have found the selenium binding protein 1 (*SELENBP1*) gene in a region showing signatures of selection. The exact function of this protein is unknown but there is some evidence to suggest that selenium is incorporated into SELENBP1 in a stable manner [30], that this selenium incorporation is necessary for at least one protein interaction [30] and that SELENBP1 levels vary in accordance with selenium availability [31]. If further work confirms that the function of SELENBP1 is dependent upon selenium levels, then it would be tempting to speculate that adaptations to changing levels of selenium in the diet in early human history are responsible for the inferred selective signatures.

7.5 The Genetic Basis of Nutritional Adaptations to Selenium

As an important environmental factor, to which all humans are exposed throughout their life, diet is a powerful selective force. Genes associated with nutrition (e.g., genes involved in the metabolism of carbohydrates and lipids and in the transport of trace nutrients) are highly represented in genome wide scans for selection highlighting their key adaptive role [32]. The selection pressure exerted by diet varies both geographically and temporally through dietary shifts. Diet is one of the main environmental factors that exhibit population differentiation even amongst recently separated populations. Thus, adaptations in genes involved in metabolism, nutrient uptake and transport are likely to be a major source of variation amongst populations. It is in this context that evolutionary adaptations to dietary selenium should be placed.

Mutations that alter the expression of an allele may be a particularly economical way to respond to changing environmental pressures. The *LCT* C13910T lactase persistence allele is one example of this. The T allele enhances the activity of the *LCT* promoter, possibly by creating a transactivating protein binding site, and allows lactase transcription to continue into adulthood [33]. This regulatory adaptation may be a common theme for other genes involved in nutritional processes [34]. It is therefore reasonable to ask whether selenoprotein genes have been subjected to regulatory adaptations in response to levels of dietary selenium.

Selenium intake occurs through the diet and is thus dependent on the levels available in the foods consumed [10]. Selenium has an uneven geographical distribution in soils [10]. Historically people have tended to eat foods that were produced in proximity to their geographical location. It is thus plausible that the differing selenium levels in local soils have exerted a differential selective pressure upon selenoprotein genes in different populations. Local shifts in diet have also occurred (and are known to leave detectable genetic signatures e.g., dairying and *LCT* in Europe). The adoption of agriculture may have altered selenium intakes and thus led to selection on selenoprotein genes because meat, a major dietary component in preagricultural populations, is a richer source of selenium than the cereals and tubers that became staple foods in agricultural societies [25, 35]. The geographical distribution of the *HFE* C282Y allele mentioned previously may be an example of a situation where culturally induced dietary shifts have influenced selection on a gene involved in nutrition.

Considering that selenium is an essential trace nutrient, its worldwide variation in availability and dietary intake might be considered a likely selective pressure on selenoproteins in humans. As discussed in Sect. 7.2, Sec and Cys residues are not functionally exchangeable in vertebrate proteins. This lack of Sec/Cys exchangeability opens the question of whether selection on selenoprotein genes could have generated regulatory adaptations instead. This seems plausible given that selenoprotein genes are hierarchically regulated in response to an individual's selenium levels (e.g., selenium deficiency leads to more degradation of GPx1 and GPx3 mRNA than GPx2 and GPx4 mRNA) [36]. This hierarchical control of selenoprotein gene expression highlights the importance of selenium levels, and raises the possibility

that consistent excess or deficiency of dietary selenium in a population could have promoted positive selection for compensatory regulatory changes at the transcriptional and/or posttranscriptional level. It is possible that, as the evolutionary importance of selenium in the diet is investigated, regulatory adaptations and not amino acid changes are shown to be the targets of natural selection. Proper evolutionary tests of this hypothesis are needed.

7.6 Concluding Remarks

Little is known about the genetic basis of adaptations to levels of dietary selenium in humans and other species. The identification of signatures of adaptation in the coding and regulatory regions of selenoprotein genes, and also in genes involved in selenium metabolism, is a first step to gain insight into this question. In turn, detecting the genetic loci that have played a major role in dietary adaptations to selenium may shed light on the mechanisms that underlie the regulation of selenium metabolism.

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Chapter 8
Selenocysteine Lyase: Mechanism, Structure, and Biological Role

Hisaaki Mihara and Nobuyoshi Esaki

Abstract Selenocysteine lyase is a homodimeric pyridoxal 5′-phosphate-dependent enzyme that specifically catalyzes the removal of selenium from L-selenocysteine to yield L-alanine and is inert to its cognate L-cysteine. The enzyme is proposed to function in the recycling of the micronutrient selenium from degraded selenoproteins that contain selenocysteine residues as an essential component. Findings from recent studies have facilitated an unprecedented understanding of how this unique enzyme distinguishes between selenocysteine and cysteine and have suggested possible directions for future research that may uncover the physiological role of the enzyme in mammals.

8.1 Introduction

The initial step of selenoprotein biosynthesis involves selenophosphate synthetase, which catalyzes the formation of selenophosphate from selenide and ATP. Selenophosphate is a precursor molecule of selenocysteyl-tRNA that decodes UGA codon [1, 2]. The metabolic pathway providing selenide for selenophosphate synthetase remains unclear. The free form of selenide is less commonly present in the body than a protein-bound form [3]. Inorganic forms of selenium, such as selenite and

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selenate, can be utilized as a source of selenium for selenoproteins. However, a considerable part of selenium uptake from foods is in the form of selenomethionine and selenocysteine residues in proteins, suggesting that a significant part of selenium in selenoprotein originates from seleno-amino acids [4, 5]. Although few reports do suggest the occurrence of free selenocysteine in mammals, it can be formed either by degradation of selenoproteins that contain selenocysteine residues or by metabolism of selenomethionine through the same metabolic pathway as the one involved in the synthesis of cysteine from methionine [6]. Free L-selenocysteine thus formed could be a specific substrate for selenocysteine lyase (SCL).

8.2 Identification of SCL in Rat Liver Homogenate

A study on selenocysteine biosynthesis [6] found that far less efficient selenocysteine formation occurred with the rat liver homogenate system than with the purified enzyme system. This finding indicated the presence of a novel enzyme that decomposes selenocysteine in the liver homogenate. On incubating DL-selenocysteine with rat liver homogenate in the presence of dithiothreitol (DTT), formation of alanine and hydrogen selenide (H_2Se) was observed. In contrast, alanine formation was not observed in control experiments with boiled rat liver homogenate, wherein water substituted for DL-selenocysteine, rat liver homogenate, or DTT and L-cysteine substituted for DL-selenocysteine. Stoichiometric studies showed that equivalent amounts of alanine and H_2Se were produced from DL-selenocysteine by using rat liver homogenate. The quantity of alanine produced was reduced by alanine dehydrogenase, which specifically acts on L-alanine. However, the remaining selenocysteine was almost completely oxidized by D-amino acid oxidase after Se-ethylation with ethyl iodide. These findings suggested that L-selenocysteine is converted into L-alanine and H_2Se and that the D-isomer of DL-selenocysteine used as a substrate remains in the reaction mixture. 2-Mercaptoethanol and 2,3-dimercapto-1-propanol could substitute for DTT, but NADH, NADPH, and ascorbic acid were inert. These findings indicated the occurrence of a new enzyme that catalyzes the degradation of selenocysteine to form alanine and H_2Se. This reaction, apparently, is a reduction reaction. However, the actual products were identified as alanine and elemental selenium (S^0). Thus, the enzyme was termed selenocysteine β-lyase (EC 4.4.1.16; at present also termed SCL) [7].

8.3 Mammalian SCL

Pig liver SCL was purified to homogeneity and characterized in 1982 [7]. The pig enzyme is a homodimer consisting of two subunits with an identical M_r of 48,000. SCL contains 1 mole of pyridoxal 5′-phosphate (PLP) per mole of the subunit as a

coenzyme, and it exhibits an absorption maximum at 420 nm. Its specific activity is 37 μmol·min^{-1}·mg^{-1}, and it exhibits maximum activity at about pH 9.0. This enzyme stoichiometrically converts L-selenocysteine to H_2Se and L-alanine in the presence of excess DTT in the reaction mixture. Further experiments confirmed that SCL catalyzes the removal of Se^0 from L-selenocysteine and that the formation of hydrogen selenide is due to the DTT-induced nonenzymatic reduction of the product Se^0. The K_m value of L-selenocysteine is 0.83 mM. L-Cysteine is a competitive inhibitor of SCL with a K_i of 1 mM. The following amino acids or their derivatives are inert: L-cysteine, L-serine, β-chloro-L-alanine, L-cysteine sulfinate, *S*-methyl-L-cysteine, Se-ethyl-DL-selenocysteine, selenohomocysteine, homocysteine, and selenocysteamine. None of the following compounds inhibits the enzyme reaction: L-serine, L-alanine, L-homocysteine, L-selenohomocysteine, H_2Se, and glutathione.

8.4 Bacterial SCL

Bacterial SCL was purified from *Citrobacter freundii* in 1985 [8]. The bacterial enzyme is markedly different from the mammalian enzyme with respect to physicochemical properties and amino acid composition. In contrast to the homodimeric pig liver enzyme with a subunit M_r of 48,000, the bacterial enzyme is monomeric with a M_r of 64,000. Nevertheless, the enzyme is very similar to the mammalian enzyme with respect to its enzymatic properties, that is, catalyzing the degradation of L-selenocysteine into L-alanine and selenium but being inert against L-cysteine. The apparent K_m for L-selenocysteine is 0.95 mM and the enzyme shows maximum activity at pH 7.0. The presence of 0.1% 2-mercaptoethanol or 1 mM DTT is essential to prevent enzyme inactivation. The enzyme is strongly inhibited by incubation with metallic divalent cations such as Zn^{2+}, Ni^{2+}, Pb^{2+}, and Hg^{2+} and with thiol reagents such as iodoacetate, iodoacetamide, and *N*-ethylmaleimide. L-Cysteine behaves as a competitive inhibitor of the enzyme with a K_i of 0.65 mM.

8.5 Distribution of SCL Activity

An enzyme assay with tissue homogenates demonstrated SCL activity in various mammalian tissues such as the liver, kidney, pancreas, adrenal, heart, lung, testis, brain, thymus, spleen, and muscles of rat, dog, mouse, pig, and some other mammals [7]. The specific activity of the liver and kidney were generally higher than those of the other tissues. However, no activity was observed in blood and fat. Western blot analysis of proteins extracted from mouse tissues also revealed relatively higher amounts of SCL in the liver, kidney, and testis [9].

SCL is also found in some bacterial strains such as *C. freundii*, *Alcaligenes viscolactis*, and *Pseudomonas alkanolytica* [10]. However, no significant activity is noted in yeasts and fungi.

8.6 Cloning and Sequence Analysis of Mammalian SCL

The first cDNA cloning of mammalian SCL was reported in 2000 for the mouse *Scly* gene [9]. The cDNA for mouse SCL is 2,172 bp in length, containing an open reading frame encoding a polypeptide chain of 432 amino acid residues (M_r=47,201). The recombinant mouse SCL overproduced in *Escherichia coli* is a homodimer with a subunit M_r of 47,000. cDNA cloning has also been performed for the rat gene [11]. Steady-state kinetic analysis of the recombinant rat SCL shows that the V_{max} and K_m values for L-selenocysteine are 26 μmol·min^{-1}·mg^{-1} and 5.5 mM, respectively.

A BLAST search analysis using the mouse SCL cDNA sequence as a query shows that a number of genes sharing sequence homology with *Scly* have been deposited in nucleotide sequence databases. These include orthologous genes in all vertebrate genomes sequenced to date such as mammals (human, chimpanzee, rhesus monkey, rat, dog, giant panda, cow, pig, horse, opossum, and platypus), birds (chicken and zebra finch), amphibians (African clawed frog and western clawed frog), and fishes (zebrafish, Japanese puffer fish, and green spotted puffer); and those in genomes of Florida lancelet (>58%), purple sea urchin (>55%), *Trichoplax adhaerens* (phylum Placozoa) (>50%), cnidarians (>49%), sea squirt (>48%), human body louse (>46%), and nematodes (>42%) (Fig. 8.1). Interestingly, the unicellular choanoflagellate *Monosiga brevicollis*, which is among the closest unicellular relatives of animals, also has a gene with moderate (>36%) sequence identity with SCL. Apart from NifS-type genes (described below), no other significant gene that is homologous to *Scly* is found in the genomic sequences of plants, fungi, bacteria, and archaea, suggesting that the SCL activities detected in some bacterial and archaeal strains are expressed by prokaryotic SCLs that are significantly different at least in primary structure from eukaryotic SCL and/or by NifS-type cysteine desulfurases.

SCL is more distantly related to NifS-type cysteine desulfurases with respect to primary structure, which catalyze the desulfurization of L-cysteine to provide sulfur for iron-sulfur clusters, thiamine, molybdopterin, and thionucleotides in tRNA [12]. Cysteine desulfurases identified to date utilize both cysteine and selenocysteine as substrates with different specificity [13–17]. Overall sequence identity (~30%) is found between SCLs and cysteine desulfurases. Generally, they are clearly classified into two distinct groups [9]. In particular, the regions corresponding to Gln105-Gly121 and Asn205-Pro214 of mouse SCL are not found in mammalian cysteine desulfurases. There are unidentified genes that have slight similarity to both known SCL and cysteine desulfurases in the genomes of diatoms, green algae, and euglenozoans such as *Trypanosoma brucei* and *Leishmania infantum* [18]. Catalytic properties of these unidentified homologs need to be investigated in detail to define a dividing line between SCLs and cysteine desulfurases.

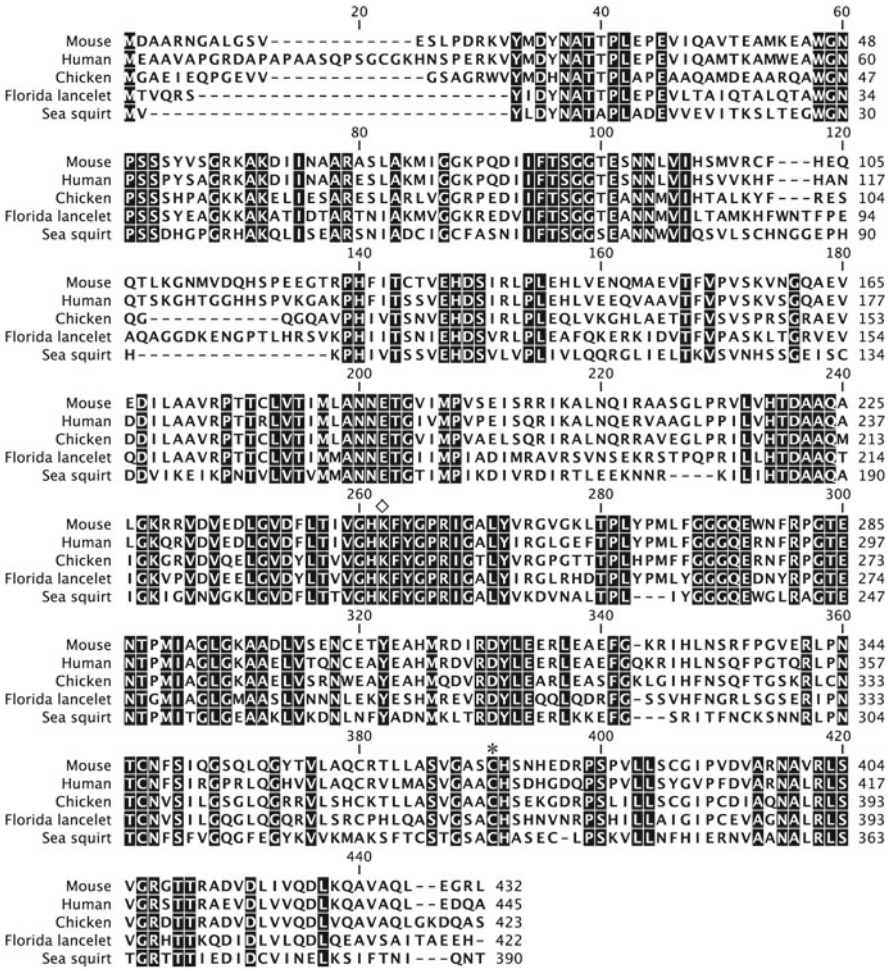

Fig. 8.1 Sequence alignment of SCL sequences predicted from mouse, human, chicken, Florida lancelet, and sea squirt genes. The *black boxes* show the residues that are completely conserved among the five sequences. The *asterisk* indicates the selenopersulfide-forming catalytic Cys residue (Cys375 in mouse and rat enzymes). The *open diamond* indicates the PLP-binding Lys residue (Lys247 in mouse and rat enzymes). The sequences were retrieved from the GenomeNet database at http://www.genome.jp/ and analyzed with a CLC Sequence Viewer software (http://www.clcbio.co.jp/)

8.7 Structure and Catalytic Mechanism

Crystal structures of mammalian SCLs have been solved and deposited into the Protein Data Bank (PDB) with accession numbers 3A9X (rat SCL in a native form), 3A9Y (rat SCL in complex with L-cysteine), 3A9Z (rat SCL in complex with selenopropionate), 3GZC (human SCL in a native form), and 3GZD (human SCL in another native form from a P1 crystal) [11]. SCL structure analyses revealed that the

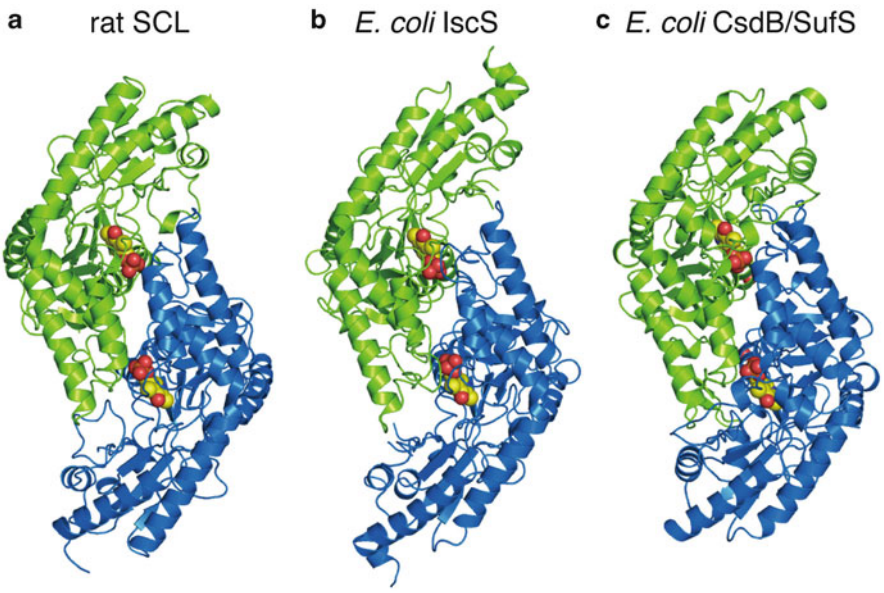

Fig. 8.2 X-ray crystal structures of (**a**) rat SCL, (**b**) *E. coli* IscS, and (**c**) *E. coli* CsdB/SufS are shown as dimeric forms in ribbon models. The coenzyme PLP located in the domain interfaces is shown in CPK models

fold of the monomer is similar to that of *E. coli* IscS [19], *Thermotoga maritima* NifS-like protein [20], *Synechocystis* sp. PCC 6803 SufS [21], *E. coli* CsdB/SufS [22–24], *Synechocystis* cysteine C-S lyase [25], and *Pseudomonas fluorescens* kynureninase [26], all of which belong to the well-described Fold type I family of PLP-dependent enzymes [27, 28]. The structure of SCL is more similar to those of group I cysteine desulfurases (IscS and NifS-like proteins) than those of group II cysteine desulfurases (SufS and CsdB proteins) (Fig. 8.2).

Mammalian SCLs have a completely conserved cysteine residue that corresponds to Cys375 in rat SCL (Fig. 8.1). Cys375 is located in a flexible extended lobe (Ser374-Ile392) that is located near the cofactor PLP forming a Schiff base with the side chain amino group of the catalytic Lys247. A mutant SCL protein (C375A) in which Cys375 is replaced by an alanine residue exhibits no activity on L-selenocysteine [11], suggesting that Cys375 is a catalytically essential residue of the enzyme similar to the persulfide-forming catalytic cysteine residue of cysteine desulfurases [12]. Electrospray ionization-mass spectrometry (ESI-MS) was performed to understand the catalytic mechanism of rat SCL; the findings revealed that selenium eliminated from L-selenocysteine is bound to Cys375 of the enzyme in the form of a cysteine selenopersulfide intermediate (SCL-S-Se$^-$) [11]. From X-ray structure analysis of the SCL•selenopropionate complex, it is proposed that the interaction of the thiol group of Cys375 with the selenolate of the substrate selenocysteine brings the substrate L-selenocysteine into a favorable arrangement to form a Schiff base with PLP.

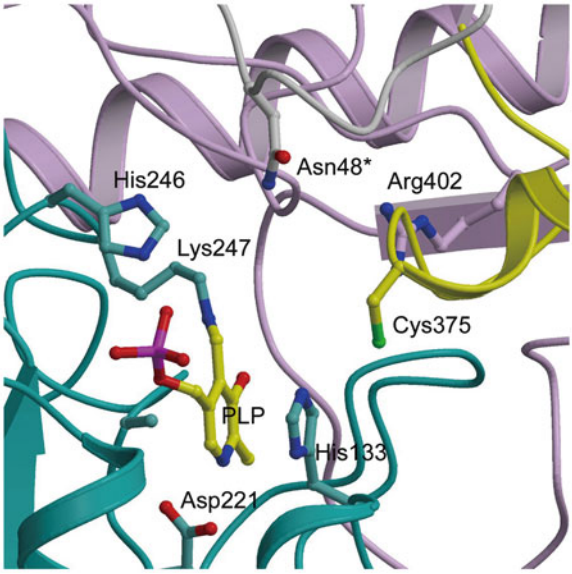

Fig. 8.3 An active-site structure of rat SCL. Although the structure is obtained from the SCL complexed with L-cysteine (PDB: 3A9Y), only the PLP moiety and active-site residues are shown as ball-and-stick models for simplification

The X-ray structure analysis of rat SCL complexed with L-cysteine or selenopropionate [11] showed that the small domain moves toward the active site, and the extended lobe including the active site Cys375 in the small domain, which is disordered in the unliganded form, exhibits an ordered structure, encapsulating substrate analogs within the active-site cavity (Fig. 8.3). This open–closed conformational change completely covers the active site like a lid and shields the bound ligands from the solvent region. The disorder–order transition of the extended lobe is highly involved in the catalytic event in that the lobe residue Cys375 is favorably oriented for interactions with the selenol group of the substrate, and oxygen-sensitive selenopersulfide is formed in the cavity shielded from the solvent region.

The reaction mechanism of SCL is now proposed as follows (Fig. 8.4). The productive L-selenocysteine is encapsulated in the active site by a small domain rotation and the ordering of the extended lobe with its Seγ atom interacting with the thiol group of Cys375. The deprotonated amino group of the substrate makes a nucleophilic attack on the C4′ of PLP to produce the external aldimine and release the neutral side chain of Lys247. The α-proton of the substrate is eliminated by Lys247 to yield a quinonoid intermediate. Lys247 adds a proton to the C4′ of the quinonoid intermediate, producing the substrate-ketimine intermediate. The substrate ketimine then transfers selenium to Cys375 to form cysteine selenopersulfide (Cys375-S-Se(H)) and the enamine.

It remains unclear whether selenium is directly released from Cys375-S-Se(H) or is trapped by a selenium-transferring protein and subsequently released by a reductant in the reaction system. If selenopersulfide selenium, which is more sensitive to oxygen than persulfide sulfur, is released directly from an intermediate and

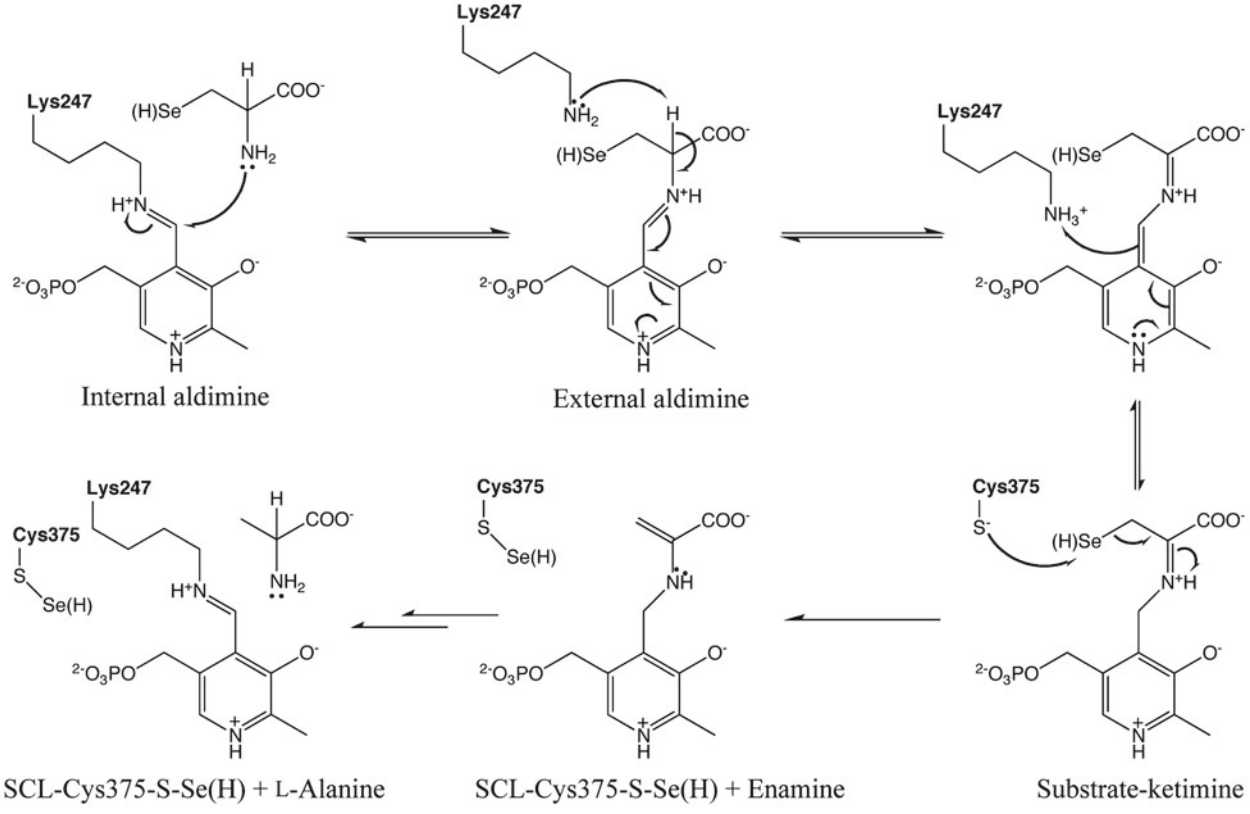

Fig. 8.4 A proposed reaction mechanism for SCL. Only a part of the catalytic reaction is shown. The protonation/deprotonation states of selenol/selenolate in the substrate moiety are not known. The cysteine selenopersulfide intermediate is shown as Cys375-S-Se(H)

diluted by the bulk solvent, then delivery to a specific acceptor molecule would be inefficient. Plausibly, selenium is shipped to the target protein through an interaction with the selenium-transferring protein in a manner similar to the reaction proposed for cysteine desulfurases [12, 29]. A potential selenium acceptor protein is discussed below in Sect. 8.9.

8.8 Discrimination Between Selenium and Sulfur

Most enzymes cannot distinguish between the sulfur and selenium atoms contained in their substrate molecules, as observed in the synthesis of selenocysteine from selenomethionine through the transsulfuration pathway involving cystathionine β-synthase and cystathionine γ-lyase [6] and in the nonspecific incorporation of selenomethionine in proteins [30]. In contrast to cysteine desulfurases catalyzing the elimination of both the selenium atom from L-selenocysteine and the sulfur atom from L-cysteine, SCL is highly specific for L-selenocysteine. A part of the mechanism for this strict specificity was solved by the analysis of ultraviolet-visible (UV-Vis) spectrum and the crystal structure of SCL complexed with L-cysteine [11]. Upon the binding of L-cysteine to the active site of rat SCL, the side chain Sγ atom of the bound cysteine, but not the α-amino group, makes a covalent bond with the C4′ atom of PLP to form a stable tetrahedral adduct with the α-amino group far away from the C4′ atom and the Cα-hydrogen directed to the solvent side, thus suggesting that the enzyme reversibly forms a nonproductive adduct with L-cysteine. On the other hand, the substrate L-selenocysteine binds to the active site in the productive form with the aid of the interaction of the substrate selenolate with Cys375 thiol as described above. This interaction thus allows the substrate to form the Schiff base with PLP, resulting in the production of the external aldimine to initiate the catalytic reaction. This finding provides an example of the molecular basis of how a selenium-containing substrate in trace quantities is distinguished from its cognate sulfur-containing compound in excess in a biological system.

8.9 Biological Role

Based on the high K_m value of SCL for L-selenocysteine, a detoxification role for the enzyme was suggested originally [7]. However, the activity of the enzyme in human liver is not related to tissue selenium concentration [31], and that in rat tissues it is not affected by dietary selenite, selenocysteine, or selenomethionine [32]. The studies of the NifS-type cysteine desulfurases that supply sulfur from L-cysteine for synthesis of a wide variety of sulfur-containing biomolecules suggested a comparable selenium transfer role for SCL [33–38]. In addition, because the substrate L-selenocysteine can be supplied by selenoprotein degradation, a role in selenocysteine recycling is also suggested for SCL. As described in Sect. 8.7, SCL was

shown to remove the selenium atom from L-selenocysteine to form an enzyme-bound selenopersulfide intermediate. The selenium in the selenopersulfide intermediate can be transferred to a yet unidentified selenium acceptor protein, presumably via specific protein–protein interactions. Selenophosphate synthetase is among the most promising candidate for such selenium acceptor proteins because a substrate of this enzyme is selenide. In vitro studies showed that NifS-type enzymes from *E. coli* [35] and *Methanococcus vannielii* [39] as well as mouse SCL (Mihara et al., unpublished results) effectively provided selenium from L-selenocysteine to selenophosphate synthetase for the production of selenophosphate. A co-immunoprecipitation experiment using a reticulocyte lysate system indicated that SCL associates with each of the two selenophosphate synthetase isozymes, SPS1 and SPS2 [40]. Although these findings imply SCL involvement in selenoprotein synthesis, further studies are needed to establish its physiological relevance.

Apart from the above-mentioned potential role of SCL in selenoprotein synthesis, a differential gene expression study showed that the expression of SCL is enhanced in acute glomerulonephritis and is diminished in chronic glomerulonephritis [41]. An enhancer element was found between bp-152 and bp-298 of the 5′-regulatory region of the SCL gene, suggesting regulation by activator protein-1 (AP-1), which modulates many processes critical for carcinogenesis, including cell proliferation, survival, transformation, invasion, and angiogenesis, and is present at elevated levels in many neoplasms, including breast tumors [42, 43]. Another differential display study of mRNA from HepG2 hepatocarcinoma with and without transient expression of hepatitis C virus core protein identified SCL gene as a candidate gene involved in the pathophysiology of hepatocellular carcinoma [44]. In a yeast two-hybrid screening of mouse cDNA libraries, major urinary proteins as well as several proteins related to spermatogenesis, protein synthesis, and cell viability/apoptosis were identified as potential interactors [40]. These studies may provide new clues for exploring the physiological function of SCL.

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Part II
Selenoproteins and Selenoproteins in Health

Chapter 9
Selenoproteins and Selenoproteomes

Vadim N. Gladyshev

Abstract Recent progress in high-throughput sequencing and analysis allowed efficient identification of selenocysteine-containing proteins in sequence databases, including full sets of selenoproteins in organisms, designated selenoproteomes. Information is currently available on selenoproteomes from all major model organisms as well as humans, which have 25 selenoprotein genes. This chapter gives an overview of selenoproteins at the level of individual proteins, protein families, and entire selenoproteomes. Comparative genomic analyses of selenoproteins offer exciting avenues for studying selenoprotein function and evolution, provide insights into the biological functions of the trace element selenium, and even allow addressing important biological questions unrelated to selenium.

9.1 Introduction

Selenium (Se) is found in biological molecules in three specific forms. It occurs in the form of selenouridine in the wobble position of certain bacterial tRNAs [1]. In addition, in some bacterial Se-containing molybdoproteins it is present as a labile cofactor that contains a Se–Mo bond that is directly involved in catalysis [2–4]. However, the major form of Se in biological systems is represented by selenocysteine (Sec), the 21st amino acid in the genetic code. It is encoded by the UGA codon and has been found in each domain of life (i.e., bacteria, archaea, and eukaryotes). It is now clear that the essential roles of Se in biology, as well as its beneficial functions in human health, are due to its presence in proteins in the form of Sec.

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In contrast to the 20 common amino acids in proteins, Sec is utilized only when it is required for protein function. Accordingly, it is normally a key functional (and almost always catalytic) group in proteins, wherein selenoproteins use Sec in redox catalysis. Therefore, information on identities and functions of selenoproteins is a key to the understanding of biological and biomedical roles of Se.

9.2 Bioinformatics Tools for Selenoprotein Identification

Over the years, researchers in the Se field developed very convenient tools for selenoprotein analyses. Selenoproteins can be analyzed by following the presence of Se in protein fractions, e.g., by inductively coupled plasma mass-spectrometry (ICP-MS) [5]. Sec-containing proteins can also be metabolically labeled with ^{75}Se, a convenient γ-emitter that remains covalently bound to proteins during the SDS-PAGE procedure and can be visualized on gels and membranes with a PhosphorImager [6]. With these techniques, a number of proteins have been identified in both prokaryotes and eukaryotes [6–8]. We suggest, however, that the approach that in recent years benefited the Se field the most with regard to selenoprotein discovery is the analysis of sequence databases.

Remarkable progress in genome sequencing and analyses offered an excellent resource for selenoprotein discovery and analysis of selenoprotein functions. All selenoprotein genes have two characteristic features: a Sec-encoding TGA codon and a Sec insertion sequence (SECIS) element. The TGA triplet that codes for Sec does not provide sufficient information at the nucleotide sequence level to identify Sec sites computationally. However, SECIS elements are amenable to these techniques as these structures are highly specific for selenoprotein genes, have conserved sequences, and possess a sufficiently complex secondary structure. Therefore, many bioinformatics analyses focused on SECIS elements, and selenoprotein discovery used the following strategy: (1) finding candidate SECIS elements; (2) analyzing upstream regions to identify coding regions; and (3) testing candidate selenoproteins for insertion of Se by metabolically labeling cells with ^{75}Se. The first selenoproteins identified using this technique were mammalian selenoproteins R (now known as methionine-*R*-sulfoxide reductase 1), N, and T [9, 10]. These searches were initially restricted to small nucleotide sequence databases, but later could be adapted to searching entire genomes [11–13]. Currently, to aid in these analyses, groups of closely related genomes are analyzed in order to identify evolutionarily conserved SECIS elements that belong to selenoprotein orthologs in these organisms [14].

A separate approach (independent of SECIS elements) was also developed that searched for in-frame TGA codons by analyzing TGA flanking sequences [13–16]. This approach is possible because the majority of selenoprotein genes have homologs (most often in organisms with reduced or lost Sec utilization), in which Sec is replaced with Cys. Thus, a strategy was developed wherein protein databases (large sets of overlapping reading frames, nonredundant protein databases, etc.) are searched

against nucleotide sequences from organisms that contain selenoprotein genes (genomes, expressed sequence tags, metagenomic projects) to identify nucleotide sequences that, when translated, align with Cys-containing protein sequences from the protein database, such that Cys residues align with candidate Sec and these pairs are flanked by conserved sequences. Although SECIS predictions could be used to guide the computational gene predictions, the Sec/Cys homology approach is completely independent of the searches for SECIS elements. Therefore, this method provides a SECIS-independent tool for selenoprotein identification. Both Sec/Cys and SECIS-based algorithms identify very similar sets of selenoprotein genes in organisms, suggesting that both tools show excellent performance and that all, or almost all, selenoproteins can be identified by these programs in completely sequenced genomes and large sequence databases.

Major currently known selenoprotein families are shown in Fig. 9.1. The majority of these proteins were discovered using bioinformatics approaches and subsequently verified, at least in the case of eukaryotic selenoproteins, experimentally. Below, the best studied selenoproteins are described, with the focus on eukaryotic selenoproteins. Additional and more detailed information on various selenoproteins can be found in various chapters throughout the book.

9.3 Mammalian Selenoproteins

9.3.1 Glutathione Peroxidases

Mammals have eight glutathione peroxidases (GPx1–GPx8), of which five are Sec-containing enzymes (GPx1, GPx2, GPx3, GPx4, and GPx6). However, GPx6 reverted back to a Cys-containing protein in many rodents, including mice and rats, so these organisms have only four selenoprotein GPxs [14]. GPx1 is the first animal selenoprotein identified [17] and it is also the most abundant one in mammals, especially in the liver and kidney. It catalyzes glutathione-dependent hydroperoxide reduction. In recent years, another GPx, GPx4, received much attention due to its essential status during embryonic development in mice and role in regulation of phospholipid hydroperoxide levels [18]. Moreover, the mitochondrial form of this protein serves a structural role in mature sperm and was implicated in disulfide bond formation during spermiogenesis [19]. Whereas GPx1 and GPx4 are expressed in all cells, GPx2 is gastrointestinal and GPx3 is primarily made in the kidney and secreted to the blood stream. It localizes to the basement membrane of the proximal tubules in the kidney [20]. However, it remains unclear how it can function in the extracellular milieu in the absence of sufficient levels of thiol reductants. Besides mammals, selenoprotein GPx homologs were identified in most animals as well as various single-celled eukaryotes and even bacteria. However, the ancestral form of these proteins is the Cys-containing form, and it is thought that Cys was replaced with Sec during evolution to make these enzymes better catalysts.

	Protein name	SEC location	Protein size	Source (representative)
Vertebrates	15 kDa selenoprotein (Sep15)	93	162	*Homo sapiens*
	Fish Sep15-like selenoprotein (Fep15)	46	138	*Danio rerio*
	Iodothyronine deiodinase (DI)	126	249	*Homo sapiens*
	Selenoprotein H (SelH)	38	116	*Homo sapiens*
	Selenoprotein I (SelI)	387	397	*Homo sapiens*
	Selenoprotein J (SelJ)	262	341	*T. nigroviridis*
	Selenoprotein N (SelN)	428	556	*Homo sapiens*
	Selenoprotein O (SelO)	667	669	*Homo sapiens*
	Selenoprotein P (SelP)	59, 300, 318, 330, 345, 352, 367, 369, 376, 378	381	*Homo sapiens*
	Methionine-R-sulfoxide reductase (MsrB, SelR)	95	116	*Homo sapiens*
	Selenoprotein S (SelS)	188	189	*Homo sapiens*
	Selenoprotein V (SelV)	273	346	*Homo sapiens*
	Selenophosphate Synthetase (SPS2)	60	448	*Homo sapiens*
Single-cell organisms	**Glutathione peroxidase (GPx)**	47	201	*Homo sapiens*
	Selenoprotein K (SelK)	92	94	*Homo sapiens*
	Selenoprotein L (SelL)	160, 163	302	*Danio rerio*
	Selenoprotein M (SelM)	48	145	*Homo sapiens*
	Selenoprotein T (SelT)	36	182	*Homo sapiens*
	Selenoprotein U (SelU)	71	210	*Gallus gallus*
	Selenoprotein W (SelW)	13	87	*Homo sapiens*
	Thioredoxin reductase (TR, TrxR, Txnrd)	498	499	*Homo sapiens*
	Methionine-S-sulfoxide reductase (MsrA)	20	160	*C. reinhardtii*
	Protein disulfide isomerase (PDI)	46	189	*Emiliania huxleyi*
	Peroxiredoxin (Prx)	44	151	Sargasso Sea database
	Proline reductase (PrdB)	12	119	Sargasso Sea database
	Prx-like protein	12	87	Sargasso Sea database
	Thioredoxin (Trx)	33	111	Sargasso Sea database
	Formate dehydrogenase (FDH)	11	264	Sargasso Sea database
	Glycine reductase selenoprotein A (GrdA)	25	135	Sargasso Sea database
	Glycine reductase selenoprotein B (GrdB)	161	187	Sargasso Sea database
	AhpD-like protein	28	132	Sargasso Sea database
	Arsenate reductase	12	119	Sargasso Sea database
	Molybdopterin biosynthesis MoeB protein	27	224	Sargasso Sea database
	Glutaredoxin (Grx)	13	252	Sargasso Sea database
	DsbA-like protein	14	199	Sargasso Sea database
	Glutathione S-transferase	13	105	Sargasso Sea database
	Thiol-disulfide isomerase-like protein	9	253	Sargasso Sea database
	CMD domain-containing protein	59	192	Sargasso Sea database
	Rhodanase-related sulfurtransferase	33	74	Sargasso Sea database
	OsmC-like protein	40	137	Sargasso Sea database
	DsrE-like protein	14	54	Sargasso Sea database
	DsbG-like protein	76	219	Sargasso Sea database
	HesB-like protein	106	33	*Desulfovibrio vulgaris*
	Formylmethanofuran dehydrogenase	121	435	*Methanocaldococcus sp*
	Methylviologen-reducing hydrogenase	456	491	*Desulfotalea sp*
	Coenzyme F420-reducing hydrogenase	15, 66	132	*Methanopyrus kandleri*
	Heterodisulfide reductase	195	666	*Thermodesulfovibrio sp.*

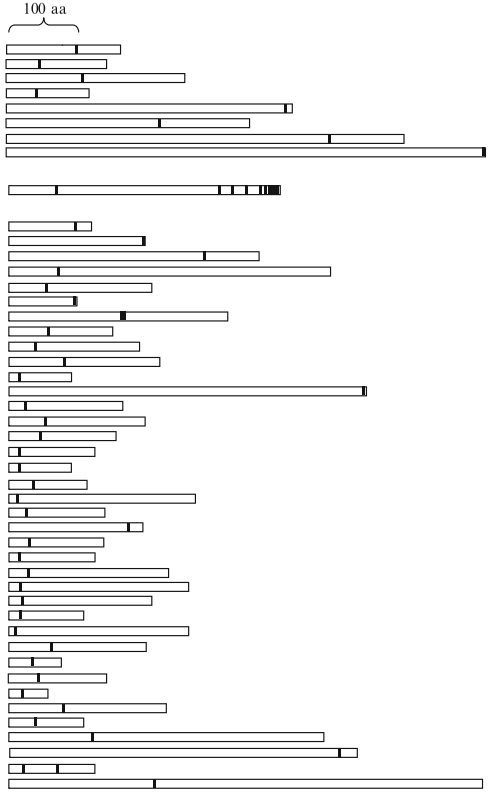

Fig. 9.1 Selenoprotein families. Selenoproteins that occur in vertebrates or single-celled eukaryotes are highlighted by *shaded boxes*, and selenoproteins found in prokaryotes are shown in *bold*. On the *right*, relative sizes of selenoproteins are shown (relative to a 100 amino acid scale) and the location of Sec within protein sequence is shown by a *black line*

9.3.2 Thyroid Hormone Deiodinases

There are three deiodinases (DI1, DI2 and DI3) in mammals, which activate and/or inactivate thyroid hormones by reductive deiodination. Deiodinases also occur in other vertebrates, and their homologs were even detected in unicellular eukaryotes and bacteria, although their function must be different in these organisms. Like GPxs and the majority of other selenoproteins, deiodinases are thioredoxin-fold proteins. These enzymes are extensively reviewed in this book.

9.3.3 Thioredoxin Reductases

The entire family of mammalian thioredoxin reductases (TRs) is dependent on Se as all three TRs in mammals are selenoproteins. Sec in TRs is located in the C-terminal penultimate position. These enzymes evolved from glutathione reductases by adding a C-terminal Sec-containing extension that became an intraprotein substrate for the classical N-terminal active center of pyridine nucleotide disulfide oxidoreductase family members [21–24]. TR1 (also known as TrxR1, TxnRd1) is a cytosolic and nuclear protein. Its main function is to control the reduced state of thioredoxin. However, it exhibits broad substrate specificity, especially with regard to low molecular weight compounds [25] and occurs in the form of at least six isoforms generated by alternative transcription initiation and alternative splicing [26–28]. A close homolog of TR1 is thioredoxin/glutathione reductase (TGR, also known as TR2, TxnRd3 and TrxR3) that, compared to other animal TRs, has an additional N-terminal glutaredoxin domain [22]. This protein was implicated in the formation/isomerization of disulfide bonds during sperm maturation [29]. TGR can catalyze many reactions specific for thioredoxin and glutathione systems. TR3 (also known as TxnRd2 and TrxR2) is a mitochondrial protein, which keeps mitochondrial thioredoxin and glutaredoxin 2 in the reduced state. TR1 and TR3 are essential for embryonic development in mammals [30, 31], while the consequences of TGR knockout have not been examined thus far.

9.3.4 Methionine-*R*-Sulfoxide Reductase 1 (MsrB1)

MsrB1 is the first selenoprotein identified through bioinformatics approaches. It was designated as Selenoprotein *R* [9] and Selenoprotein X [10], but after it was found to catalyze stereospecific reduction of methionine-*R*-sulfoxide residues in proteins, it was renamed as MsrB1 [32]. Mammals have two additional MsrBs (MsrB2 and MsrB3), which contain catalytic Cys in place of Sec and reside in mitochondria and the endoplasmic reticulum, respectively [33]. At least in liver and kidney of mammals, MsrB1 has the highest activity of all MsrBs, so the protein reductive repair

function is dependent on Se in mammals. MsrB1 is located in the cytosol and nucleus [33]. MsrB1 knockout mice are viable, but are characterized by oxidative stress [34].

9.3.5 15 kDa Selenoprotein (Sep15)

Sep15 is a conserved eukaryotic selenoprotein that occurs in most animals as well as in some unicellular eukaryotes, such as algae [7]. It resides in the endoplasmic reticulum where it binds UDP-glucose:glycoprotein glucosyltransferase, a sensor of protein folding [35]. Sep15 is composed of a N-terminal ER signal peptide, a Cys-rich domain responsible for binding UDP-glucose:glycoprotein glucosyltransferase, and a C-terminal domain characterized by the thioredoxin-like fold. Sep15 is implicated in the cancer prevention effect of dietary Se [36, 37]. Sep15 knockout mice are viable, but develop cataracts (MV Kasaikina and VN Gladyshev, unpublished).

9.3.6 Selenophosphate Synthetase 2 (SPS2)

By analogy to bacterial selenophosphate synthetase SelD [38], SPS2 was thought to synthesize selenophosphate, a Se donor compound. It was recently shown to be essential for selenoprotein biosynthesis in mammals, whereas the function of SPS1, a paralog of SPS2, remains unknown [39].

9.3.7 Selenoprotein P (SelP)

SelP is the only selenoprotein with multiple Sec residues [40]. For example, human and mouse SelP have ten Sec residues and zebrafish SelPa has 17 [41]. However, the number of Sec residues in SelP homologs varies greatly (e.g., 7–15 in mammals) [41]. SelP is the major plasma selenoprotein, which is synthesized primarily in the liver and delivers Se to certain other organs and tissues [42, 43]. The SelP knockout mouse was particularly useful in examining Se metabolism in mammals as discussed elsewhere in this book.

9.3.8 Selenoproteins W (SelW) and V (SelV)

SelW is the smallest mammalian selenoprotein [44]. Although it was one of the first identified (more than 20 years ago), its function remains unknown. SelW homologs were identified in lower eukaryotes and even bacteria, but these findings did not help identify SelW function [16]. A SelW paralog, SelV, is a larger protein due to an additional N-terminal sequence of unknown function [14]. This protein is expressed exclusively in testes. Its function is also not known.

9.3.9 Selenoproteins T (SelT), M (SelM), and H (SelH)

Functions of these three proteins are not known. They are clustered here because they belong to a group of thioredoxin-like fold proteins (together with Sep15, SelW and SelV). SelT is among the first selenoproteins identified through bioinformatics [9]. SelM is a distant homolog of Sep15 and, like Sep15, it resides in the endoplasmic reticulum [37, 45]. SelH was first identified as BthD in fruit flies [12, 14]. It resides in the nucleus. Several studies have found that knockdown of these proteins leads to oxidative stress suggesting roles, at least partially, as antioxidants.

9.3.10 Selenoproteins O (SelO) and I (SelI)

SelO is a widely distributed protein with homologs in animals, bacteria, yeast and plants, but the functions of any members of this protein family are not known [14]. Only vertebrate homologs of SelO have Sec, which is located in the C-terminal penultimate position. In SelO homologs from other organisms, Sec is replaced with Cys. SelI is a recently evolved selenoprotein specific to vertebrates [14]. This membrane selenoprotein has no known function.

9.3.11 Selenoprotein K (SelK) and S (SelS)

SelK and SelS are unusual among selenoproteins in that they do not have a pronounced secondary structure [14]. These small selenoproteins contain a single transmembrane helix in the N-terminal sequence that targets them to the ER membrane. SelK homologs were detected in many eukaryotes, but no information is available on the function of any of these proteins. In contrast, recent studies revealed the role of SelS in retrotranslocation of misfolded proteins from the ER to the cytosol, where these proteins are further degraded [46]. SelS binds Derlin 1, an ER membrane-resident protein. In addition, SelS was implicated in inflammation and the immune response. A SelK knockout mouse model was recently developed [47] and is discussed elsewhere in the book.

9.3.12 Selenoprotein N (SelN)

One of the first selenoproteins discovered through bioinformatics approaches [10], SelN remains a selenoprotein of unknown function. This protein was implicated in the role of Se in muscle function through biochemical and genetic analyses, as well as through analyses of knockout mice [48], and was found to serve as a cofactor for the ryanodine receptor [49].

9.4 Additional Selenoproteins in Eukaryotes

The following selenoproteins that are absent in mammals were identified in eukaryotes: methionine-*S*-sulfoxide reductase (MsrA), protein disulfide isomerase (PDI), selenoproteins U (SelU), L (SelL), J (SelJ), Fep15, MCS, plasmodial selenoproteins Sel1, Sel2, Sel3 and Sel4, and a selenoprotein SelTryp in Trypanosoma. MsrA is a widely distributed protein family, whose function is to repair methionine residues in proteins. Like MsrB, it catalyzes a stereospecific reduction of methionine sulfoxides, but is specific for methionine-*S*-sulfoxides. MsrA was initially found in the green algae, *Chlamydomonas* [8], but later was also identified in other eukaryotes as well as in some bacteria. PDI is also very narrowly distributed in eukaryotes [50], in contrast to Cys-containing PDIs, which are essential for formation of disulfide bonds in the ER of eukaryotic cells. SelU [51], SelJ [52], Fep15 [53], and SelL [54] were only found in fish and/or invertebrates. The four *Plasmodium* selenoproteins (Sel1–Sel4) show no detectable homology to any other proteins [55]. However, Sel1 and Sel4 have Sec in the C-terminal regions and may be related to SelK and SelS.

9.5 Prokaryotic Selenoproteins

Several selenoproteins discussed above, including selenophosphate synthetase, deiodinase homologs, glutathione peroxidase and SelW, occur in both prokaryotes and eukaryotes. Below, we briefly discuss selenoproteins specific for prokaryotes.

9.5.1 Formate Dehydrogenase (FDH)

FDH is the most widespread prokaryotic selenoprotein. Sec in this protein is coordinated to molybdenum and directly involved in the oxidation of formate to carbon dioxide [56, 57]. In many bacteria, FDH is the only selenoprotein, which may be responsible for maintaining the Sec trait in these organisms [58].

9.5.2 Hydrogenase

Several hydrogenases are known that contain Sec. In these proteins, Sec is bound to nickel and is directly involved in catalysis [59]. Two different hydrogenase subunits may contain Sec, including one which may have two Sec residues [60].

9.5.3 Formylmethanofuran Dehydrogenase (FMDH)

FMDH is a distant homolog of FDH and catalyzes a similar reaction (with formylmethanofuran as the substrate) [61]. As in FDH, Sec in FMDH is coordinated to molybdenum in the enzyme active site.

9.5.4 Selenoproteins A (GrdA) and B (GrdB)

GrdA is a selenoprotein component of a multiprotein glycine reductase complex in certain bacteria [62]. This is currently the only known prokaryotic selenoprotein for which no Cys homologs have been detected [38]. GrdB is a selenoprotein component of multiprotein complexes involved in the reduction of glycine, sarcosine, betaine, and other substrates [63–65]. GrdB proteins are substrate-specific and bind a single GrdA.

9.5.5 Thioredoxin-Like Selenoproteins

Peroxiredoxins (Prxs), thioredoxins (Trxs), and glutaredoxins (Grxs) are abundant Cys-containing proteins that are present in essentially all organisms. However, some bacteria contain Sec-containing forms of these proteins [15, 16, 66–68]. Especially in bacteria, there are a variety of selenoproteins of the thioredoxin fold with distant homology to Prx, Trx, or Grx.

9.5.6 HesB-Like

This distant homolog of HesB proteins (also known as IscA) is a selenoprotein only present in certain archaea and bacteria [15]. HesB/IscA proteins are involved in iron-sulfur cluster biosynthesis, but the function of their selenoprotein homolog has not been characterized.

9.5.7 Additional Prokaryotic Selenoproteins

Additional prokaryotic selenoproteins are listed in Fig. 9.1. Most of these proteins are homologs of thiol-dependent oxidoreductases, in which the catalytic Cys is replaced with Sec [15, 16]. There are also numerous predicted bacterial selenoproteins of unknown function [68, 69].

9.6 Selenoprotein Functions

From the brief description of selenoprotein functions, it is apparent that selenoproteins for which functions are known are oxidoreductases. In these proteins, Sec is the catalytic residue that is employed because it is superior to Cys in this function [38]. In selenoproteins, Sec reversibly changes its redox state during catalysis. Functions of many selenoproteins, particularly those found in vertebrates, are not known. However, by analogy to proteins with known functions, it may be expected that the majority of these uncharacterized selenoproteins are also oxidoreductases.

All selenoproteins may be loosely clustered into three protein groups. The most abundant selenoprotein group includes proteins containing Sec in the N-terminal region or in the middle of the protein. Many of these selenoproteins exhibit thioredoxin or thioredoxin-like folds, but some proteins (e.g., SelD, MsrA) show different folds. In these proteins, Sec is the catalytic group, which often works in concert with a resolving Cys.

In the second group, Sec is located in the C-terminal sequences. These proteins so far have been described only in eukaryotes and include selenoproteins K, S, O, I, and TRs. Except for TRs, the function of Sec in selenoproteins in this group is not known.

Selenoproteins in the third group utilize Sec to coordinate redox metals (Mo, W, Ni) in the active sites of these proteins. This protein class includes hydrogenase, FDH, and FMDH.

However, non-catalytic functions of Sec, while rare, may be expected. Known examples include Sec residues in the C-terminal region of SelP (they function to transport Se) and recently evolved Sec residues in the *Metridium senile* MsrB homolog (the function of these Sec residues is not known) [70].

9.7 Selenoproteomes

Bioinformatics analyses allowed the identification of all or almost all selenoproteins in a variety of organisms [71]. The data involving full sets of selenoproteins in organisms (selenoproteomes) provide an opportunity to address numerous questions relevant to the biology of Se. This information helps explain the biological and biomedical effects of dietary Se. This is because it is now possible to link individual selenoproteins or selenoprotein groups with the specific effects of dietary Se. In this respect, Se is ahead of the studies involving other trace elements (as well as vitamins and other biofactors) where new proteins are still discovered biochemically (and often by accident) and where full sets of proteins dependent on a particular biofactor is difficult to ascertain.

Searches of the nematode selenoproteomes revealed that *C. elegans* and *C. briggsae* have only a single UGA codon that codes for Sec in their genomes [72]. This codon is present in the TR1 gene, and phylogenetic analyses suggested that

other selenoprotein genes were lost in these nematodes during evolution. Recently, the first selenoproteinless animals were identified, all of which are arthropods (mostly insects, such as beetles and silkworms) [73, 74]. Information about such animals (or other organisms that lost selenoproteins, such as yeast and higher plants) helps explain the changing requirements for Se during evolution. For example, selenoproteinless insects lost the entire Sec insertion machinery, but preserved SPS1, suggesting that this protein does not provide a Se intermediate for Sec biosynthesis [73]. It is an exciting possibility that SPS1 is involved in some other Se-dependent pathway.

Recent characterization of selenoproteomes of nematodes [71], fruit flies [12, 13], mammals [14], other vertebrates [51, 52], Apicomplexan parasites [54], and numerous other organisms including bacteria and archaea [15, 68–75], provided many clues with regard to the use of Se in these organisms. A recent study identified 57 selenoproteins in the harmful alga *Aureococcus anophagefferens*, which is currently the eukaryote with the largest number of selenoprotein genes [76]. Among prokaryotes, this record belongs to a symbiotic deltaproteobacterium of the gutless worm *Olavius algarvensis*, which also has 57 selenoprotein genes [77].

Rapid progress in genome sequencing should allow application of bioinformatics tools to many additional genome projects. It should be noted that environmental genome projects are also amenable to these applications [16]. For example, one recent study characterized the selenoproteome of the microbial marine community derived from the Global Ocean Sampling expedition [69]. More than 3,600 selenoprotein gene sequences belonging to 58 protein families were detected. Geographic location had little influence on Sec utilization, but higher temperature and marine (as opposed to freshwater and other aquatic) environments were associated with the increased use of this amino acid. This study provided insights into global trends in microbial Se utilization in marine environments.

Selenoproteome analyses also are capable of uncovering trends in the use of Sec [78], although some limitations of this approach have been described [79]. An analysis of selenoproteomes of several model eukaryotes detected 26–29 selenoprotein genes in two species of *Ostreococcus*, 5 in the social amoebae *Dictyostelium discoideum*, and 16 in the diatom *Thalassiosira pseudonana*, including several new selenoproteins [78]. Further analyses identified massive, independent selenoprotein losses in land plants, fungi, nematodes, insects, and some protists. Comparative analyses of selenoprotein-rich and -deficient organisms revealed that aquatic organisms generally have large selenoproteomes, whereas several groups of terrestrial organisms reduced their selenoproteomes through loss of selenoprotein genes and replacement of Sec with Cys. These observations suggested that many selenoproteins originated at the base of the eukaryotic domain and showed that the environment may play a role in selenoproteome evolution. In particular, aquatic organisms apparently retained and sometimes expanded their selenoproteomes, whereas the selenoproteomes of some terrestrial organisms were reduced or completely lost. It is an interesting possibility that aquatic life supports Se utilization, whereas terrestrial habitats lead to reduced use of this trace element [78].

In a separate study involving vertebrates, reconstruction of evolutionary changes in the Se transport domain of SelP revealed a decrease in the Sec content specifically in the mammalian lineage via replacement of Sec with Cys [41]. Compared to mammals, fish showed higher Sec content of SelP, larger selenoproteomes, elevated SelP gene expression, and higher levels of tissue Se. In addition, mammals replaced Sec with Cys in several proteins and lost several selenoproteins altogether, whereas such events were not found in fish. These data suggested that evolution from fish to mammals was accompanied by decreased use of Sec and that analyses of SelP, selenoproteomes, and Sec/Cys transitions provide a genetic marker of utilization of this trace element in vertebrates. The evolved reduced reliance on Se raises questions regarding the need to maximize selenoprotein expression by Se dietary supplements in situations when pathology is not imminent, a currently accepted practice.

9.8 Applications of Selenoproteome Analyses to Biology

9.8.1 Genetic Code Supports Targeted Insertion of Two Amino Acids by One Codon

Strict one-to-one correspondence between codons and amino acids was thought to be an essential feature of the genetic code. However, a recent selenoproteome analysis of the ciliate *Euplotes crassus* revealed that one codon can code for two different amino acids with the choice of the inserted amino acid determined by a specific 3′-untranslated region and location of the dual-function codon within the mRNA [80]. It was found that the codon UGA specified insertion of Sec and Cys in *E. crassus*, that the dual use of this codon could occur even within the same protein, and that the structural arrangements of *Euplotes* mRNA preserved the location-dependent dual-function of UGA when expressed in mammalian cells. Thus, the genetic code supports the use of one codon to code for multiple amino acids. This finding challenged one of the foundations of the code, i.e., that a genetic codeword is used only for one amino acid in an organism.

9.8.2 High-Throughput Identification of Catalytic Redox-Active Cysteine (Cys) Residues

Cys residues often play critical roles in proteins; however, identification of their specific functions has been limited to case-by-case experimental approaches. A recent study developed a procedure for high-throughput identification of catalytic redox-active Cys in proteins by searching for sporadic Sec/Cys pairs in sequence databases [81]. This method was independent of protein family, structure, and taxon. It was used to selectively detect the majority of known proteins with redox-active Cys and

to make additional predictions, one of which was verified. Rapid accumulation of sequence information from genomic and metagenomic projects, coupled with selenoproteome analyses, should allow detection of many additional oxidoreductase families as well as identification of redox-active Cys in these proteins.

9.9 Conclusions

Fifteen years ago, only several selenoproteins were known. Largely due to remarkable progress in genomics, we now know approximately 100 selenoprotein families. This information allows researchers to study various aspects of Se biology and selenoprotein functions and address questions, not even imaginable until only recently, such as geographical distribution of selenoprotein utilization or expansion of the genetic code. In selenoproteins with known functions, Sec is a key functional group that carries out redox catalysis. Further studies of selenoproteins and selenoproteomes should help explain known biological and biomedical effects of Se and identify new biological processes and pathways dependent on this trace element.

Acknowledgements This work was supported by National Institutes of Health grants.

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Chapter 10
Structural Characterization of Mammalian Selenoproteins

Stefano M. Marino, Vadim N. Gladyshev, and Alexander Dikiy

Abstract Structural analysis of proteins is a highly informative approach to assess protein function and regulation. It can help establish catalytic mechanisms of enzymes and visualize the structural basis for their interactions with substrates and partner proteins. To date, the following mammalian selenoproteins have been structurally characterized either by X-ray crystallography or nuclear magnetic resonance spectroscopy: the 15 kDa selenoprotein, glutathione peroxidases 1, 2, 3 and 4, selenophosphate synthetase 2, selenoproteins M and W, methionine sulfoxide reductase B1, and thioredoxin reductases 1 and 3. For structural analysis of most of these proteins, the catalytic selenocysteine was mutated to cysteine or glycine, allowing high protein expression. These structures and dynamic properties of selenoproteins verified the dominance of thioredoxin fold in mammalian selenoproteins and yielded critical insights into their functions and catalytic mechanisms.

10.1 Structure-Function Characterization of Proteins

Numerous genome sequencing projects yielded tens of millions of protein sequences. Some of these proteins are well known and characterized; however, the majority correspond to unknown proteins (both structurally and functionally). One of the major challenges scientists face (and will continue to face in the future) is to assign functions to each protein and protein family identified through genome sequencing projects. Analysis of protein function is considerably more complicated

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than gene and genome sequencing, as it requires integration of many different, yet synergistic, technologies.

Structural biology emerged as one of the core areas of the life sciences because three-dimensional structures of proteins provide many functional insights [1, 2]. Functional assignments through their structural comparison with proteins of known structure and function prove particularly valuable, since many functional similarities escape detection by sequence-based approaches [3]. The concept of determining protein structure on a genome-wide scale is called structural proteomics. Its methods include X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy on the experimental side, and molecular modeling (e.g., homology modeling, docking to identify substrates for proteins of unknown function) and functional inferences (e.g., functional associations, such as using the STRING database) on the computational side.

10.2 Structural Characterization of Selenoproteins

Mammalian genomes contain 24 or 25 selenoprotein genes. Their selenium (Se)-containing amino acid, selenocysteine (Sec), is co-translationally inserted into nascent proteins in response to the UGA codon, which is also the signal designating translation termination. UGA codes for Sec only if the mRNA contains a characteristic stem-loop structure, the SECIS element, in its 3′-untranslated region, and this process also requires several components of the Sec insertion machinery, including $tRNA^{[Ser]Sec}$, elongation factor, EFsec, and SECIS-binding protein 2. In bacteria, SECIS elements are located immediately downstream of UGA (i.e., within coding regions of selenoprotein genes). Thus, the majority of eukaryotic selenoproteins cannot be expressed in bacteria without changes in the coding sequences creating SECIS elements. Exceptions are selenoproteins with Sec in the C-terminal regions, such as thioredoxin reductase, which can be expressed in bacterial cells (its Sec is a C-terminal penultimate amino acid, allowing introduction of the SECIS element downstream of the stop codon). Undoubtedly, this limitation considerably affected structure analyses of selenoproteins: both X-ray crystallography and NMR spectroscopy require large amounts of proteins (at least 10–20 mg of a protein are typically needed for full structural analysis). The inability to use bacterial hosts for production of sufficient amounts of selenoproteins led to the use of their cysteine (Cys)-containing forms (or glycine-containing forms). The only exceptions include thioredoxin reductase 1 (TR1) and glutathione peroxidase 1 (GPx1), whose structures include the actual Sec.

To date, the following mammalian selenoproteins have been structurally characterized: the 15 kDa selenoprotein (Sep15), glutathione peroxidases 1 (GPx1), 2 (GPx2), 3 (GPx3), and 4 (GPx4), selenophosphate synthetase 2 (SPS2), selenoproteins M (SelM) and W (SelW), methionine sulfoxide reductase B1 (MsrB1), and thioredoxin reductases 1 (TR1, also called TrxR1 or TxnRd1) and 3 (TR3, also called TrxR2 or TxnRd2). A graphical overview of all mammalian selenoprotein structures is shown in Fig. 10.1.

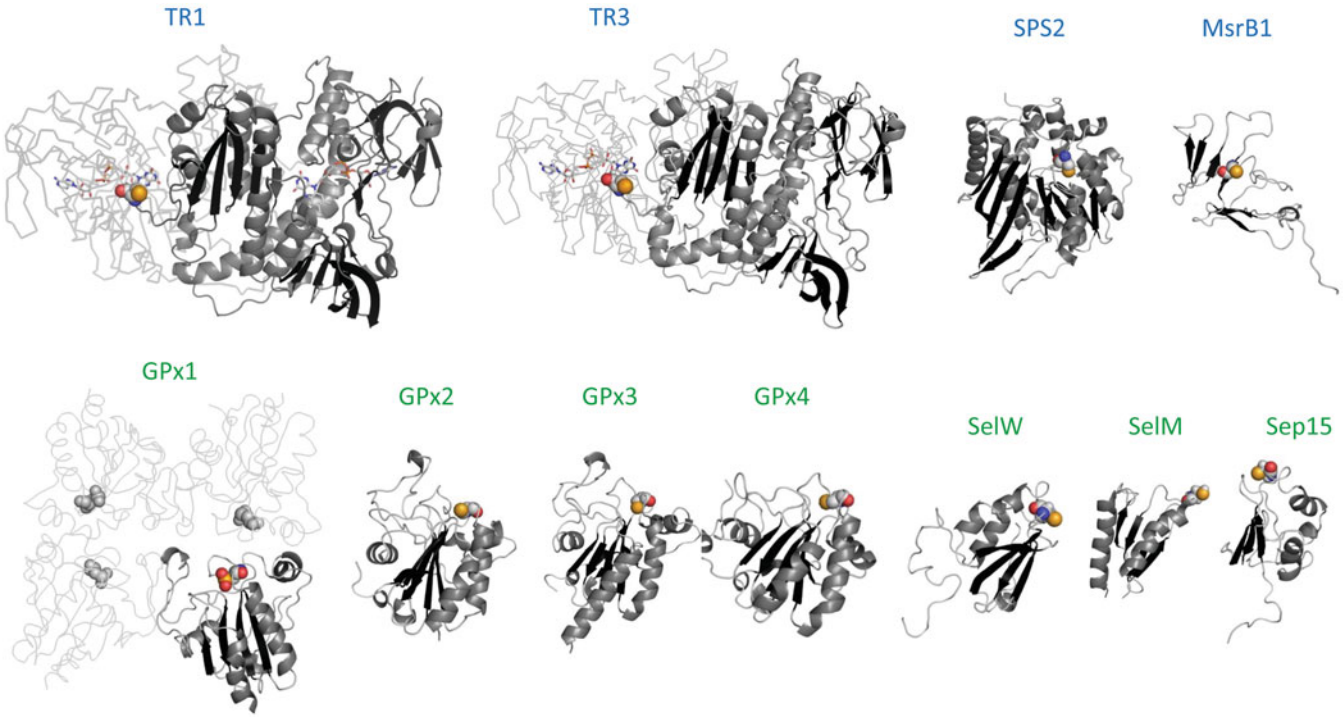

Fig. 10.1 Three-dimensional structures of mammalian selenoproteins. Selenoprotein names are shown above each structure. Helixes are shown in *dark gray*, strands in *black* and coils in light *gray*. For each protein, the catalytic Sec/Cys is shown as space fills. Selenoproteins with the thioredoxin fold are labeled with names in *green*, and their structures are reported in the *bottom panel*. Mammalian TR1 and TR3 are homodimers. In the figure, one monomer is reported in ribbon representation and a color scheme, while the backbone atoms of the other monomer are shown in *gray*. The C-terminus of TR3 (pdb code 1zdl) was modeled based on the structure of human TR1 (3ean). The GPx1 quaternary structure is derived from the assembly (1gp1). GPx2 and GPx3 are also natural homo-tetramers (not shown in the figure). GPx1 has been crystallized with its Sec overoxidized

The most represented structural fold among selenoproteins is the thioredoxin fold (SelW, SelM, Sep15 and all four GPxs), followed by pyridine nucleotide disulfide oxidoreductases (all TRs). Below, we review each mammalian selenoprotein with a known 3D structure.

10.2.1 Thioredoxin Reductases

TRs maintain their thioredoxin substrates in the reduced state and also reduce a variety of small compounds with NADPH as the electron donor. The thioredoxin system, along with the glutathione system, are the major systems of cellular redox homeostasis [4, 5]. All mammalian TRs are selenoproteins, so the entire mammalian thioredoxin system is dependent on Se.

10.2.1.1 Cytosolic TR1

Initially, an X-ray structure of rat cytosolic TR1 (truncated enzyme lacking Sec) was described [6]. More recently, a recombinant TR1 expressed in bacteria was structurally analyzed that had Sec inserted with the help of an engineered SECIS element [7]. TRs are homodimers with two subunits arranged in a "head-to-tail" manner. Their overall topology is similar to that of other pyridine nucleotide disulfide oxidoreductases, particularly glutathione reductases [6–8]. The structural features revealed by crystallographic analyses of both truncated (pdb code 1h6v) [6] and Sec-containing (pdb codes for reduced and oxidized forms are 3ean and 3eao, respectively) [7] forms are very similar: root mean square deviation (RMSD) value from backbone atom comparison is 0.4–0.5 Å. These studies showed, besides the obligatory "head-to-tail" arrangement, that the redox-active C-terminal tail of one subunit interacts with the N-terminal active site of the other subunit (Fig. 10.1). Each subunit of TR1 consists of three domains: FAD-binding (residues 1–163 and 297–367, rat TR1 numbering), NADPH-binding (residues 164–296), and the interface (residues 368–499) domains. The FAD- and NADPH-binding domains have similar structural organizations: each contains a central five-stranded parallel β-sheet and a three-stranded β-meander that is packed against the larger β-sheet. The other side of the parallel sheet is covered by several α-helices. These domains bind FAD and NADPH in a manner which is characteristic of this fold. The active disulfide is formed between Cys59 and Cys64: it is located in helix α2, within the FAD domain, a feature in common with other pyridine nucleotide disulfide oxidoreductases [8]. Interestingly, structural analyses confirmed that mammalian TR1 is more closely related to glutathione reductases than to prokaryotic TRs, in which the redox-active disulfide is located within the NADPH-binding domain. This observation is in excellent agreement with previous findings, derived from amino acid sequence analyses and evolutionary comparison of pyridine nucleotide disulfide oxidoreductases [9].

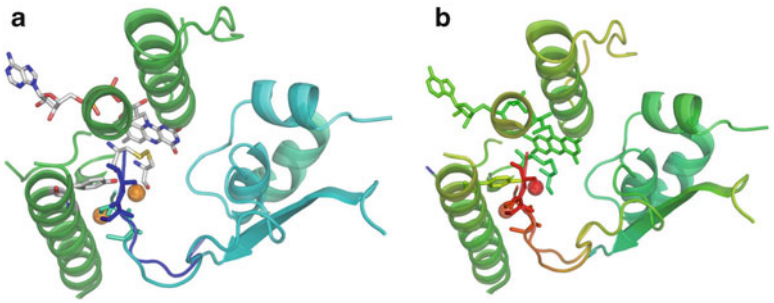

Fig. 10.2 The active site of TRs and the mobility of the C-terminal region. (**a**) Simplified view (only amino acids within 20 Å from the catalytic Sec are shown) of the "head-to-tail" arrangement of rat TR1. The two monomers are shown in cartoon representation. The N-terminal part of one monomer is shown in *green ribbons* (the N-terminal redox center is shown in *sticks*). The C-terminal part of the second monomer is shown in *cyan colored ribbons*. The C-terminal redox center (GCUG) is shown in *sticks*, colored in *cyan* (C and U in *cis*-conformation, oxidized protein, pdb code 3eao) or in *blue* (C and U in *trans*-conformation, reduced protein, pdb code 3ean). (**b**) The same arrangement as in (**a**), but colored according to B-factors. The color scheme ranges from *red* (high mobility, reflected by high B-factor values), to *blue* (low mobility, low B-factor), passing through *yellow* and *green*

The third domain of mammalian TR1, the interface domain, contains an antiparallel five-stranded β-sheet flanked on both sides by four helices. This domain participates in subunit–subunit interaction and forms a large part of the dimer interface.

The C-terminal extension with the characteristic Gly-Cys-Sec-Gly motif carrying the essential Sec residue is a signature feature of mammalian TRs. The structural peculiarities of this C-terminal motif can be elucidated by the analysis of structures corresponding to the native protein in its reduced and oxidized forms (pdb codes 3ean and 3eao, respectively). As it appears from this analysis, the last six residues are highly flexible (Fig. 10.2) with an ensemble of possible conformations, particularly in the reduced form of the enzyme. It was suggested that the mobile C-terminal region not only serves as a third redox-active group, but it also blocks access of oxidized glutathione to the N-terminal redox Cys couple [10]. However, only weak electron density was observed for the C-terminal motif, and thus its positioning is not well determined, which is reflected by very high B-factor values of its atoms. Besides the intrinsic mobility of this region, an additional important feature is the *cis* to *trans* movement of Sec, in respect to the flanking Cys residue. In the reduced state, the functional group of Sec498 points away from the thiol of Cys497 (Fig. 10.2), whereas in the oxidized form, Sec flips on the side of Cys497 so that different redox states of TR1 (reduced or oxidized) are coupled with a significant relocation of Sec498 relative to Cys497. This movement has been proposed as a sort of shuttling from the N-terminal redox center to the exterior part of the protein. In this way, the enzyme can interact with its substrate, thioredoxin [11]. A closely located Tyr116 assists in this interaction. Based on the analysis of available X-ray structures of native rat TR1, a catalytic mechanism was proposed for this important oxidoreductase [7].

10.2.1.2 Mitochondrial TR3

Crystallographic structures of the Sec to Cys variant of mouse mitochondrial TR3 in its reduced and oxidized forms (pdb codes are 1zdl and 1zkq, respectively) were determined by molecular replacement [12] using rat cytosolic TR1 as a probe [6]. This fact by itself indicates that the structures of TR1 and TR3 are rather similar. Indeed, the RMSD value for comparison of protein backbones is 0.79 Å, indicating that the main structural features are well maintained in both enzymes (Fig. 10.1), particularly in relation to the overall architecture of the active sites. The Gly-Cys-Sec-Gly motif in the TR3 structure was modeled into the active site of the enzyme [12]. As expected, this model showed that the C-terminal region is positioned in the active site of the opposing subunit, as is in the case of TR1 [6, 7]. According to this model, the Se atom of selenocysteine is nearly equidistant from the sulfur of Cys86 (numbering based on mouse TR3 sequence) and the epsilon nitrogen of His497* (asterisk denotes that this residue belongs to the other subunit of TR3). Similarly, the sulfur of Cys522* is adjacent to the epsilon nitrogen of His143. The model of the C-terminal tail of TR3 is consistent with Sec523* interacting with the active site dithiol/disulfide via Cys86. After reduction of the selenenylsulfide, the resulting selenolate attacks the disulfide of the oxidized thioredoxin. Overall, the catalytic mechanism of TR3 is very similar to that proposed for TR1 [7, 12].

10.2.2 SPS2

Incorporation of Sec into proteins requires the generation of an intermediate reactive Se donor compound, selenophosphate [13, 14]. Selenophosphate synthetases (SPSs) are enzymes responsible for the synthesis of selenophosphate by activating selenide with ATP. These proteins are strictly conserved in organisms that utilize Se, i.e., organisms that use Sec or selenouridine. Animals possess two SPSs (SPS1 and SPS2); however, only the latter is capable of synthesizing selenophosphate [15, 16]. Interestingly, in most animals, SPS2 is itself a selenoprotein. It exhibits a di-kinase activity wherein selenide and ATP are converted to selenophosphate, orthophosphate, and AMP. Mutational analyses revealed that Cys17 (or Sec13, numbering of *Escherichia coli* and *Aquifex aeolicus* sequences, respectively) and Lys20 (Lys16) are essential for SPS activity. In the case of SPS2, structural analysis was only possible with the Cys mutant [17]. Nevertheless, these studies provided important insights into the structure and function of this protein. SPS2 exhibits a mixed α/β fold, which is typical of PurM superfamily members. It consists of two halves represented by N-terminal and C-terminal domains (residues 1–156 and 157–336, respectively) (pdb code 2yye) [17]. The N-domain is composed of a six-stranded mixed β-sheet flanked by three α-helices and two 3^{10}-helices. The C-terminal domain has a seven-stranded mixed β-sheet, eight α-helices, and three 3^{10}-helices. A steep 30 Å-long channel is formed between the two domains, which seems to serve as the binding/catalytic site for the substrates. The channel is covered by the N-terminal turn-rich segment (residues 1–45), which shows high intrinsic flexibility,

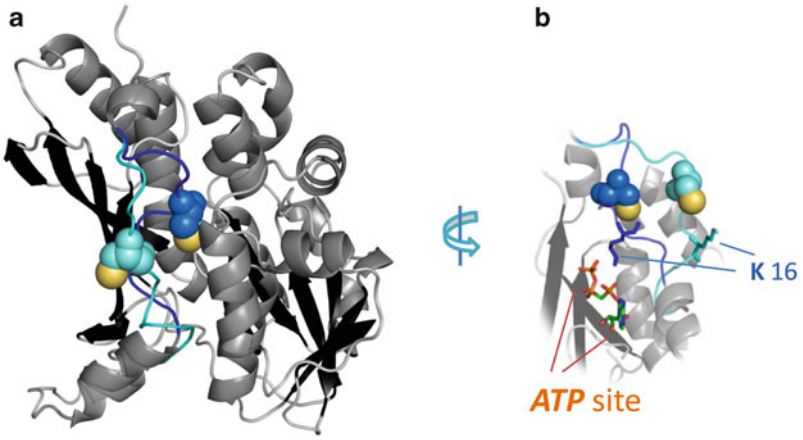

Fig. 10.3 Mobility of the N-terminal region of SPS2. (**a**) The structure of SPS2 is shown in *cartoon*, with the catalytic Sec shown in space fills. (**b**) Detailed view of the active site (*blue* colored loop, with Sec in space fills) and of the ATP (AMP-PP, represented in *sticks*) binding site. The catalytic N-terminal loop can shuttle between two "extreme" positions: (1) inward, going towards the ATP binding site, and (2) outward, reaching the solvent and going further away from the ATP, as shown by the *cyan colored loop* in (**a**) and (**b**). A fundamental feature of the shuttling trajectory is that it moves Sec13 and Lys16 (numbering of *Aquifex aeolicus* SPS2, pdb code 2yye) in and out of the phosphate donor pocket. These residues are essential for catalysis: Lys16 stabilizes the interaction with ATP, and Sec is the catalytic nucleophile [15]

with large portions of this segment being disordered. The catalytically crucial residues Sec/Cys13 and Lys16 reside on this mobile segment. During the movement, these residues can approach ATP accommodated in the channel (Fig. 10.3). Interaction of the charged nitrogen of Lys16 provides a crucial binding for one of the negatively charged phosphate groups of ATP. According to the proposed reaction mechanism, the ability of Sec/Cys to move would be an essential feature of catalysis, as Sec could attack hydrogen selenide from the outside and then, after the Sec(Se)-Se bond is formed, bring in the newly bound Se, which subsequently nucleophilically attacks one of the phosphodiester bonds of ATP. As in the case of TRs, the structural analysis of SPS2 provided very valuable information on the reaction mechanism of this selenoprotein.

10.2.3 MsrB1

Methionine sulfoxide reductases (Msr) are thiol-based oxidoreductases in which either Cys or Sec function as catalytic residues. These enzymes are classified into two families, MsrA and MsrB, according to their substrate specificity. MsrA catalyzes the reduction of the s-form of methionine sulfoxide (Met-s-SO), whereas MsrB can only reduce the R-form (Met-R-SO). These two enzyme families reduce methionine sulfoxides in proteins, but MsrA can also reduce free Met-s-SO. Msrs

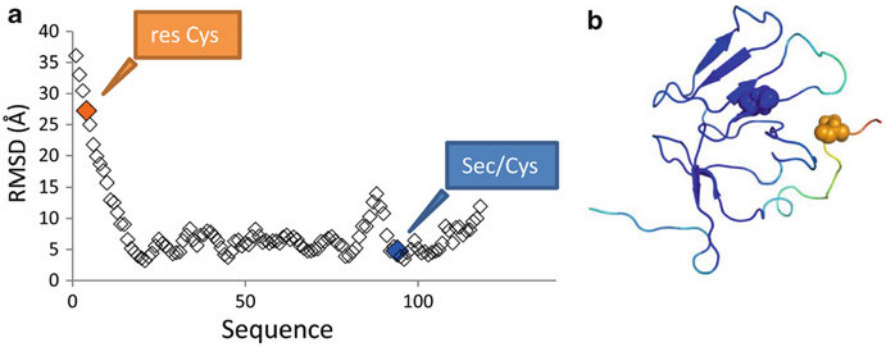

Fig. 10.4 Mobility of the N-terminal region of MsrB1. (**a**) The average RMSD, calculated for carbon α atoms, in the pairwise comparison of all structures present in the NMR ensemble of mouse MsrB1 (18). The N-terminal region, including Cys4 (the resolving Cys, "res Cys" in the figure), is considerably more mobile than the rest of the protein. (**b**) Visual representation of the values calculated in (**a**), as plotted on the structure of mouse MsrB1 (shown in *cartoon*; color code: *red* higher mobility, *blue* lower mobility, *green* intermediate). The N-terminal resolving Cys is shown in space fill

are important repair proteins that protect cells against oxidative stress and have been implicated in delaying the aging process and protecting against neurodegeneration [18, 19]. One of four mammalian MsrBs, MsrB1 (also called SelR or SelX), is a selenoprotein.

Recently, a family of NMR solution structures of mouse selenoprotein MsrB1, in which Sec95 was replaced with Cys, was reported (pdb code 2kv1) [20]. The structure is characterized by an overall β-fold consisting of eight all antiparallel β-strands. The structure is of ellipsoidal shape and consists of two antiparallel β-sheets. The first β-sheet is three-stranded, forming the backside of the structure, while the second sheet has five strands forming the front side. The active site is situated in the second β-sheet. The N-terminal region contains two hinge sections defined by Gly8-Gly9 and Pro18-Gly19 pairs followed by the backside β-sheet consisting of strand β_1 (residues 19–23), β_8 (residues 100–104), and β_2 (residues 28–30) in the listed order. The C-terminal flexible region comes out of the middle of the backside β-sheet. The front side β-sheet is connected in the following order: β_3 (45–48), β_7 (93–96), β_6 (77–82), β_5 (66–72), and β_4 (55–60). This sheet forms the hydrophobic core through residues Leu67, Val69, Phe94, and Ile96, linking to the backside β-sheet hydrophobic Tyr21, Phe31, and Phe103 at the bottom of the structure. The top part of MsrB1 is held together through tetrahedral structural zinc, in which the metal ion is bound to the protein matrix by Cys23, Cys26, Cys71, and Cys74. A disordered region consisting of 13 residues (amino acids 31–44) between β_2 and β_3 strands connects their front side and backside β-sheets of the protein.

MsrB1 mobility data indicate that its N- and C-terminal regions are largely unstructured (since they are intrinsically flexible), with the N-terminus being particularly mobile (Fig. 10.4). It was suggested that the increased mobility of these

regions is needed during catalysis to form an intermediate selenenylsulfide [20]. It is also possible that flexibility of these regions promotes interactions between MsrB1 and its substrates.

On the basis of the MsrB1 structure, mobility studies and analysis of interactions of the enzyme with its substrate and inhibitor, the N-terminus was proposed to play a crucial role in MsrB1 structure and function: its unstructured nature and hydrophobic compatibility with the active site [20] allow it to move toward the reaction center in response to substrate binding. Therefore, MsrB1 can regulate the completion of its catalytic cycle by modulating access of the resolving Cys4 to the active site. Thus, even though the high mobility is not related to Sec (as in TR and SPS2, i.e., it relates to the N-terminal resolving Cys), high flexibility seems to be a common structural feature associated with Se-based enzymatic catalysis.

10.2.4 GPxs

GPxs are critical components of the mammalian system that controls hydroperoxide levels [21]. These enzymes reduce peroxides at the expense of reduced glutathione and/or other reductants. Mammalian GPxs are classified into several subfamilies, which are numbered consecutively from 1 to 8 [22, 23]. GPx1, GPx2, GPx3, and GPx4 are invariantly Se-containing enzymes (i.e., they possess the active site Sec nucleophile). GPx6 is a selenoprotein in most mammals, but in many rodents it is a Cys-containing protein. GPx5, GPx7, and GPx8 are Cys-containing proteins in all mammals. X-ray structures were determined for native bovine GPx1 (pdb code 1gp1), Sec to Gly mutants of human GPx1 (2f8a), GPx3 (2r37) and GPx4 (2gs3), and Sec to Cys mutants of human GPx2 (2he3) and GPx4 (2obi). All GPxs have a rather similar structure; therefore, in the following paragraphs, we focus on just one of them, human GPx4, which is one of the best characterized GPxs.

The structural model characterizes GPx4 (Fig. 10.1) as a monomeric protein composed of 170 amino acids. In contrast, GPx1, GPx2, and GPx3 are tetramers (Fig. 10.1, note that the structure of only one the fully assembled tetramer is available, i.e., the structure of GPx1). Structures of all GPxs show a typical thioredoxin fold [24, 25] consisting of four α-helices on the protein surface and seven β-strands, five of which are clustered to form a central β-sheet (Fig. 10.1). The catalytic triad consisting of Sec46, Gln81, and Trp136 is positioned on the protein surface [25, 26].

Sequence alignments of GPxs indicated strict conservation of the catalytic triad: mutations of any of these residues impaired enzymatic activity [26, 27]. Additionally, a fourth residue (Asn137, numbering based on human GPx4) is also strictly conserved in GPxs and was proposed to assist in catalysis, forming the catalytic tetrad [22]. This amino acid is found in close proximity to the catalytic thiol/selenol of GPxs.

Structural analysis confirmed the importance of the tetrad residues in the modulation of GPx activity by establishing direct interactions (e.g., H-bonds) with the catalytic Cys (or Sec) [22]. Indeed, it was proposed that this H-bond network is responsible not only for structure stabilization, but also for lowering the pK_a of

the catalytic residue [22]. However, this hypothesis does not appear to justify the observation that Sec-containing GPxs, in spite of the intrinsic acidity of Sec (i.e., Sec pK_a, estimated in the range 5–6, is considered to be ~3 pH units lower than that of Cys), show complete conservation of all residues belonging to the catalytic tetrad. Thus, it is still not completely clear why the tetrad residues are strictly conserved in GPxs, regardless of whether they do or do not contain the very reactive Sec (i.e., Sec does not need the H-bond network with tetrad residues to lower its pK_a).

Irrespective of the overall structural similarity, some important differences between various Sec-containing GPxs were found. In particular, comparison of their structures revealed differences between GPx4 and other GPxs. GPx4 lacks an internal stretch of about 20 amino acids, which is present in all other GPxs [24]. This sequence forms a surface exposed loop (loop 1) that does not exist in GPx4. Interestingly, this loop structure lines the active site of other GPxs and partially shields Trp136, a constituent of the catalytic tetrad. It may be concluded that the occurrence of this loop structure limits the accessibility of complex lipid substrates to the active site of GPxs. Instead, the lack of this structural element in GPx4 may allow efficient binding of these molecules in the active site.

10.2.5 SelW

Rdx is a recently identified family of selenoproteins and Cys homologs [28]. Mammalian members of this family include selenoproteins SelW, SelT, SelH, SelV, and a Cys-containing Rdx12 protein. Functions are not known for any of these proteins. Through sequence analysis and structural modeling, it was predicted that these proteins possess a thioredoxin-like fold, suggesting a possible catalytic and redox-related function for their Sec (Cys) residues.

Among Rdx proteins, the best characterized is SelW. It is a 9 kDa selenoprotein, abundant in muscle and brain [29–32]. The structure of the Sec13Cys variant of mouse SelW was determined by high resolution NMR spectroscopy (pdb code 2npb, Fig. 10.1) [33]. It consists of a four-stranded β-sheet with two extended α helices and a short 3^{10} helix, all located on one side of the β-sheet. Besides these secondary structures, the protein contains two external loops and a type II turn. The molecule is characterized by a β_1(3–9)-α_1(15–28)-β_2(34–40)-β_3(48–52)-β_4(54–60)-3^{10}(61–63)-α_2(70–88) secondary structure pattern, wherein β_1 and β_2 are parallel strands forming a classical β_1-α_1-β_2 motif, typical of thioredoxin fold proteins. The axis of the α_2-helix is at an approximately 45° angle with respect to the axis of the α_1-helix as a consequence of the β-sheet topology. The CxxU motif is located in the loop (residues 10–14) between β_1 and α_1. The second loop (residues 40–47) separates β_2 and β_3 strands. β_3 and β_4 strands form a β-hairpin with a type II turn (residues 52–54) in between. The decreased structural resolution observed for the two loops may be a consequence of increased mobility of these regions with respect to the rest of the protein. Considering the already described role of high flexibility for other selenoproteins (TRs, SPS2, MsrB1), the latter observation suggests that, also in SelW, structural mobility has a functional role. Currently, however, the function of

SelW is unclear, with no experimental evidence supporting a model for its catalytic mechanism. Nevertheless, structural and computational analyses suggested a functional association with 14-3-3, wherein SelW might control the redox state of this protein [28, 33, 34].

10.2.6 Sep15 and SelM

The structures of Cys versions of two thioredoxin-like selenoproteins, Sep15 and SelM (Fig. 10.1), were determined using NMR spectroscopy [35] (pdb codes 2a4h and 2a2p, respectively). These proteins have Sec-containing CxxC-like motifs, suggesting that they may function as redox proteins within the endoplasmic reticulum. Both Sep15 and SelM contain a central α/β domain composed of three α-helices (α1–3) and a mixed parallel/antiparallel four-stranded β-sheet (β1–4). Structure-based multiple sequence alignments illustrate that SelM has a short N-terminal extension that precedes strand β1 and a flexible C-terminal extension after helix α3. In contrast, Sep15 has an elongated Cys-rich N-terminal extension before strand β1 and a shorter C-terminal extension after helix α3 that does not adopt a regular secondary structure. The highly flexible C-terminal regions of SelM (residues 121–145) and Sep15 (residues 150–178) may participate in binding protein substrates or other redox proteins and may assume a defined conformation after such interactions.

Sep15 and SelM are structurally related to the Rdx family members in having an overall thioredoxin-like fold and lacking the thioredoxin helix following the β_1-α_1-β_2 structural motif and two functionally relevant external loops. The second loop appears to be shorter in Sep15 and SelM, similarly to that in SelW. One difference between Rdx proteins on the one side and Sep15 and SelM on the other is that the α_1-helix in the latter proteins is divided into two smaller α-helices with a kink in between.

Sep15, SelM, and SelW lack charged and hydrophobic residues within the two external loops, which appears to be a characteristic feature of these proteins. On the other hand, thioredoxin, Sep15 and SelM, in contrast to SelW, do not have the aromatic cluster. Since the fold of all these proteins is roughly the same, it is possible to exclude the role of aromatic residues in maintaining their topology. Although the structures of SelM and Sep15 are available, their functions remain unknown.

10.3 Conclusions

The structures reviewed in this chapter clearly show the many significant contributions that structural biology brought to the Se field. In some cases, advancements in selenoprotein function would not have been possible in the absence of structural data. By employing a variety of tools, both experimental (X-ray crystallography, NMR spectroscopy) and computational (modeling, docking), structural biologists provided important insights into selenoprotein functions, physico-chemical properties and catalytic mechanisms. As an example, only through structural approaches it

was possible to detect (and characterize) the crucial role of protein mobility in the modulation of catalytic properties, as well as in the control of substrate specificity within selenoproteins. Altogether, structural biology provided substantial contributions to the current understanding of this very important class of proteins. Structural characterization of the remaining mammalian selenoproteins is the major objective for structural biologists involved in the Se field and, more broadly, redox biology.

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Chapter 11
Selenoproteins: Hierarchy, Requirements, and Biomarkers

Roger A. Sunde

Abstract Studies of the full selenoproteome have found that five, four, and one of 24 rodent selenoprotein transcripts in liver, kidney, and muscle, respectively, decrease in Se deficiency to <40% of Se-adequate levels, but that the majority of selenoprotein mRNAs are not regulated by Se deficiency. These differences match with the hierarchy of selenoprotein expression, helping to explain this hierarchy, and also showing that selenoprotein transcripts can be used as molecular biomarkers for assessing Se status. The similarity of the response curves for regulated selenoproteins suggests one underlying mechanism is responsible for the downregulation of selenoprotein mRNAs in Se deficiency, but the heterogeneity of UGA position in regulated and nonregulated selenoprotein transcripts now indicates that current nonsense-mediated decay models cannot explain which transcripts are susceptible to mRNA decay in Se deficiency.

11.1 Introduction

In 1972, Rotruck and Hoekstra [1] discovered the first identified biochemical role for selenium (Se) in the mammalian enzyme, glutathione peroxidase (Gpx). The activity of this enzyme decreases dramatically in Se deficiency, and increases during Se repletion, making Gpx1 a useful biochemical biomarker for Se status [2]. This was followed rapidly by identification of Se-dependent enzymes in bacteria [3] and then by the identification of selenocysteine (Sec) as the Se cofactor present and incorporated into the peptide backbone of these selenoproteins [4, 5]. These discoveries were followed grudgingly over the next 30 years by the identification of 17 additional selenoproteins in higher animals as well as in bacteria, including plasma

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selenoprotein P (Sepp1) containing ten Sec, all three mammalian deiodinases (Dio), all three mammalian thioredoxin reductases (Txnrd), and three additional Gpxs with unique tissue distribution or substrate utilization [6, 7]. In 2003, the elegant uncovering of the complete human and rodent selenoproteomes by Gladyshev and coworkers [8], including seven newly discovered selenoproteins, provided us with the blueprint to investigate Se regulation of the full selenoproteome [9].

Long before most of these selenoproteins were identified, Behne [10] recognized a "hierarchy" in the distribution of ^{75}Se between tissues, especially in Se-deficient animals, and furthermore recognized that within a tissue there is a "molecular hierarchy" such that Gpx1 is less labeled than other selenoproteins in Se deficiency. Hill and Burk [11] reported that Sepp1 and Dio1 mRNAs levels decrease less than Gpx1 mRNA levels in Se deficiency, and we reported that liver Gpx4 mRNA is not downregulated by Se deficiency, whereas Gpx1 mRNA falls to ~10% of Se-adequate levels [12], suggesting that transcript regulation might underlie this hierarchy. This review will discuss our recent studies using biochemical selenoenzyme activities as conventional biomarkers and selenoprotein transcript levels as molecular biomarkers to characterize the hierarchy of Se requirements, discuss current understanding of the mechanism(s) underlying this regulation of selenoprotein expression by Se status, and show the efficacy of using panels of molecular biomarkers (RNA levels) to predict Se status.

11.2 Se Requirements

11.2.1 Chemical and Biochemical Biomarkers of Se Status

When weaning rats are fed an Se-deficient diet (0.005 μg Se/g), liver Se concentrations fall to <3% of levels in Se-adequate (0.24 μg Se/g diet) rats (Fig. 11.1a). Se supplementation results in a sigmoidal response in liver Se concentration, with a plateau breakpoint at 0.08 μg Se/g diet (Table 11.1), and a plateau in liver Se extending from 0.08 to 0.24 μg Se/g diet. With supernutritional Se supplementation at 0.4 and 0.8 μg Se/g diet, however, liver Se levels increase above the plateau and are 70% higher at 0.8 μg Se/g than in rats fed 0.08–0.24 μg Se/g. In kidney, tissue Se falls to 12% of Se-adequate levels, and then increases hyperbolically to a concentration 70% higher than concentrations at 0.8 μg Se/g diet in Se-adequate kidney, and 50% higher than that in liver in the same animals [13]. Testes Se in Se deficiency, in contrast, only falls to only 60% of Se-adequate levels and is not significantly affected by dietary Se in the same animals [14]. These distinct Se-response curves clearly illustrate the distinct regulation of Se metabolism among these tissues.

Liver Gpx1 activity in Se-deficient rat liver falls to 2% of Se-adequate levels. With graded Se supplementation, liver Gpx1 activity rises sigmoidally to a defined plateau with a breakpoint at ~0.09 μg Se/g diet (Fig. 11.1b). These studies [13, 14] and many preceding studies [15–21] have established a Se requirement of 0.1 μg Se/g diet (1× requirement) as the minimum dietary Se necessary to achieve plateau levels of

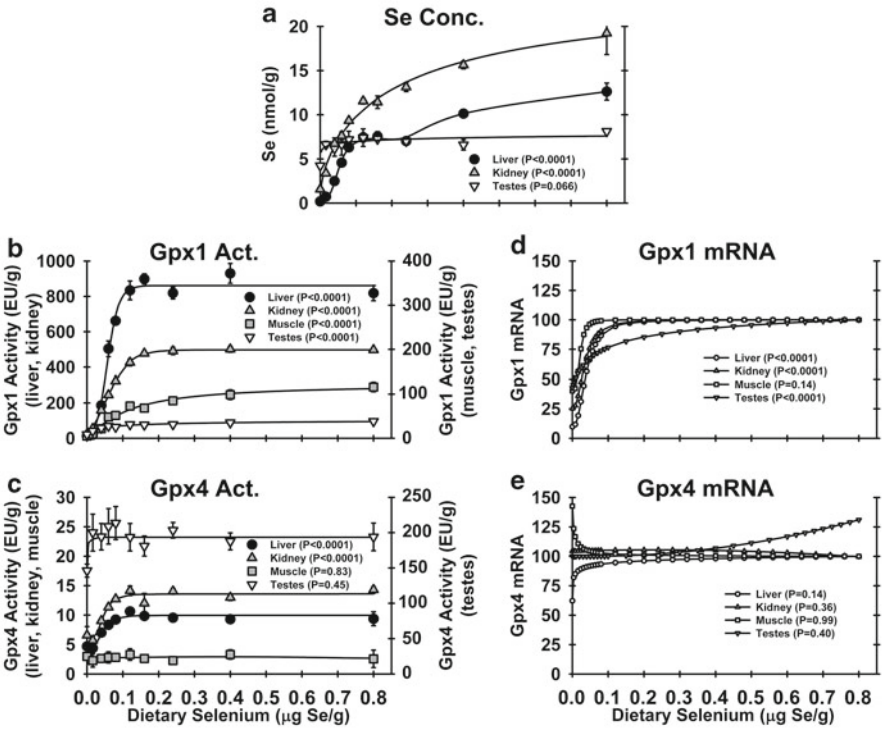

Fig. 11.1 Liver Se concentration (**a**), Gpx1 activity (**b**), Gpx4 activity (**c**), Gpx1 mRNA (**d**), and Gpx4 mRNA (**e**) in male weanling rats supplemented with the indicated levels of dietary Se (as Na_2SeO_3) for 28 days. Values for Se (**a**) and enzyme activities (**b**, **c**) are means ± SEM; *lines* for relative selenoprotein transcript levels (**d**, **e**) are resulting response curves calculated using sigmoidal or hyperbolic regression analysis. Legends indicate the level of significance by ANOVA. Figures redrawn from Barnes et al. [13] and Schriever et al. [14]

Table 11.1 Selenium requirement hierarchy in growing rats[a]

Biomarker	Minimum requirement[b] (μg Se/g diet)
Conventional biomarkers	
Growth	<0.01
Muscle Gpx4 act.	<0.01
Testes Se conc.	<0.01
Testes Gpx4 act.	<0.01
Testes Gpx1 act.	0.04
Plasma Gpx3 act.	0.06
Liver Gpx4 act.	0.06
Liver Txnrd act.	0.06
Kidney Gpx4 act.	0.07

(continued)

Table 11.1 (continued)

Biomarker	Minimum requirement[b] (μg Se/g diet)
Liver Se conc.	0.08
RBC Gpx1 act.	0.08
Liver Gpx1 act.	0.09
Kidney Se conc.	0.11
Kidney Gpx1 act.	0.12
Muscle Gpx1 act.	0.13
Molecular biomarkers	
Liver Gpx4 mRNA	<0.01
Kidney Gpx4 mRNA	<0.01
Muscle Gpx4 mRNA	<0.01
Testes Sepw1 mRNA	<0.01
Blood Gpx4 mRNA	<0.01
Kidney Sephs2 mRNA	0.02[c]
Muscle Gpx3 mRNA	0.02
Kidney Sepn1 mRNA	0.02
Liver Selk mRNA	0.03
Liver Selt mRNA	0.03
Liver Gpx3 mRNA	0.04
Liver Sepn1 mRNA	0.04
Liver Sepp1 mRNA	0.04
Testes Sepp1 mRNA	0.04
Muscle Selh mRNA	0.04
Kidney Selh mRNA	0.05
Muscle Sepw1 mRNA	0.05
Muscle Gpx1 mRNA	0.05
Liver Txnrd3 mRNA	0.05
Kidney Selk mRNA	0.05
Kidney Txnrd1 mRNA	0.05
Liver Selh mRNA	0.06
Kidney Sepw1 mRNA	0.06
Kidney Gpx3 mRNA	0.06
Kidney Gpx1 mRNA	0.06
Liver Sep15 mRNA	0.06
Liver Dio1 mRNA	0.06
Liver Sepw1 mRNA	0.07
Liver Gpx1 mRNA	0.07
Testes Gpx1 mRNA	0.08
Blood Gpx1 mRNA	0.08

[a]Table revised from Barnes et al. [13], Schriever et al. [14], and Sunde et al. [26]. Representative plots and ANOVA are provided for Gpx1 and Gpx4 biomarkers in Fig. 11.1

[b]Minimum dietary Se requirement for the growing rat as determined for each indicated biomarker. Requirements are the minimum dietary Se necessary for the indicated parameter to reach plateau levels when Se-adequate weanling rats are fed these diets from weaning, as determined by plateau breakpoint analysis

[c]Kidney Sephs2 mRNA was significantly upregulated by Se deficiency

Gpx1 activity in rodent liver. In contrast to liver Se, supernutritional Se supplementation above this requirement does not further elevate liver Gpx1 activity. Similarly, kidney and muscle Gpx1 activities fall to 5 and 6%, respectively, of Se-adequate levels, and reach plateau breakpoints at 0.12 and 0.13 μg Se/g diet, respectively. Similar to liver Gpx1 activity, supernutritional dietary Se does not raise Gpx1 activity above Se-adequate levels in kidney or muscle. Se response curves similar to those in Fig. 11.1b are also found for plasma Gpx3 activity [17], liver Txnrd activity [18], Sepp1 levels [19], Dio activity [20], and selenoprotein W (Sepw1) levels [21], indicating that the minimum dietary Se requirement is 0.1 μg Se/g diet.

Assay of testes Gpx1 activity is problematic because of the high Gpx4 activity (see below). Specific analysis for Gpx1 activity in testes shows that Gpx1 activity falls in Se deficiency to 27% of Se-adequate levels (Fig. 11.1b) with a plateau breakpoint of 0.04 μg Se/g, clearly showing unique regulation of Gpx1 expression in testes compared to these other tissues [14].

Gpx4 activity in liver, in contrast to Gpx1 activity (Fig. 11.1c), only decreases in Se deficiency to 25–50% of Se-adequate levels, and reaches the plateau at 0.05–0.06 μg Se/g diet [12]. Kidney Gpx4 activity is ~50% higher than liver Gpx4 activity but is similarly regulated by dietary Se. Muscle Gpx4 activity is not significantly decreased in Se-deficient rats, and in Se-supplemented rats, muscle Gpx4 activity is ~25 and 20% of levels in liver and kidney, respectively. In distinct contrast, Gpx4 activity in rat testes reaches 200 EU/g protein or 20-fold higher than in liver, and thus can contribute significantly to apparent Gpx1 activity unless corrected for [14]. As in muscle, testes Gpx4 activity is not significantly downregulated by Se deficiency in this rat model. This further demonstrates the hierarchy of Se distribution that Behne first described, both between tissues and within tissues.

11.3 Molecular Biomarkers of Se Status

11.3.1 Se Regulation of Selenoprotein Transcriptome

In 1988, we reported [22] that Gpx1 mRNA levels also drop dramatically in Se deficiency in rats, increase sigmoidally with increasing dietary Se, and reach well-defined plateaus [12, 15, 16, 23], providing a molecular biology-based biomarker for Se status. To evaluate Se requirements and Se regulation of the full selenoprotein transcriptome, we fed weanling rats ten graded levels of dietary Se from 0 to 0.8 μg Se/g diet for 28 days and used qRT-PCR to assess Se regulation of mRNA levels [13, 14]. Initial screening for all 24 rodent selenoprotein mRNAs for potential regulation by Se status found that five, four, and one of 24 rodent selenoprotein mRNAs in liver, kidney, and muscle, respectively, decrease to <40% of Se-adequate levels in Se deficiency, but that the majority of selenoprotein transcripts are not regulated by Se status in liver, kidney, muscle, or testes. In this study, liver Gpx1 mRNA in Se deficiency decreases to 10% of Se-adequate (0.24 μg Se/g diet) levels, and increases

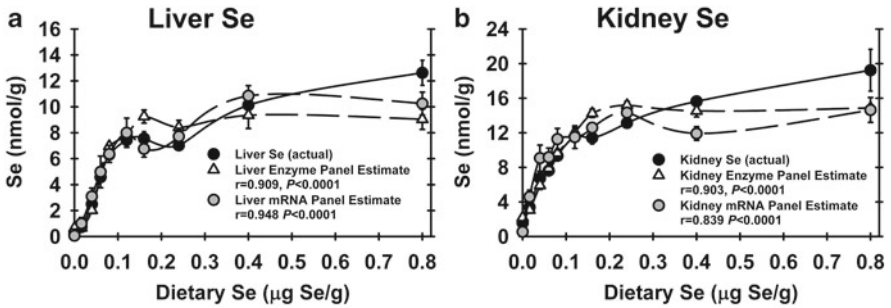

Fig. 11.2 Prediction of liver Se (**a**) and kidney Se (**b**) concentration by biomarker panels. Liver and kidney Se concentrations are shown as determined by actual measurement or calculated using biomarker panels based on tissue selenoenzyme activity, or based on the 4-transcript (liver) or 2-transcript (kidney) molecular biomarker panel identified by multiple regression analysis, as described previously [67]. Values are means ± SEM and legends indicate the level significance determined by ANOVA and the correlation coefficient for each panel relative to measured tissue Se concentration. Figure redrawn from Sunde [67]

sigmoidally with increasing dietary Se with a plateau breakpoint of 0.07 μg Se/g diet, similar to previous studies [12, 15, 16] (Fig. 11.1d). Supernutritional dietary Se up to 0.8 μg Se/g diet does not further increase (or decrease) levels of liver Gpx1 mRNA. In the same animals, kidney Gpx1 mRNA falls to 25% of Se-adequate levels with a plateau breakpoint of 0.06 μg Se/g diet whereas muscle Gpx1 mRNA levels are not significantly affected by Se status. In contrast, testes Gpx1 mRNA levels fall significantly to 40% of Se-adequate levels in Se-deficient rats. Notably, the plateau breakpoint for testes Gpx1 mRNA is 0.08 μg Se/g diet, or twice the minimum requirement for Gpx1 activity. The most logical explanation is that this is due to the unusual compartmentalization in testes [14] as well as receptor-mediated uptake of Se by a subset of cells in testes.

In liver, kidney, muscle, and testes, as in previous studies [12], Gpx4 mRNA is not significantly regulated by Se status in these tissues (Fig. 11.2e). Thus limited Se arriving in liver, kidney, and testes will be preferentially incorporated into Gpx4 relative to Gpx1, as Gpx1 mRNA is significantly downregulated in Se deficiency. This differential regulation of transcript level certainly plays an important role in the hierarchy of Se incorporation into selenoproteins in these tissues.

Sepw1, and Selh mRNA levels in Se deficiency are also reduced to 16 and 19%, respectively, of Se-adequate levels in rat liver, reach plateau breakpoints at 0.07, and 0.06 μg Se/g diet, respectively (Table 11.1), and are not further increased by supernutritional Se supplementation (up to 8× requirement). In addition, liver Gpx3 and Selk mRNAs are decreased to 20–40% of Se-adequate levels, and Dio1, Sepn1, Sepp1, Selt, Sep15, and Txnrd3 mRNA levels are decreased significantly to 40–70% of Se-adequate levels. In contrast, 12, 15, 20, and 21 selenoprotein transcripts in liver, kidney, muscle, and testes, respectively, are not regulated by dietary Se across the full range from Se deficient to 0.8 μg Se/g, with response curves similar to Fig. 11.1c [13, 14].

11.3.1.1 Blood Selenoprotein Transcripts

Some selenoprotein mRNAs, notably Gpx1 mRNA, are found in total RNA isolated from whole blood and are expressed at levels comparable to levels found in liver, kidney, and heart [24]. The red cell fraction rather than the white cell fraction is the predomininant source of mRNA isolated from whole blood [24, 25]. Whole blood Gpx1 mRNA falls in Se-deficient rats to as low as 10% of Se-adequate levels, with a plateau breakpoint of 0.08 μg Se/g diet [26], showing at least in the rat that the resulting minimum requirements are comparable to those determined using liver and kidney transcripts [26].

In our initial attempt to use whole blood transcripts to assess Se status in human subjects, we were readily able to detect mRNA in total RNA from human blood for Gpx1 and other selenoproteins that are regulated in rodents [27]. These Reading UK subjects, however, had plasma Se and Gpx3 activity levels on the plateau of the Se response curves even though they were consuming <50% of the daily US Se dietary intake, and there was no clear indication as well that any of the selenoprotein transcripts in blood were at less than plateau levels. Thus, whole blood transcripts have potential for use as a less invasive biomarker in assessing Se status and Se requirements, but further study in a more Se-deficient population will be needed to better evaluate efficacy for use in humans.

11.4 Hierarchy of Se Requirements in Rats

Table 11.1 shows the resulting minimum dietary requirements, based on the plateau breakpoints in the Se response curves for 15 conventional biomarkers (growth, 3 tissue Se concentrations, 9 selenoenzyme activities) and for 31 molecular transcript biomarkers, as described previously [13, 14, 26]. The resulting hierarchy of requirements range, where breakpoints are found, from 0.06 to 0.13 μg Se/g diet for conventional biomarkers and from 0.02 to 0.07 μg Se/g diet for molecular biomarkers in liver, kidney, and muscle. Thus requirements based on the molecular biology biomarkers are generally slightly lower than the requirements based on biochemical markers, and slightly higher than early reported requirements of 0.04 and 0.05 μg Se/g diet based on prevention of disease or maintenance of growth [28, 29]. Unlike early studies, today the Se requirement for growth is <0.01 μg Se/g diet using pups from Se-adequate dams with diets supplemented with vitamin E and sulfur amino acids.

The data in Table 11.1 also nicely illustrate the tissue hierarchy and molecular hierarchy first observed by Behne [10]. The minimum requirements listed in Table 11.1 show that with increasing dietary Se, Gpx1 is generally the last selenoprotein to reach plateau levels and no selenoprotein mRNA requirement is higher than the Gpx1 mRNA requirement in the same tissue. The tissue hierarchy is similarly observed as the selenoprotein transcript-based requirements in liver and kidney are grouped between 0.04 and 0.07 μg Se/g diet whereas these mRNA-based requirements in muscle are slightly lower, ranging between 0.03 and 0.05. Higher breakpoints of

up to 0.13 μg Se/g diet were observed in this young, rapidly growing rat model based on kidney and muscle Gpx1 activity; the basis for these higher breakpoints is unclear, but may be related to a role of Gpx1 as a Se store or buffer [30] such that these storage pools are not fully saturated in the young developing rodent. Studies with adult rats now indicate that the dietary Se requirement decreases in mature rodents relative to young rodents [23], suggesting that kidney and muscle Gpx1 activity should not be the basis for a higher Se requirement.

The unique regulation of selenium, selenoproteins, and selenoprotein transcripts in testes dramatically illustrates the hierarchy of selenoprotein expression. Testes Se in today's weanling rats, initially with adequate Se status, only falls nonsignificantly in Se deficiency to 60% of Se-adequate levels and is clearly on the plateau with supplementation of as little as 0.016 μg Se/g diet. The requirement for testes Gpx1 and Gpx4 activities is 0.04 and <0.01 μg Se/g diet (Table 11.1) [14], even though testes have 20-fold higher Gpx4 activity than found in liver. Minimal dietary Se required for plateau levels of selenoprotein mRNA levels in testes is also lower than in other tissues, insuring selenoprotein synthesis when Se is present.

11.4.1 Targeted Delivery of Se

This relatively normal Se status in testes in rats with profound Se deficiency in other tissues clearly is the result, in part, of targeted delivery of Se to testes mediated by Sepp1 from liver, and by Sepp1 receptors (ApoER2) in testes [31–34]. Under Se-limiting conditions, the relative lack of downregulation of liver Sepp1 mRNA facilitates continued incorporation of limited Se into Sepp1, which is then secreted. ApoEr2 receptors in testes and brain then mediate preferential delivery of Se to these tissues under Se-deficient conditions. In addition, the presence of the ApoER2 receptors in testes and brain appears to be the basis for retention of Se by testes and brain during Se deficiency, as ApoER2-receptor knockout eliminates the ability of these tissues to retain Se during progressive Se deficiency [33]. Heterogeneous expression of selenoproteins and selenoprotein receptors within the testes is likely to result in additional abnormal distribution of Se and selenoproteins within the testes [14]. In addition, a second Sepp1 receptor, megalin, appears to mediate Sepp1 uptake in kidney [35], perhaps mediating the increases in kidney Se under supernutritional Se conditions.

11.5 Supernutritional Supplementation

A most important observation from these studies is that not one of the biochemical or molecular biomarkers is increased above Se-adequate levels by supernutritional dietary Se levels (8× requirement, Table 11.1). Thus, these selenoprotein transcripts have little value as biomarkers for supernutritional or anticarcinogenic levels of Se. As rodent anticancer studies often use 2 μg Se/g diet (20× requirement) [36] and

human anticancer studies often use supplements of 200 μg Se/day (4× requirement) [37], these studies further reinforce the idea that the anticarcinogenic activity of Se is mediated by effects not directly related to selenoprotein activity [38, 39].

Note also that the Se response curves for both the conventional biochemical biomarkers and the molecular biomarkers do not match with the increases in liver and kidney Se concentration (Fig. 11.1a) at supernutritional Se supplementation above 0.24 μg Se/g diet. This implies that this accumulation of tissue Se is not associated with conventional selenoproteins such as Gpx1 (Fig. 11.1b) or Gpx4 (Fig. 11.1c) or with other well-studied selenoproteins [13, 17, 18, 20, 21]. Burk and colleagues [19] reported an ~40% increase in plasma Sepp1 levels with supplementation of 2 μg Se/g diet to rats for 8 weeks as compared to levels in rats fed 0.1 or 0.5 μg Se/g diet, so this increase might be related to Sepp1 synthesis and secretion even though Sepp1 mRNA levels are unchanged over this range. Alternatively, the increase in tissue Se above 0.24 μg Se/g diet might be associated with increased synthesis of excretory forms of Se [40]. Whatever the case, this discrepancy offers opportunity for further investigation that might help to better define Se metabolism as well as Se's anticancer activity.

11.5.1 Species Differences

Se requirements appear to be remarkably similar across species [41], presumably reflecting common underlying molecular mechanisms. One notable exception is the turkey, where minimal Se requirements are at least twice those for other higher animals [42]. We recently found that levels of both Gpx4 and Gpx1 activities fall dramatically in Se-deficient turkeys in multiple tissues, and that Se requirements in male turkey poults based on liver Gpx1, gizzard Gpx4, and gizzard Gpx1 activities are ~0.3 μg Se/g diet. Cloning of turkey Gpx1 and Gpx4 cDNAs revealed that both mRNAs are regulated by Se status and fall to ~36% of Se-adequate levels [42]. These results indicate that the differences in dietary Se requirements are associated with differences in the underlying regulatory mechanisms.

11.6 Mechanisms Underlying Se Regulation of the Selenoprotein Transcriptome

11.6.1 Nonsense-Mediated Decay

The similarity of minimal Se requirements among transcripts and tissues (Table 11.1) and the similarity of the response curves for Se-regulated transcripts strongly suggests that there is one underlying mechanism in play in Se regulation of selenoprotein mRNA levels, but the mechanism has not been identified. For Gpx1 and Gpx4

mRNA, at least, it is clear that this regulation is neither due to transcriptional regulation nor due to mRNA processing and export from the nucleus [43, 44]. To date, the best experimentally supported hypothesis explaining the downregulation of selenoprotein mRNA levels in Se deficiency is that selenoprotein transcripts in Se deficiency are degraded via nonsense-mediated decay (NMD) [45, 46]. NMD occurs in eukaryotic cells as a mechanism for eliminating mRNAs in which translation terminates prematurely, where a nonsense codon is positioned more than 50–55 nucleotides (nt) upstream from a post-splicing exon–exon junction, and where NMD depends on an exon junction complex (EJC) of protein, deposited during pre-mRNA splicing and located ~20–25 nt upstream of an exon–exon junction [47–49]. Under these conditions, the translation complex stalls at the nonsense codon during the initial or pioneering round of translation before it can sweep the EJC off the mRNA [48]. This allows the EJC to recruit proteins from the translation complex, including Upf and Smg proteins, promotes ATP hydrolysis, and subsequent recruitment of mRNA degrading activities [48, 49].

11.6.2 Selenoproteins and NMD

For selenoproteins under Se-adequate conditions, the concentration of Sec-tRNA is sufficiently high such that NMD does not occur because translation continues past the UGA, sweeping the EJC off the mRNA and thus preventing mRNA decay. Under Se-deficient conditions, however, the hypothesis is that insufficient Sec-tRNA concentrations result in stalling of translation at the UGA codon; when UGA is more than 50–55 nt upstream of an exon–exon junction, such as for rodent Gpx1 mRNA with its UGA located 105 nt from the exon–exon splice junction (Table 11.2), the EJC is not dislodged from the mRNA during the pioneering round of translation, resulting in Gpx1 mRNA decay in Se deficiency. In contrast, when the UGA codon is located closer to the exon–exon junction, such as for rodent Sepx1 with its UGA located 34 nt from the exon–exon splice junction (Table 11.2), the translation complex also stalls at UGA in Se deficiency but is close enough to dislodge the EJC, thus preventing NMD. This hypothesis is supported by studies showing that positioning of an in-frame UGA codon sufficiently upstream of a splice junction in B-globin plus a SECIS element in the 3′UTR will confer Se regulation onto B-globin mRNA [45].

11.6.3 Limitations of the NMD Hypothesis

SelH fits the >55 nt rule, with its UGA 136 nt upstream of the exon–exon junction, but Sepw1's UGA only lies 15 nt upstream and yet this mRNA is dramatically degraded in Se deficiency. For moderately regulated transcripts, the UGA positions for Dio1 and Selt fit the >55 nt rule, but the UGAs for Sepn1, Selk, Gpx3, and Sep15

Table 11.2 Effect of UGA codon position on selenium regulation of selenoprotein transcripts

Se gene	UGA exon[a] (nth of n exons)	Distance[b] (nt)	Liver Se regulation[c]	Kidney Se regulation[d]
Dio3	1st of 1	–	Low	Low
Sephs2	1st of 1	–	Low	Mod-up[d]
Gpx1	1st of 2	105	High	High
Gpx2	1st of 2	102	Low	Low
Dio2	2nd of 2	–	Low	Low
Dio1	2nd of 4	103,303	Mod	Low
Selh	2nd of 4	136,265	High	High
Gpx3	2nd of 5	22,140,240	High	High
Selm	2nd of 5	21,56,135	Low	Low
Sepp1	2nd of 5	39,252,370	Mod	Low
Sepw1	2nd of 6	15,69,144,242	High	High
Selt	2nd of 6	101,228,316,470	Mod	Low
Selv	2nd of 6	15,69,144,282	np	np
Sepx1	3rd of 5	34,66	Low	Low
Sep15	3rd of 5	28,78	Mod	Low
Gpx4	3rd of 7	105,257,282,342	Low	Low
Selk	4th of 5	5	High	Mod
Sels (H47)	6th of 6	–	Low	Low
Selo	9th of 9	–	Low	Low
Sepn1	9th of 12	1,114,216	Mod	Mod
Seli	10th of 10	–	Low	Low
Txnrd1	15th of 15	–	Low	Mod
Txnrd3	16th of 16	–	Mod	Low
Txnrd2	17th of 18	150	Low	Low

[a] Exon location of the UGA codon in each selenoprotein gene. Data from National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/)

[b] Distance in nucleotides from the UGA (A) and the downstream 3′ exon/intron splice junction(s)

[c] Susceptibility of all 24 rat selenoprotein transcripts to Se regulation in Se-deficient rat tissue, as assessed by qRT-PCR in Se-deficient vs. Se-adequate (0.24 μg Se/g diet) rat samples; high = 10–41% of Se-adequate; mod = 41–70% of Se-adequate; Low = >70% of Se-adequate; *np* not detected by qRT-PCR (data from Barnes et al. [13])

[d] Kidney Sephs2 transcript is moderately upregulated in Se deficiency

are 1, 5, 22, and 28 nt, respectively, upstream of the closest exon–exon junction. This model, however, is further complicated because NMD studies indicate that the required protein–protein interactions for NMD can be facilitated in some cases by long-distance interactions over the full length of the transcript, not just within the 55 nt region between the nonsense codon and the exon–exon junction [48, 49]. For selenoprotein transcripts with multiple exons downstream of UGA, additional EJCs could be located at these additional exon–exon junctions and mediate NMD (Table 11.2). Thus, moderate Se regulation of transcript levels by NMD might be mediated via additional downstream EJCs for Sepn1, Gpx3, and Sep15, but this is not the case for highly regulated Selk with its UGA located 5 nt upstream of the exon–exon junction and with no additional downstream exon–exon junctions.

Most importantly, the NMD hypothesis as currently stated is completely disproved by the stability of Gpx4 mRNA in Se-deficient animals, as the Gpx4 UGA codon lies 105 nt upstream of the first exon–exon junction, and with three additional downstream exon–exon junctions. Thus, Gpx4 mRNA should be targeted for NMD but it is not. Overall, these studies show that the downregulation of selenoprotein transcripts in Se deficiency cannot be explained by NMD as currently hypothesized, but these studies do provide a number of new selenoprotein mRNAs that can be used in future studies to better understand this process.

11.7 Cell Culture Models vs. Intact Animals

Our understanding of the mechanism underlying the Se regulation of selenoprotein mRNA level is further complicated because there is often a huge discrepancy between Se metabolism in intact animals vs. cultured cell models. Overexpression of selenoprotein mRNA in cultured cells typically results at best in a doubling of the selenoprotein itself when conducted in cells with modest or higher endogenous levels of the selenoprotein [45]. At the same time, the extent of Se regulation of Gpx1 mRNA in cultured cells by Se deficiency, however, is much smaller than in the whole animal (twofold vs. up to tenfold) [45]. More importantly, Gpx4 mRNA levels fall dramatically in Se-deficient cultured cells and appear to be regulated by NMD [50], whereas Gpx4 transcripts are not regulated in intact animals [45, 51], indicating that additional factors are likely to be involved. Thus, extrapolations and models based on studies conducted in cultured cells need to be made with caution unless confirmed in intact animals or tissues.

11.8 Se Regulation of Selenoprotein Translation

It was clear that Sec incorporation results in less efficient translation immediately after the discovery that UGA encodes Sec; Böck and colleagues [52] found that replacement of UGA with serine or cysteine codons in fusion constructs results in increased amounts of polypeptide synthesis in their bacterial system. Berry [53] in her early studies with chimeric mRNA in cultured cells or oocytes found that SECIS elements differ in their ability to facilitate Sec incorporation in translation (Sec translational efficiency), with the efficiency of the first Sepp1 SECIS threefold greater than that of Dio1 SECIS. Use of a nifty dual reporter construct, with UGA separating the two reporter coding regions and with replaceable SECIS elements, found that the Gpx4 SECIS has threefold higher translational efficiency relative to the Dio1 SECIS [54], whereas the Gpx2 SECIS has relatively low Sec efficiency [55]. Using the same reporter, comparison of five SECIS elements in CHO cells resulted in an over fourfold higher translational efficiency of a Sep15 SECIS relative to Txnrd1 and Txnrds SECIS elements [56]. More recently using a recombinant luciferase with an in-frame UGA, all 26 human selenoprotein SECIS elements

(2 in Sepp1) were compared directly for translational efficiency in Hek293 and HepG2 cells and in an in vitro translation system [57]; these three systems showed over 1,000-fold differences between different SECIS elements, but the relative rankings between systems were fairly varied suggesting that other model system-specific factors are important. Additionally, Gpx1 and Gpx4 SECIS elements had similar, moderate translational efficiencies in this system [57], and there was no consistent pattern between these results and the stability of the full selenoprotein transcripts in Se-deficient rats (Table 11.2).

The UGA position itself relative to the start codon or to the SECIS element, or the local UGA context, all can affect translational efficiency and thus might affect mRNA sensitivity to NMD [58]. Differential affinities of SECIS elements for isoforms of the Sec-tRNA also influence translational efficiency and are accompanied by changes in mRNA levels, and so may influence mRNA stability [59]; these shifts in relative levels of Sec-tRNA isoforms, however, are modest in Se-deficient mice [60], making it unlikely that this alone regulates selenoprotein mRNA stability in intact animals.

11.8.1 Alternative Hypotheses

A number of hypotheses, in addition to NMD, have been proposed to explain the hierarchy of susceptibility of selenoprotein mRNAs to degradation [45, 46, 53, 59, 61–63]. As discussed above, varied translational efficiency due to differences in SECIS elements, UGA location, and local UGA context might confer differential sensitivity to NMD [53, 55]. Some studies using chimeric constructs expressed in cultured cells suggest that regions in both the coding region and in the 3′UTR are involved in making selenoprotein mRNAs susceptible to decay in Se-deficient cells [61]. Differential affinity of SECIS elements for SECIS-binding proteins, such as SBP2, has also been proposed to explain the hierarchy of sensitivity to NMD [62, 63], but the recent cultured cell studies with 1,000-fold differences in Sec translational efficiency also found that these differences were not explained by SBP2-binding affinity [57]. Levels of another SECIS-binding protein, nucleolin, also have differential effects on translation of UGA/SECIS-containing recombinant constructs in cultured cells but nucleolin does not seem to affect transcript levels, only translation [64]. Even the eukaryotic initiation factor, eIF4a3, has been shown to differentially bind to Gpx4 vs. Gpx1 SECIS elements in cultured cells and differentially inhibit translation [65]. Other studies, however, have shown that the Gpx4 SECIS is just as sufficient as the Gpx1 SECIS in conferring NMD sensitivity in cultured cells [45]. Lastly, the presence of a putative second stem-loop immediately downstream of UGA in the coding region in some selenoprotein mRNAs, termed the Sec codon redefinition element (SRE), is thought to influence translational efficiency [66, 67] but only Sepn1 mRNA has been studied so far. Thus, there are multiple levels where differential expression of selenoproteins occurs, including efficiency of translation, but the details of the mechanism(s) involved in Se regulation of selenoprotein transcript levels remain unclear.

11.9 Molecular Biomarkers for Se Status

A molecular biomarker can be defined as an mRNA transcript that indicates the (nutrient) status of an organism or tissue, as distinguished from biochemical biomarkers, such as enzyme activity, or chemical biomarkers, such as the concentration of an element, vitamin, or metabolite [25]. To illustrate the potential of molecular biomarkers for predicting Se status across the full spectrum of Se, we used the 4 liver and 4 kidney conventional biomarkers and 13 liver and 9 kidney selenoprotein transcript levels (Table 11.1) to predict liver Se concentration [68]. Multiple regression analysis against liver Se concentration, with step-wise single elimination of biomarkers that did not significantly contribute, was used to identify biomarker panels with significant ($P < 0.05$) regression coefficients. The resulting 4-selenoprotein mRNA biomarker panel (Gpx1, Gpx3, Selt, Selk) predicted liver Se concentration with a correlation of 0.948 which was nominally higher and statistically the same as the correlation of 0.909 for the panel based on Gpx1 activity (Fig. 11.2a), and the resulting 2-selenoprotein mRNA panel (Sepw1, Selk) predicted kidney Se concentration with a correlation of 0.839 (Fig. 11.2b). These panels, however, predict essentially flat Se response curves after ~0.2 μg Se/g diet, as compared to the continued but slow further increase in measured liver and kidney Se between 0.24 and 0.8 μg Se/g diet. This observation clearly illustrates that additional, orthogonal biomarkers for supernutritional status are needed to more accurately predict this increase in tissue Se [68].

11.10 Concluding Remarks

This review has discussed the Se regulations of selenoprotein expression in rodents over the spectrum from Se deficient to supernutritional Se status. Over this range, the underlying regulation of selenoprotein transcript levels, along with targeted delivery of Se to various tissues, appears to explain much of the hierarchy of selenoprotein expression. Newly identified Se-regulated and non-Se-regulated transcripts offer new models to help unravel the mechanism responsible for this Se regulation. A larger question is emerging, however, as none of these biochemical or molecular biomarkers can serve as biomarkers for high Se status. Our recently completed microarray studies on rats fed 0–5 μg Se/g diet [69] found few transcriptional changes in rats fed up to 20× requirement, but a vastly expanded number of transcriptional changes in rats fed 50× requirement. This observation suggests that non-selenoprotein transcripts have potential for use in molecular biomarker panels that can accurately predict supernutritional and toxic Se status [69].

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Chapter 12
Selenoproteins and the Thioredoxin System

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Abstract The thioredoxin system, comprising NADPH, flavoprotein thioredoxin reductase (TrxR), and 12 kDa thioredoxin (Trx) with a catalytic dithiol/disulfide, is the ubiquitous biological cellular disulfide reduction system with major functions in DNA synthesis, defense against oxidative stress, and thiol redox control. In mammalian cells Trx system activity is controlled by the three TrxR isoenzymes which are large homodimeric selenoproteins. The availability of selenium affects not only the Trx system activity but also the existing form of TrxR. Selenium-deficient conditions cause the increase of a low activity form of TrxR, in which a cysteine residue substitutes for selenocysteine (Sec). On the other hand, the Trx system can reduce selenite into selenide, which is required for Sec residue synthesis in proteins. Trx system activity in turn may regulate the redox state and subcellular translocation of Sec insertion sequence element-binding protein 2 (SBP2) and Sec incorporation efficiency in all selenoproteins. The overall structure of TrxR is similar to that of glutathione reductase, but with a C-terminal elongation of 16 residues containing the conserved C-terminal active-site sequence –Gly–Cys–Sec–Gly. In oxidized TrxR, the active site is a selenenylsulfide, which is reduced to a catalytic selenolthiol by electrons from the redox-active disulfide/dithiol of the other subunit, as revealed by three-dimensional structures of the rat TrxR1 enzymes. The critical role of Sec in TrxR and its accessible location and reactivity in the C-terminal active site provide promising pharmaceutical drug targets for various human diseases such as malignant cancer and rheumatoid arthritis.

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12.1 Introduction

The thioredoxin (Trx) system, composed of thioredoxin reductase (TrxR), Trx, and NADPH, is one of the main thiol dependent electron donor systems in the cell and is ubiquitously present from archaea and bacteria to man [1–4]. Trx with a redox-active dithiol/disulfide is an electron donor for essential enzymes such as ribonucleotide reductase and a general protein disulfide reductase with numerous functions in control of intracellular redox potential, defense against oxidative stress, and signal transduction by thiol redox control [4]. TrxR from mammalian cells and higher eukaryotes are selenoenzymes [3, 5] and very different from the smaller selenium-independent enzymes of archaea, bacteria, yeast, and plants [6]. This chapter discusses the relationships between selenoproteins and the Trx system and some of the structure–function relationships of mammalian TrxRs.

12.2 General Properties of Thioredoxin Systems

All thioredoxin reductases reduce oxidized thioredoxin (Trx-S_2) at the expense of NADPH [1, 2] (Reaction 12.1). Reduced Trx [Trx-$(SH)_2$] is reoxidized by disulfides in proteins generating thiols (Reaction 12.2):

$$\text{Trx - } S_2 + \text{NADPH} + H^+ \xrightarrow{\text{TrxR}} \text{Trx - } (SH)_2 + \text{NADP}^+ \tag{12.1}$$

$$\text{Trx - } (SH)_2 + \text{Protein - } S_2 \rightarrow \text{Trx - } S_2 + \text{Protein - } (SH)_2 \tag{12.2}$$

$$\text{Net : Protein - } S_2 + \text{NADPH} + H^+ \xrightarrow{\text{TrxR + Trx}} \text{Protein - } (SH)_2 + \text{NADP}^+ \tag{12.3}$$

Generally, the K_m-value for NADPH is low or in the range below 10 μM and that of Trx-S_2 is typically from 1 to 3 μM.

Isolation and characterization of mammalian Trx and TrxR started about 35 years ago [7, 8]. As shown in Table 12.1, there are some major differences between the Trx systems of prokaryotes like *Escherichia coli* and those of mammals.

E. coli and mammalian cytosolic Trxs are homologous proteins with a conserved –Cys–Gly–Pro–Cys-active site. Interestingly, mammalian Trxs must be purified in the fully reduced form since they contain structural SH-groups which form additional disulfides upon oxidation. This may have an autoregulatory function on Trx activity in resting cells or upon oxidative stress, yet this is incompletely understood in vivo. TrxRs from mammalian cells have very different properties when compared with the enzymes from *E. coli*, yeast, or plants [6]. The cytosolic enzyme has subunits with 55 kDa or larger instead of the 35 kDa subunits in the *E. coli* enzyme [6]. As is described below, the mammalian enzyme also has a very broad substrate specificity entirely different from the generally species-specific enzymes only reducing Trx-S_2 present in prokaryotes, yeast, and plant cytosol.

Table 12.1 Properties of Trx systems

	E. coli Trx1	Human Trx	*E. coli* TrxR	Human TrxR
Molecular weight	12,000	12,000	70,000	110,000 or larger
Amino acid residues	108 aa	104 aa	321 aa	499 aa
Subunits	1	1	2	2
Active sites	–CGPC–	–CGPC–	–CATC–	-CVNVGC-(N)-GCUG-(C)
Substrate specificity	Broad	Broad	High	Broad
Stability upon aerobic storage	Stable	Activity reversibly lost for additional disulfide formation among three structural cysteines	Stable without NADPH	Stable without NADPH

12.3 Sec Incorporation Mediated by the Trx System

Selenium is incorporated into the selenoprotein polypeptide chain as Sec by a complex machinery including a Selenocysteine (*Sec*) *I*nsertion *S*equence (SECIS) element, Sec-specific elongation factor (eEFSec), $tRNA^{[Ser]Sec}$, SBP2, and other components in mammalian cells [9]. Selenium from selenite cannot be utilized directly and must be reduced into selenide, a precursor for selenophosphate and Sec synthesis. The observation that selenite is a substrate for the Trx system indicates that the Trx system may play critical roles in selenium assimilation [10, 11]. With 200 μM NADPH and 50 nM calf thymus TrxR, addition of 10 μM selenite caused oxidation of 40 μM NADPH in 12 min and 100 μM NADPH after 30 min demonstrating a direct reduction of selenite with redox cycling by oxygen [10]. This was shown by incubation under anaerobic conditions where only 3 mol of NADPH were oxidized per mol of selenite according to Reaction (12.4):

$$SeO_3^{2-} + 3NADPH + 3H^+ \xrightarrow{TrxR} Se^{2-} + 3NADP^+ + 3H_2O \qquad (12.4)$$

Addition of Trx stimulated the reaction further since selenite rapidly reacts with Trx-$(SH)_2$ to oxidize it to Trx-S_2 [12–14]. Since glutathione reductase will not react with selenite [14], Reaction (12.4) should provide cells with selenide for Sec synthesis [15]. Selenite and glutathione react to form selenodiglutathione (GS-Se-SG) which has been suggested to be a major metabolite of inorganic selenium salts in mammalian tissues [16]. Reaction of selenodiglutathione by NADPH and glutathione reductase was demonstrated by Ganther [17], and it has been proposed to be a source of selenide in cells as well as an inhibitor of neoplastic growth [18]. We synthesized GS-Se-SG [12, 19] and discovered that this compound is a direct efficient substrate for mammalian TrxR and a highly efficient oxidant of reduced Trx. Since GSSG is not a substrate for mammalian TrxR [7, 8], the insertion of the selenium atom in the GSSG molecule to form GS-Se-SG makes this molecule highly reactive with the enzyme.

Reduction of GS-Se-SG to yield selenide by glutathione reductase requires 2 mol of NADPH. We found only the first stoichiometric reduction to be fast with GS-Se^- as a product [12]. The second reaction was slow and relatively inefficient. These results strongly suggest that the major selenide generation in cells is via TrxR and Trx. Thus, these enzymes may be also responsible for the generation of selenide required for its own synthesis. The direct evidence that TrxR is essential for selenoprotein synthesis was found in the bacterial system [20]. Disruption of *trxB* gene to produce a TrxR knockout mutant *E. coli* caused the diminishment of the activity of selenoprotein formate dehydrogenase H (FDH_H). ^{75}Se was incorporated in FDH_H in wild-type or glutathione reductase knockout mutant strains, but not in the TrxR mutant [20].

In mammalian cells, knockdown of TrxR or Trx did not affect selenoprotein synthesis in A549 cells [21], but the treatment with TrxR inhibitors auranofin and arsenic trioxide [22] led to a block in selenoprotein synthesis. One explanation for this observation may be that a low activity of TrxR and Trx is sufficient for selenium incorporation, which is a critical process in the cells. The TrxR inhibitors, auranofin and arsenic trioxide, caused not only the TrxR activity to decrease, but also resulted in Trx oxidation [22]. The disruption of the whole Trx system may thus be responsible for the inhibition of selenium incorporation, while knockdown of TrxR alone does not lead to oxidation of Trxs [23].

Another selenoprotein synthesis key factor, SECIS-binding protein 2 (SBP2), also has a close link with Trx system. Oxidative stress induced the shuttling of SBP2 from cytosol into the nucleus through the CRM1 pathway [24] and the decrease of selenoprotein synthesis efficiency [9]. This process correlated with the oxidation of cysteine residues in the cysteine-rich domain of SBP2. The oxidized SBP2, containing disulfide bonds and/or glutathione mixed disulfides, could be reduced by the Trx system, indicating that Trx may participate in the regulation of SBP2 and thus may affect selenoprotein synthesis efficiency [24].

12.4 Effects of Disruption of Selenium Incorporation on the Trx System

As described above, Sec incorporation is regulated by the Trx system. On the other hand, since TrxR is a housekeeping selenoprotein and involved in many biological processes such as maintaining redox balance, disruption of the selenoprotein synthesis machinery affects the Trx system as well as other selenoproteins, and leads to damage to the cells. The depletion of SBP2 by using antisense oligonucleotides (ASO) resulted in oxidative stress, leading to DNA damage, stress granule formation, lipid oxidation, cell cycle arrest, and induction of caspase- and cytochrome c-dependent apoptosis [25]. SBP2 depletion by ASO caused a decrease in expression of selenoprotein cytosolic TrxR1 and mitochondrial TrxR2, as well as GPx4, whereas Trx1 remained at the same level [25]. The critical roles of SBP2 in diverse biological processes have been verified recently by identification of several subjects with heterozygous defects in the SBP2 gene [26]. The SBP2 mutations caused a

lack of expression of many selenoenzymes including TrxR and an elevation of cellular reactive oxygen species (ROS) levels. The multisystem selenoprotein deficiencies resulted in disorders in humans, including azoospermia, axial muscular dystrophy, photosensitivity, T lymphocyte proliferation impairment, abnormal mononuclear cell cytokine secretion, telomere shortening, and an elevated systemic and cellular insulin sensitivity [26].

Very interestingly, severe dietary selenium deficiency caused a dramatic loss of TrxR activity in liver, although the TrxR1 mRNA was expressed at nearly unchanged levels [27]. The TrxR protein from control rat liver was shown to have a higher specific activity and contain much more Sec than that from selenium deficient rat liver. There was no truncated enzyme and mass spectra experiments demonstrated a low active form of TrxR with a Cys residue substituted for the penultimate Sec [27]. A recent finding confirmed that dietary selenium and availability of thiophosphate regulated the insertion of Cys in place of Sec in TrxR1 [28]. The thiophosphate which was synthesized by selenophosphate synthetase 2 could react with phosphoseryl-tRNA$^{[ser]sec}$ to produce Cys-tRNA$^{[ser]sec}$ for the insertion of Cys [28].

12.5 Structure and Mechanism of Mammalian TrxR

Mammalian TrxRs, surprisingly, are selenoproteins and entirely different from the corresponding enzymes in bacteria, yeast, and plants (review in [6]). Stadtman and coworkers serendipitously discovered that human tumor cell TrxR is a selenoprotein using labeling of selenoproteins with radioactive selenite [29]. This also explained [30] why a previously putative clone of the human enzyme [31], where the TGA codon for Sec was interpreted as the stop codon, gave no recombinant enzyme activity. The TGA acts as a stop codon in *E. coli* due to the species-specific machinery for synthesis of selenoproteins which is different in bacteria and mammalian cells [15].

By sequencing large parts of the cytosolic TrxR enzymes, the C-terminal peptide was identified as containing Sec [30, 32]. The peptides were used to identify a rat cDNA clone which was sequenced [32]. The results showed a polypeptide chain with a high homology to glutathione reductase [32, 33] including an identical active-site disulfide (CVNVGC) but with a 16-residue elongation containing the conserved C-terminal sequence, Gly–Cys–Sec–Gly. A SECIS element was identified in the 3′-untranslated region [32]. Furthermore, digestion of TrxR by carboxypeptidase after reduction by NADPH released Sec with loss of activity; the oxidized form of the enzyme was resistant to carboxypeptidase digestion [32]. Redox titrations with dithionite and NADPH demonstrated that the mechanism of the human placenta enzyme is similar to that of lipoamide dehydrogenase and glutathione reductase and is distinct from the mechanism of TrxR from *E. coli* [34]. The results demonstrated that the Sec residue of human TrxR is redox active and communicates with the redox-active disulfide since more than four electrons per subunit are required to completely reduce the FAD of the oxidized enzyme. Furthermore, the Sec residue is alkylated with loss of activity only after reduction by NADPH [32, 35, 36].

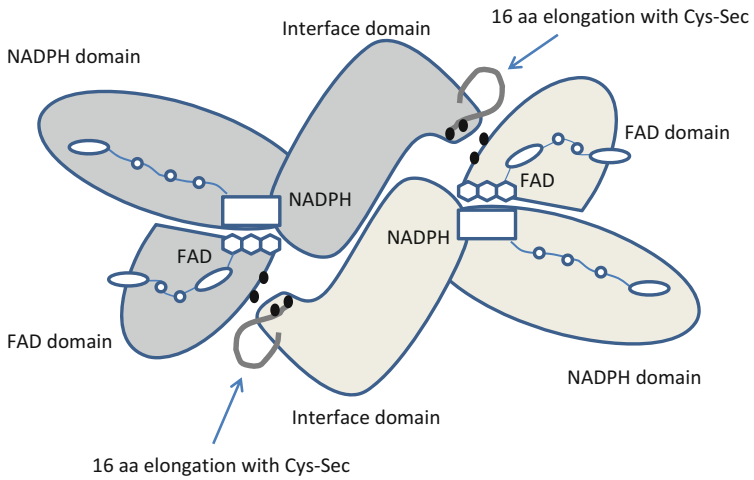

Fig. 12.1 Structural model of mammalian TrxR based on the homology to glutathione reductase. The 16-residue C-terminal extension with the active site is shown as well as the head-to-tail arrangement of the subunits in the dimer. Taken from [37], the FAD, NADPH, and Interface domains are shown (see also Fig. 12.2)

Based on the homology to glutathione reductase, we proposed a model of mammalian TrxR (Fig. 12.1). The enzyme is a head to tail dimer with the 16-residue elongation in principle taking the place of GSSG in glutathione reductase. The active site of the enzyme is a selenolthiol in its reduced form and a selenenylsulfide formed from the conserved Cys-Sec sequence in the oxidized form [37]. The selenenylsulfide was isolated by peptide sequencing and also confirmed by mass spectrometry [37]. Mechanisms of the enzyme have also been postulated involving a reductive half-reaction similar to that of glutathione reductase leading to reduction of the active-site disulfide (Figs. 12.1 and 12.2). Electrons are thereafter transferred from the redox-active dithiols to the selenenylsulfide of the other subunit generating the selenolthiol. Characterization of the Cys mutant enzyme revealed that the selenium atom with its larger radius is critical for the formation of the unique selenenylsulfide [37] since the C-terminal dithiol stays reduced in the Cys mutant [37]. Similar results have also been published [38]. The structure of the rat TrxR1 enzyme has been solved by X-ray crystallography [39]. The structure of the $Sec_{498} \rightarrow Cys$ mutant of rat TrxR1 in complex with $NADPH^+$ was determined to 3.0-Å resolution. The overall structure is similar to that of glutathione reductase, including the conserved amino acid residues binding the cofactors FAD and NADPH. The redox-active disulfide in the N-terminal is identical to that of glutathione reductase. Residues directly binding the substrate GSSG in glutathione reductase are conserved despite the fact that GSSG is not a substrate for TrxR [39]. The 16-residue C-terminal tail, a unique feature on mammalian TrxRs, folds in such a way that it can approach the active-site disulfide of the other subunit in the dimer (see schematic drawing in Fig. 12.1). A unique feature of the $Sec_{498} \rightarrow Cys$ mutant of rat TrxR1 is

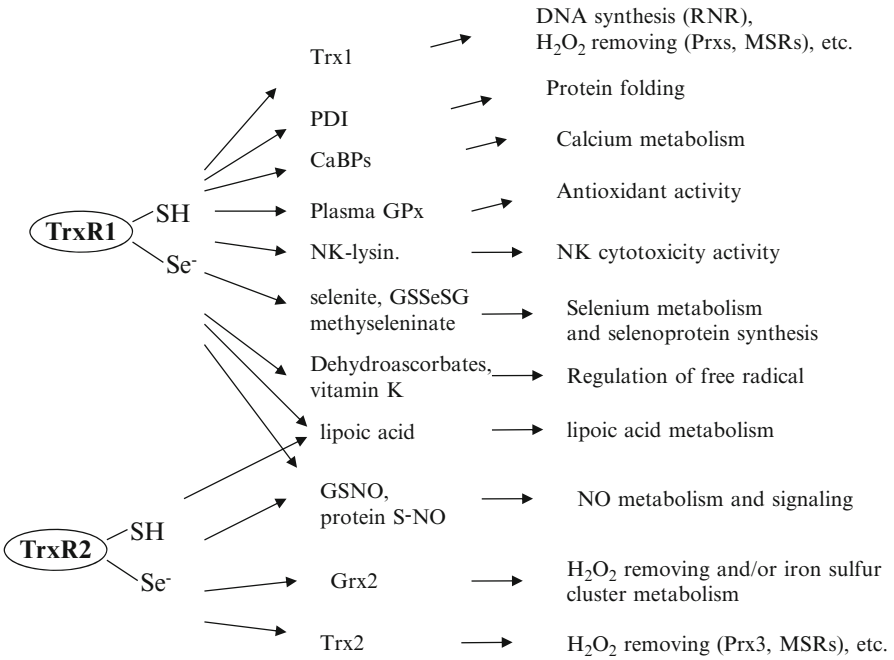

Fig. 12.2 Mammalian cytosolic TrxR1 and mitochondrial TrxR2 play critical roles in different biological processes. TrxR1 and TrxR2 alone or with Trx1 or Trx2 exert numerous functions by reducing different biological substrates. Sec is essential or critical in most of the processes

that the thiols in the Gly–Cys–Cys–Gly sequence do not form a disulfide [39]. A model of the complex of TrxR allows docking of oxidized Trx to the structure without large conformational changes [39]. This is in great contrast to the large conformational change required for the prokaryotic TrxR enzymes [6]. The model suggests specific interactions between Trx (D60, D61, and K72) and TrxR forming electrostatic interactions. The X-ray structure particularly explains the function of the 16-residue C-terminal extension conserved in all three mammalian isoenzymes of TrxR. It extends the electron transport chain from the catalytic disulfide to the enzyme surface, enabling reaction with Trx and a range of other substrates (Table 12.2). It acts to prevent the enzyme from serving as a glutathione reductase by blocking excess to the redox-active disulfide. The results of the X-ray study strongly suggest that mammalian TrxR evolved from a glutathione reductase scaffold rather than from its prokaryotic counterpart. Such an evolutionary switch will render cell growth dependent on selenium in the form of Sec and it may have advantages for cells using ROS like hydrogen peroxide or nitric oxide in cell signaling [39]. Most recently, the crystal structure of recombinantly produced Sec-containing rat TrxR1 was determined [40]. This structure demonstrates that a selenenylsulfide is formed at the C-terminal end in oxidized TrxR1 and Tyr116 may be involved in the electron transfer between the active sites, which was verified by site-directed mutagenesis [40].

Table 12.2 Role of Sec in the reduction of substrate by cytosolic and mitochondrial mammalian TrxRs[a]

Substrates	Cytosolic TrxR1	Mitochondrial TrxR2
Thioredoxin-S_2	Essential [7, 33]	Essential [49]
Selenite, Sec	Critical [33]	Not required [50]
5,5-dithiobis-(2-nitrobenzoic acid) (DTNB)	Critical [33]	Critical [49, 51]
Lipoic acid or lipoamide	Critical [33]	Not required [50]
Juglone	Not required[b]	Not required [49]

[a]Critical: large loss of activity; essential: no activity
[b]J. Lu and A. Holmgren, unpublished data

12.6 Role of Selenium in Isoenzymes of Thioredoxin Reductase

Apart from the cytosolic TrxR1, two additional genes encoding novel forms of human and mouse selenoprotein TrxRs have been identified [41]. One is a mitochondrial enzyme [42, 43] and the other is thioredoxin-glutathione reductase carrying an N-terminal glutaredoxin domain and preferentially expressed in testis. All these enzymes have extensions in the N-terminal region but share the C-terminal active-site sequence. Additional complexity is given by the identification of enzymes with mRNA variants differing in the 5′-untranslated region [44] and by 5′-exon splicing [45, 46].

The essential role of selenium in the catalytic activities of mammalian TrxR1 was revealed by characterization of recombinant enzymes with Sec mutations (Table 12.2) [33]. This was done by removing the SECIS element in the rat gene and changing the Sec_{498} encoded by TGA to Cys or Ser codons by mutagenesis. The truncated protein having the C-terminal dipeptide deleted was also engineered. All three mutants were successfully overexpressed in *E. coli* and purified to homogeneity with 1 mol of FAD per monomeric subunit. All three mutant proteins rapidly generated the A_{540} absorbance resulting from the thiolate-flavin charge transfer complex characteristic of mammalian TrxR. Only the $Sec_{498} \rightarrow$ Cys enzyme showed catalytic activity in reduction of Trx, with a 100-fold lower K_{cat} and a tenfold lower K_m compared to the wild-type rat enzyme. The pH-optimum of the Sec-containing wild-type enzyme was 7 whereas the $Sec_{498} \rightarrow$ Cys enzyme showed a pH optimum of 9. This strongly suggested the involvement of the low pK_a Sec selenol in the enzyme mechanism. Selenium was also required for hydrogen peroxide reductase activity [33].

Mammalian TrxR1 displays a surprisingly wide substrate specificity as first observed during purification [7, 8]. This is in contrast to the smaller prokaryotic TrxRs, which do not use mammalian Trxs as substrates despite their conserved active sites and closely related three-dimensional structures [47]. As shown in Fig. 12.2, a truly wide range of direct biological reductions are catalyzed by mammalian cytosolic TrxR1. Trx from *E. coli* is a substrate with a similar K_{cat}, but with a 15-fold higher K_m-value (35 μM) compared with the rat liver protein [8]. Mammalian cytosolic Trxs generally show full cross reactivity with TrxRs from different mammalian sources and vice versa.

Crystal structures of oxidized and NADPH-reduced mouse TrxR2 have a similar overall structure as rat TrxR1 [48]. Mitochondrial TrxR2 also has a wide range of substrates (Fig. 12.2); however, the role of Sec in cytosolic and mitochondrial TrxR activity shows some differences for different substrates [49, 50]. Sec is essential or critical for both TrxRs to reduce its biological substrate Trxs, but for some small molecules, Sec or even the C-terminal active site is not required for the activity of mitochondrial TrxR2 (Table 12.2) [33, 49–51]. Sec in TrxR is critical for reduction of almost all substrates except juglone. The Sec498→Cys form of rat TrxR has a similar activity as the wild-type enzyme. These results strongly suggest that mitochondrial and cytosolic TrxRs have some differences in catalytic mechanism. In the case of reduction of certain small molecular substrates, probably only the N-terminal active site participates in the catalytic reaction, especially for mitochondrial TrxR2.

12.7 Regulation of Thioredoxin Reductase and Its Medical Applications

ROS are generated as by-products of the respiratory chain or by NADPH oxidases. ROS are implicated in the pathogenesis and pathophysiology of a variety of human diseases, such as cardiovascular and degenerative disorders and cancer. ROS is also implicated in cellular signaling. Peroxiredoxins working together with Trxs and TrxRs control the levels of ROS and free radicals. A complete Trx system, including TrxR2, Trx2, and peroxiredoxin (Prx III), is present in mitochondria. To address the function of mitochondrial TrxR2, a ubiquitous Cre-mediated inactivation of TrxR2 was shown to be associated with death at embryonic day 13 [52]. TrxR2$^{-/-}$ embryos are smaller and severely anemic and showed increased apoptosis in liver [52]. Also, the size of hematopoietic colony cultures ex vivo was dramatically reduced. TrxR2-deficient embryologic fibroblasts showed a high sensitivity to endogenously produced oxygen radicals when glutathione synthesis was inhibited [52]. Also, the ventricular heart wall of the mitochondrial TrxR knockout embryos was thinner and the proliferation of cardiomyocytes was decreased. Cardiac-specific ablation of TrxR2 resulted in fetal lethality and cardiomyopathy with symptoms similar to those of Keshan disease and Friedreich's ataxia. Thus, mitochondrial TrxR2 plays an essential role in hematopoiesis, heart development, and heart function [52].

A similar study on the cytoplasmic TrxR1, using a conditionally targeted deletion of exon 15 of the *Txnrd1* gene including C-terminal Cys and Sec, showed that the gene was essential for embryogenesis [53]. Ubiquitous Cre-mediated inactivation of Txnrd1 leads to early embryonic lethality around E10.5 [53]. Embryos of the homozygous mutant displayed severe growth retardation and failed to turn. Also, *Txnrd1*-deficient embryonic fibroblasts do not proliferate in vitro in line with growth impairment. Surprisingly, in contrast, ex vivo-cultured embryonic *Txnrd1*-deficient cardiomyocytes were not affected and mice with a heart-specific inactivation of TrxR1 developed normally and appeared healthy [53]. Another *Txnrd1* knockout study targeting exons 1 and 2 including all the functional ATGs and two N-terminal

Table 12.3 Classification of TrxR inhibitors

	Substrate class	Examples
Metal or metalloid-containing compounds	Gold compounds	Auranofin, aurothioglucose, Au(III) –dithiocarbamato complexes, etc.
	Platinum compounds	Cisplatin, terpyridine platinum (II) complexes, etc.
	Mercury compounds	$HgCl_2$ and MeHg
	Arsenic compounds	Arsenic trioxide, $As(GSH)_3$, $As(Cys)_3$, etc.
	Other organometallic complexes	Texaphyrins motexafin gadolinium (MGD), etc.
	Other metal ions	Lead, zinc, calcium, ruthenium ions, etc.
Sulfur, selenium, or telluride-containing compounds	Sulfur compounds	Isothiocyanates
	Selenium compounds	1, 2-[bis(1, 2-Benzisoselenazolone-3(2H)-ketone)]ethane (BBSKE)
	Telluride compounds	Cyclodextrin-diorganyl telluride
Michael acceptors (α,β-unsaturated carbonyl compounds)	Flavonoids	Myricetin, quercetin, EGCG, etc.
	Quinols	PMX464, BW114, etc.
	Quinones	5-Hydroxy-1,4 naphthoquinone (Juglone), napthoquinone, etc.
	Other Michael acceptors	Curcumin, 4-hydroxynonenal, etc.
Alkylation agents	Nitrosureas	Carmustine (BCNU)
		Cyclophosphamide
Nitroaromatic compounds		DNCB
Other compounds	Cationic triphenylmethanes	Brilliant green, gentian violet

active sites Cys59 and Cys64 yielded more severe phenotype. Mouse embryos with the *Txnrd1* mutation died at E9.5 [54]. But the mice in which all hepatocytes were *Txnrd1*$^{-/-}$ were fully viable for more than 1 year [55]. Only 0.3% of the liver transcriptome had been changed in the TrxR1-deficient liver [55]. The TrxR1 deficiency resulted in the induction of Nrf2 pathway and caused an effective compensatory response [55], which was similar to that caused by selenium deficiency [27, 56].

A range of human diseases and conditions are now known or suspected to be related to the activity and function of TrxR (for in-depth review see [57]). This involves diseases like rheumatoid arthritis, Sjögren's syndrome, AIDS, and malignancies. TrxR has been found to be overexpressed in many aggressive cancer cells [58]. The central role of TrxR in various cancer biosynthetic reactions and defense against oxidative stress (as shown in Fig. 12.2) makes the enzyme a major target for drug development. The C-terminal active-site Sec confers a higher selectivity on the enzyme over other proteins due to its low pK_a value and easily accessible location. The inhibition of TrxR blocked the TrxR activity and also resulted in modified TrxR. The modification of TrxR may yield rapid induction of cell death [59]. Obviously, TrxR is a novel and important molecular target for cancer therapy [9, 46, 58, 60].

A number of compounds have been found to serve as TrxR inhibitors [46, 61]. We classified these compounds as listed in Table 12.3. Many of them are clinically used anticancer compounds, including alkylating and platinum-containing drugs,

arsenic trioxide [22], and chemoprevention agents such as flavonoids [62] and curcumin [63]. These compounds selectively inhibit TrxR, but not glutathione reductase [46] and show inhibitory activity in vitro and in vivo. For example, cyclophosphamide specifically inhibited TrxR activity in Lewis lung carcinomas in mice, while the other antioxidant enzymes, such as GPx, GST, SOD, and CAT, as well as GSH levels were not affected [64]. Some compounds are the environmental toxins such as mercury compounds. Though the metal or metalloid-containing compounds have a high priority to attack the C-terminal Sec, the metal or metalloid may be bound not only through the C-terminal Sec-containing active site, but also via the N-terminal active sites, as is evident from the analysis of crystal structure and mass spectra [22]. Very interestingly, we found that a mercury compound formed the complex with TrxR and inhibited TrxR activity. A selenite supplement recovered TrxR activity by replacing a mercury atom bound to the active site [65, 66]. Some of the compounds like lead, zinc, or calcium compounds only show their inhibitory effects in the absence of EDTA in vitro. In cells or in vivo, they did not display selective inhibition of TrxR. Cationic triphenylmethanes, such as brilliant green, inhibit TrxR in vitro, but in cells, the compounds target mitochondria and cause Trx2 oxidation and degradation [67]. Since TrxR is regulated by several redox-sensitive transcription factors, such as Nrf2, NF-κB, etc., the inhibition of TrxR always caused the TrxR mRNA level to increase in cells, and in some cases, TrxR activity was recovered [64].

The fact that the Trx system is ubiquitous and present in highly variant forms in pathogenic bacteria makes the enzyme a particularly attractive drug target. There is a surprising diversity in the structure and mechanism of the enzyme in several important pathogenic bacteria as reviewed in [57]. This may lead to the development of specific inhibitors of bacterial infections as in Lepra, parasitic diseases, and malaria. Treatment of an inflammatory disease like rheumatoid arthritis with drugs like gold thioglucose and auranofin, which are strong inhibitors of TrxR, likely occurs by binding to the reduced Sec residue in the enzyme.

12.8 Concluding Remarks

Human TrxR is a reducing enzyme with a wide substrate specificity contributing to cellular redox homeostasis and is a major pathophysiological factor and drug target. Together with Trx, it is involved in prevention, intervention, and repair of damage caused by hydrogen peroxide-based oxidative stress. As a key reducing enzyme with a selenol-containing active site, human TrxR plays a central role in various biological processes including selenium metabolism. Many different types of clinical drugs and environmental toxins exhibit the inhibition and regulation on TrxR. These results pinpoint mammalian TrxR as a critical target for pharmacological application.

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Chapter 13
Selenoproteins of the Glutathione Peroxidase Family

Leopold Flohé and Regina Brigelius-Flohé

Abstract The glutathione peroxidase (GPx) family is spread over the entire living kingdom. In humans, eight distinct molecular paralogs coexist, five of which (GPx1, 2, 3, 4, and 6) contain Sec as the active-site residue. The selenoperoxidases (SecGPx) prevail in vertebrates, while GPx homologs having the active-site Sec replaced by Cys (CysGPx) are found in terrestrial plants, yeasts, protozoa, and bacteria. The typical signature of GPxs is an active-site tetrad composed of Sec or Cys, Trp, Gln, and Asn. SecGPx efficiently reduces hydroperoxides with rate constants, k_{+1}, beyond 10^7 $M^{-1}s^{-1}$, while the CysGPxs rarely reach a k_{+1} near 10^6 $M^{-1}s^{-1}$. The scope of accepted hydroperoxides appears to broaden from GPx1 and GPx2 to GPx3 and phospholipid hydroperoxide GPx (GPx-4), while the specificity for GSH declines in this order. Most of the non-mammalian CysGPxs use redoxin-type proteins as reducing substrate. The scope of biological functions of GPxs comprises detoxification of hydroperoxides, inhibition of apoptosis and inflammatory processes, modulation of signaling cascades, sensing of H_2O_2 for activation of transcription factors, and using ROOH for the synthesis of structural proteins.

13.1 Introduction

Glutathione (GSH) had been recognized as maintaining cellular redox balance long before the discovery that it does so only as substrate of an efficient peroxidase. Glutathione peroxidase, now termed GPx1, was indeed the first non-heme peroxidase shown to specifically use a thiol for the reduction of H_2O_2 (13.1) [1].

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$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \quad (13.1)$$

It was also the first, and for a long time the only, mammalian enzyme that was recognized to utilize selenium for catalysis. As recently reviewed [2], the history of GPx was instrumental in unraveling the role of the essential trace element selenium and fertilized the entire field of selenium biochemistry. It was the first selenoenzyme to be kinetically [3, 4] and structurally analyzed [5] and sequenced [6], and the insights thereof disclosed the magic catalytic power of selenium, allowed first concepts on the redox catalysis of selenium in proteins, and critically contributed to the discovery that the opal codon TGA can code for selenocysteine incorporation [7, 8].

Meanwhile more than 700 GPx-related sequences have entered the databases revealing that GPx, as discovered back in 1957 [1], is just one prototype of a huge enzyme family which is spread over all domains of life [9]. Only a minority of the GPxs are selenoproteins. The latter prevail in mammals and other vertebrates and have only sporadically been detected in lower animals such as *arthropoda* and *trematoda*, exceptionally in bacteria, so far not in *protozoa*, and not, of course, in fungi and green plants that are generally devoid of the machinery for selenoprotein synthesis [9]. An intriguing exception is the green alga, *Chlamydomonas reinhardtii*, which contains ten selenoproteins including a SecGPx [10].

Another surprising finding was that the GPxs changed their specificity during evolution. As first detected in *Plasmodium falciparum,* a protein that by sequence is a *glutathione* peroxidase may adopt the function of a *thioredoxin* peroxidase [11], thus sharing function with most of the peroxiredoxins [12]. This specificity switch has so far mainly been observed in CysGPxs of plants, *protozoa* and *arthropoda* [13]. Likely, the *glutathione* peroxidase activity, which gave name to the entire family, is rather the exception than the rule. Inversely, glutathione peroxidase activity may be associated with a variety of proteins. The reaction of GSH with ROOH has been reported to be catalyzed by, e.g., GSH-S-transferases B [14], selenoprotein P [15, 16], and peroxiredoxins [12, 17, 18] sharing with the GPx family no or marginal sequence similarity, respectively.

In this chapter, we restrict ourselves to the "real" glutathione peroxidases, as defined by sequence homology, and since several chapters of this volume are devoted to particular types of GPx, we will here focus on basic aspects of GPx catalysis and their biological relevance.

13.2 Structure and Peroxidatic Activity

A common feature of the GPx family is a conserved catalytic tetrad composed of Sec or Cys, Gln, Asn, and Trp residues (Fig. 13.1). Sec is present in the five prototypes of GPx prevailing in, yet not restricted to, vertebrates: (1) in "cytosolic" or "classical" GPx (GPx1) [6, 19, 20]; (2) in the extracellular GPx (GPx3) [21]; (3) in phospholipid hydroperoxide GPx (PHGPx; GPx4) [22, 23]; (4) in the gastrointestinal GPx (GI-GPx; GPx2) [24]; and (5) in man at least in GPx6 [25]. Among the

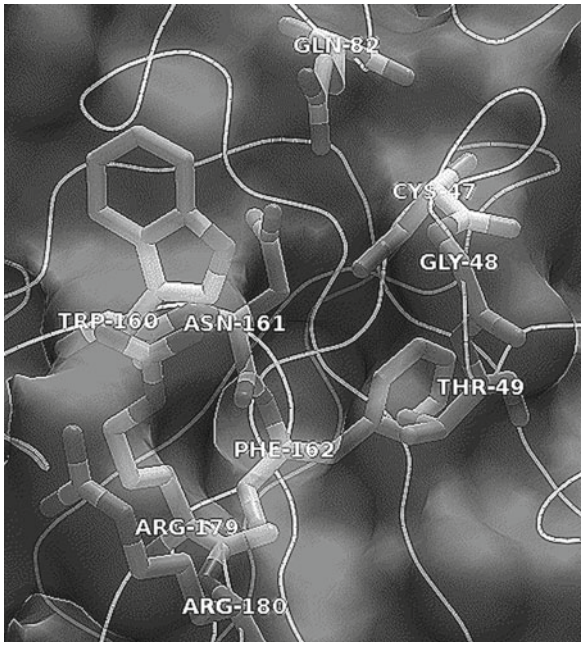

Fig. 13.1 Active site of human GPx1. The Se of selenocysteine (Cys47) is surrounded by the tetrad residues Gln82, Trp160, and Asn161. These residues, as well as Gly48 and Phe162, are highly conserved in the entire GPx family, while Arg179 and Arg180 are characteristic for GPx1. The model was generated by Stefano Toppo (Padova, Italy) from the structure of a Sec→Gly variant of GPx1 (pdb code: 2F8A)

Cys-containing congeners of vertebrates, GPx5 is related to GPx3, while GPx7 and 8 are closer to GPx4 [9]. The conserved Sec or Cys is the redox-active moiety, as was first shown by replacement of Sec in GPx1 by Cys which caused a substantial decrease in activity, while replacement by Ser abrogated activity completely [26]. This Sec or Cys, respectively, is oxidized by H_2O_2 or other hydroperoxides during the catalytic cycle and therefore called the peroxidatic Cys (C_p) or Sec (U_p). Irrespective of the active residue being Sec or Cys, the intimate environment, as shown in Fig. 13.1 for GPx1, is conserved in the entire family with a vanishing number of exceptions [27, 28]. The selenium (or sulfur) is localized in the center of the catalytic tetrad and is coordinated to the carboxamide groups of the Gln and Asn and the indole nitrogen of the Trp. A possible contribution of Gln and Trp to catalysis had already been inferred from the first X-ray analysis of a GPx [5] and was confirmed by mutagenesis studies [29–31]. The critical role of the Asn that immediately follows the Trp was later disclosed by comparison of GPx structures and mutagenesis [27]. Its carboxamide function reaches the active-site sulfur or selenium from the protein core, while the Gln carboxamide does so from the opposite site. As is evident from the position numbers in Fig. 13.1, the active site is built up by three remote loops of the GPx proteins: The redox-active Sec or Cys is embedded in a $Gx_6NvAx_2U(C)g$ motif near the N-terminus; the typical Gln is the last highly conserved one in an fPcnQFgxq motif some 20 residues downstream, and the Trp and Asn mark the start of a strictly conserved WNF motif ($WNFxkxlvx_3Gx_2vxry$) closer to the C-terminus.

The catalytic relevance of the tetrad residues is seen in activating the Se (or S) for reaction with a hydroperoxide, as well as in facilitating the cleavage of the hydroperoxy bond. The carboxamide amido groups and the imino group of the Trp have often been suspected to form hydrogen bonds to the active-site selenium (or sulfur) [5, 27], thereby forcing the thiol or selenol, respectively, into dissociation. Lowering the pK_a of C_P in CysGPxs is indeed a prerequisite for a fast reaction with peroxides, while for a U_P the pK_a of about 5 is low enough anyway. Neither sulfur nor selenium, however, is particularly prone to build stabile hydrogen bonds and, more correctly, the contribution of the tetrad residues to catalysis is interpreted by shuttling labile protons toward the selenium (or sulfur, respectively) and the substrate. The residue most important in lowering C_P in GPx of *Drosophila melanogaster* was shown to be the tetrad Asn. Its replacement by Asp led to a calculated upward shift of the pK_a of C_P by three units and complete loss of enzymatic activity [27]. Also the exchange of the tetrad Trp and Gln to apolar or negatively charged residues was reported to increase the pK_a of C_P and to decrease activity of the artificial porcine CysGPx4 [29] in the GPx-type Orp1 protein of yeast [31] and in a GPx-type thioredoxin peroxidase of *Trypanosoma brucei* [30], although to a minor extent. In fact, the Gln could even be exchanged by Gly in Chinese cabbage GPx without any loss of activity [32] and is naturally replaced by Glu in some plant GPxs [33] and by Ser in human GPx8 [9]. Thus, the tetrad Gln tolerates some variability, while the Asn and Trp are strictly conserved. Working in concert, the two surface exposed residues Gln and Trp may polarize the hydroperoxide substrate, which is equally important, since dissociation of the active-site SeH or SH is by no means sufficient to explain the efficiency of GPxs. The nonenzymatic reaction of a fully dissociated low molecular mass thiol with H_2O_2 hardly proceeds with a rate constant higher than 50 $M^{-1}\,s^{-1}$ [34] and the corresponding (unknown) rate constants for selenolates cannot reasonably be estimated to be much faster. By contrast, the rate constants for oxidation of the C_P or U_P in GPxs range from 10^3 to 10^6 $M^{-1}\,s^{-1}$ and 10^6 to 5×10^7 $M^{-1}\,s^{-1}$, respectively [28]. The substantial discrepancy between the spontaneous and enzymatic reaction velocities reveals catalytic increments beyond lowering the pK_a of C_P or U_P. Polarization of Se (S) to facilitate a nucleophilic attack on the peroxide, hydrogen bonding to the substrate to polarize the hydroperoxy bond for easier cleavage, and, again, proton shuttling to generate H_2O or ROH as good leaving groups likely synergize for the enzymes' extreme efficiencies [27, 28, 35], while the relative contribution of the residues involved remains uncertain and appears to vary between individual GPxs.

The products of the first catalytic step are H_2O or an ROH and an oxidized enzyme (F in Fig. 13.2), which then has to react with thiols to complete the catalytic cycle. The chemical nature of the catalytic intermediate F has been clarified by mass spectrometry in the case of CysGPxs [31]: Like in the other large thiol peroxidase family, the peroxiredoxins [36], it is the sulfenic acid form of the enzyme (13.2).

$$\text{GPx - S}^- + \text{H}_2\text{O}_2 \rightarrow \text{GPx - SO}^- + \text{H}_2\text{O} \tag{13.2}$$

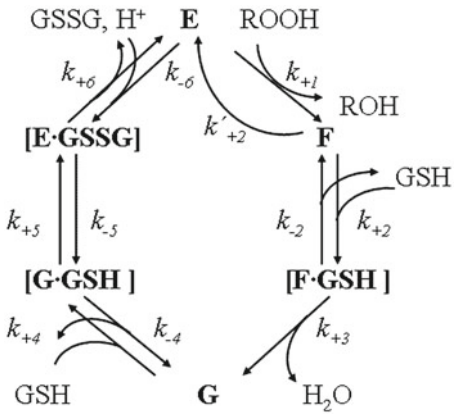

Fig. 13.2 Catalytic cycle of a typical GPx. E is the ground-state enzyme with Sec (Cys) being present as selenolate (thiolate). F represents the oxidized enzyme of unclear structure in case of SecGPx (see text; Sect. 13.3.1); G is a Se-glutathionylated intermediate. The rate constants k_{+1} and k'_{+2} are those obtained experimentally. They describe the oxidation of E to F and the regeneration of E from F, respectively. Other microscopic rate constants are introduced for qualitative consideration

The sulfenic acid function of C_P then readily reacts with a thiol, which may be GSH to form the glutathionylated enzyme form G from which the ground-state enzyme E is regenerated by a second GSH molecule (Fig. 13.2).

Alternatively, the oxidized C_P can form an intramolecular disulfide bond with another Cys residue as in atypical 2-Cys-peroxiredoxins, thereby creating an ideal basis for reduction by redoxin-type proteins (Fig. 13.3), which is likely the case in most of the nonvertebrate GPx-type proteins [13]. Like in peroxiredoxin catalysis [36, 37], the second Cys is assumed to be essential for completion of the catalytic cycle and is therefore called the resolving cysteine C_R.

By analogy to CysGPxs and peroxiredoxins catalysis, formation of a selenenic acid is commonly inferred for the initial oxidative step of SecGPxs catalysis (13.3).

$$\text{GPx - Se}^- + \text{H}_2\text{O}_2 \rightarrow \text{GPx - SeO}^- + \text{H}_2\text{O} \tag{13.3}$$

However, all attempts to verify this straightforward hypothesis have so far failed. Instead, the oxidized form of SecGPx4 presented as an enzyme with the molecular mass of the reduced one minus two mass units [38] and identical results were obtained with SecGPx1 (F. Ursini, personal communication). Evidently, the selenenic acid form, if it is formed at all, immediately splits off a water molecule. Available structures do not show any Cys that could react with the active-site Sec in the common SecGPxs and, thus, the likely solution of the enigma is the formation of a selenylamide bond in analogy to the catalytic cycle of GPx mimics such as ebselen [39]. With respect to the downstream events, such selenylamide would be equally suited to be reduced by GSH to form G, the Se-glutathionylated enzyme form which has been verified by mass spectrometry [38], and further to the ground state E to start the next catalytic round (Fig. 13.2).

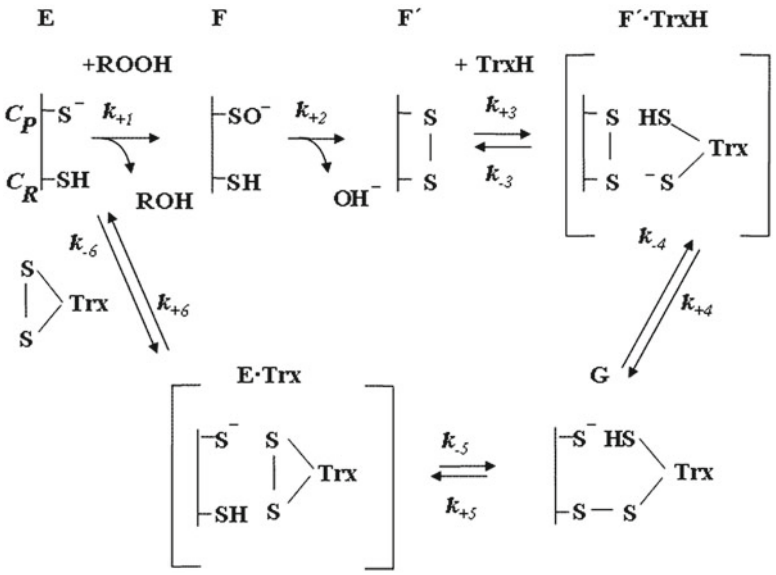

Fig. 13.3 Catalytic cycle of a 2-CysGPx with thioredoxin specificity. The active-site cysteine (C_P) is oxidized to a sulfenic acid (F) which, like in peroxiredoxin catalysis, forms an intramolecular disulfide (F′) with a resolving cysteine (C_R) before E is regenerated by reduced thioredoxin (TrxH) through thiol/disulfide exchange reactions

13.3 Structures and Substrate Specificities

13.3.1 Hydroperoxide Specificity

So far all members of the GPx family proved to be thiol-dependent peroxidases acting on H_2O_2 [1], peroxynitrite [40], and a broad range of organic hydroperoxides [41] but not on dialkyl peroxides. In line with this poor selectivity, a classical binding pocket for the hydroperoxide substrate is not detectable in any of the GPx structures. The reacting Se (or S) is surface-exposed and the ROOH specificity is primarily determined by the accessibility of the reaction center by the substrate's OOH function. The latter appears to be modulated by the quaternary structure of the enzymes. The tetrameric vertebrate GPxs are known (GPx1–3 and 5) or presumed (GPx6) to accept H_2O_2 and soluble low molecular mass hydroperoxides such as t-butyl hydroperoxide, cumene hydroperoxide, hydroperoxy fatty acids [41], and even hydroperoxy lysophosphatides [42]. However, these tetrameric vertebrate GPxs either do not (GPx1, GPx2) accept, or only comparatively poorly (GPx3) [16] accept hydroperoxides of more complex lipids such as phosphatidylcholine hydroperoxide (PCOOH) or cholesterol hydroperoxide that form micelles or are integrated in biomembranes [22, 24, 27]. The reduction of hydroperoxides of

complex lipids is the domain of the monomeric SecGPx4 of mammals [22, 27] and has also been documented for a related SecGPx of *Schistosoma mansoni* [43] and a monomeric CysGPx from *D. melanogaster* [13], while no or poor activities with PCOOH were found with an equally monomeric CysGPxs from *P. falciparum* [11] and *T. brucei* [44]. The hydroperoxide specificity of GPxs, thus, does not strictly depend on the oligomerization state. The pronounced preference of mammalian GPx4 for PCOOH has *inter alia* been attributed to a positively charged surface area near the active site which may interact with the negative charges of phospholipids [28]. As a rule, however, a monomeric nature appears to facilitate the interaction with OOH groups of lipids in biomembranes, as its reactive Se (S) is freely exposed, while in the tetrameric GPxs the active site is located in a kind of flat valley built up by the subunit interface [5, 28]. Determinants of oligomerization [9] may therefore be taken as reasonable predictors of the ROOH specificities of GPxs that have only been identified by sequence. Essentially, the oligomerization depends on the "oligomerization loop" which comprises about 20 residues between the third α-helix and the WNF motif and a PGGG motif downstream of the second α-helix. These structural elements characterize the tetrameric enzymes and are deleted in monomeric GPxs. Accordingly, among the poorly investigated mammalian GPxs, GPx6 is predicted to be tetrameric and having a restricted hydroperoxide specificity, whereas GPx7 and 8 should be monomeric and might be able to reduce complex lipid hydroperoxides.

13.3.2 Thiol Specificity

A characteristic binding pocket for the reducing substrates of vertebrate GPxs cannot be detected either. Instead, the pattern of surface charges, which is characteristic for the individual types of GPxs, appears to accommodate the substrate in a way that its thiol can react with the enzyme's oxidized Se or S. In the GPx1 subfamily, which is highly specific for GSH [45], the U_p is surrounded by four Arg residues (R57, 103, 184, and 185 in the bovine sequence) and a Lys (K91) of an adjacent subunit. These residues have been suggested to bind to the carboxylic functions of GSH by X-ray studies [5], docking experiments [12], and molecular dynamics calculations [35]. Depending on the approach, however, the proposals of substrate binding differed and likely there are different possibilities to direct the substrate's SH into a position suitable for the reaction with the selenium.

Irrespective of the relative contribution of the five basic residues to GSH binding by GPx1, it is intriguing to find them gradually decreased in the other subfamilies in parallel with a trend toward lower GSH specificity. In GPx2, only Lys91 and Arg185 are replaced by uncharged residues. The specificity of GPx2, which has not been systematically investigated, may be considered to be similar to that of GPx1; in GPx3 and the similar GPx5 family only Arg103 and Arg185 are conserved, and reactivity with thioredoxin and glutaredoxin has been reported for GPx3 [13, 46]; in GPx4 none of the residues binding GSH in GPx1 is conserved. GPx4 is nevertheless

a *glutathione* peroxidase. Here, Lys residues near the reaction center substitute for the Arg residues in attracting GSH [28, 38]. Beyond, however, GPx4 readily reacts with many low molecular mass thiols [47] and peptide thiols with PPCCPP motifs [48], thiols of chromatin [49] and of GPx4 itself [38, 50] (for more details see Chap. 14). No structural feature that would suggest specificity for GSH is conserved in the newly discovered GPxs 6–8.

With the vertebrate SecGPx3 and the nonvertebrate CysGPxs, the GPx family expands its activity spectrum into the thioredoxin system (see Chap. 12). Typically, thioredoxin reduces protein disulfide bonds, as they are also created in F´ of the catalytic cycle of many CysGPxs (Fig. 13.3). This variation of GPx catalysis usually depends on the presence of a C_R that is localized in a flexible loop 9–13 residues downstream of the tetrad Gln and, thus, may be considered as a predictor of redoxin specificity. As in 2-Cys-peroxiredoxin catalysis, the disulfide formation in the "2-CysGPxs" requires substantial conformational changes including unwinding of the α-helix in the flexible loop and complete disruption of the catalytic tetrad to build an interface for the reaction with the redoxin [12]. Neither the substrate interacting sites of these GPxs nor those of the redoxins look particularly characteristic and might be designed for interaction with different proteins, as was demonstrated for the Orp1/GPx of *Saccharomyces cerevisiae* which "specifically" interacts either with thioredoxin or with the transcription factor Yap1 [51].

13.4 GPx Kinetics

The GPx1 kinetics were worked out almost 4 decades ago [3, 4], and confirmed for other SecGPxs such as GPx4 [22] and GPx3 [16, 52]. But the implications for activity determination and physiological role are still widely ignored. As with superoxide dismutases, catalase, and many of the peroxiredoxins, the kinetics of SecGPxs cannot be described in terms of the Michaelis–Menten theorem such as V_{max} and the Michaelis constant K_M. These constants do not exist in SecGPx kinetics: they are infinite. As a consequence, the rather complex general rate equation for an enzymatic reaction involving three substrates, in case of SecGPxs, shrinks to the simple (13.4):

$$[E_0]/v_0 = 1/k_{+1}[ROOH] + 1/k'_{+2}[GSH] \tag{13.4}$$

Therein, $[E_0]$ is the total enzyme concentration, v_0 the initial velocity, k_{+1} the rate constant for the reaction of the reduced enzyme with the ROOH, and k'_{+2} the net forward rate constant for the reduction of the oxidized enzyme by GSH (Fig. 13.2). The rate constants k_{+1} and k'_{+2} are experimentally available from steady-state kinetics, while the remaining microscopic ones shown in Fig. 13.2 are not. Equation (13.4) describes a reaction mechanism involving a sequence of bimolecular reactions, as is in line with the scheme shown in Fig. 13.2. This mechanism corresponds to an "enzyme substitution mechanism" or "ping-pong" mechanism without interim formation of enzyme–substrate complexes, as had been anticipated to occur in Keith

Dalziel's systematic compilation of multisubstrate reaction mechanisms as the reaction type IVii previously described in 1957 [53]. Figure 13.2 indeed assumes that the reaction of a SecGPx with ROOH does not involve an enzyme–substrate complex because of the structural heterogeneity of accepted hydroperoxide substrates and lack of kinetic evidence. However, with GPx1 at least, the reductive part is highly specific, which implies that GSH must be specifically complexed to the oxidized enzyme forms F and/or G. That these enzyme–substrate complexes are not reflected in the kinetic pattern is easily explained by the efficiency of selenium catalysis as well as by rotational freedom of the substrate GSH. It likely takes more time to force the flexible GSH molecule into a productive conformation and location at the enzyme surface (see Sect. 13.3.2) than to glutathionylate the highly reactive Se within the complex [F•GSH] to form the second catalytic intermediate G. Just by chemical reasoning, $k_{+2} - k_{-2}$ should be much smaller than k_{+3} and, therefore, the complexes [F•GSH] will never accumulate. The same consideration applies to the regeneration of E from G. Formation of the symmetric disulfide GSSG from the selenylsulfide in G should be faster than formation of the complex [G•GSH]. Thus, the speed of the intra-complex reactions of SecGPxs precludes the Michaelis–Menten-type saturation kinetics observed in most of the enzymatic reactions.

This interpretation of the SecGPxs kinetics is supported by a revealing difference observed with 2-CysGPxs acting on thioredoxins (Trx; see Fig. 13.3). They also display ping-pong kinetics, but with some of these enzymes saturation kinetics are observed and the rate equation, thus, is enriched by the term $1/k_{cat}$ (13.5).

$$[E_0]/v_0 = 1/k_{cat} + 1/k_{+1}[ROOH] + 1/k'_{+2}[Trx] \tag{13.5}$$

This shift in the kinetic pattern likely reflects the lower reactivity of S vs. Se and easier binding of the more rigid Trx compared to GSH. Thereby, the intra-complex reactions may become rate limiting, as in the Michaelis–Menten case. Alternatively, though, another monomolecular reaction might become rate limiting: the formation of the disulfide in F′ from the sulfenic acid form F, which, as mentioned, requires substantial conformational changes. The assignment of k_{cat} in these enzymes, thus, remains ambiguous. The shift to saturation kinetics is, however, not always observed in 2-CysGPxs. While, e.g., the *P. falciparum* GPx and a tryparedoxin peroxidase of *Leishmania major* have defined K_M values for their redoxins of 10 and 3 μM, respectively, lack of saturation was again obtained with homologous 2-CysGPx proteins from *D. melanogaster* and *T. brucei.* Such changes in kinetic patterns are also observed in the mechanistically related peroxiredoxin family [54]. These observations demonstrate that there is not a fundamental difference between the peroxidases working with sulfur or selenium catalysis. Available quantitative data on GPx kinetics have recently been compiled by Toppo et al. [28].

The practical relevance of the unusual kinetics of SecGPxs is that their activity cannot be determined according to IUPAC/IFFC rules, because there are no "saturating" substrate concentrations. Commonly, similar concentrations of ROOH and GSH are used for activity measurements. Because k_{+1} is much faster (~10^7 $M^{-1}\,s^{-1}$) than k'_{+2} (10^5 $M^{-1}\,s^{-1}$), the enzyme is completely oxidized under these conditions and, accordingly, the limiting rate of enzyme reduction is measured (13.6).

$$v_0 = -d[H_2O_2]/dt = k'_{+2}[E_0][GSH] \quad (13.6)$$

This implies that, irrespective of the method applied, the turnover depends on the total GSH concentration. Further, the rate is determined by k'_{+2}, a constant that unfortunately is dramatically affected by pH, ionic strength, and kind of ions. The resulting complications have been extensively and repeatedly discussed [41, 55, 56]. Yet, the growing GPx literature keeps being flooded with activity data that, due to undisclosed testing conditions, are almost meaningless.

With respect to physiology, the kinetics reveal that, in contrast to common feelings and the in vitro testing conditions (see above), the GPx turnover is usually independent from the GSH concentration. Although k'_{+2} is about two orders smaller than k_{+1}, the cellular GSH in the range of 2–10 mM guarantees that the enzyme is practically 100% reduced ($E_0 = E_{red}$). At estimated physiological steady states of 1 μM H_2O_2, (13.3) then shrinks to (13.7):

$$v_0 = -d[H_2O_2]/dt = k_{+1}[E_0][H_2O_2] \quad (13.7)$$

As is easily estimated with (13.3), the GPx turnover will not respond to a decrease in GSH to less than 10% of normal. Such dramatic drop in GSH is, however, only observed when the generation rate of a hydroperoxide exceeds the regeneration of GSH from GSSG, a condition that typically marks the transition from physiology to pathology and physiologically occurs only in special situations such as spermiogenesis [57].

13.5 One Catalytic Principle, Many Functions

GPxs, together with peroxiredoxins, thioredoxins, thioredoxin reductases, selenoprotein P, and other selenoproteins, are commonly subsumed under the term "antioxidant proteins," which are believed to collectively fight "oxidative stress." The peroxidase nature of GPxs seems to justify this classification. Progress over the last 2 decades, however, reveals that nature uses the catalytic principle of GPxs for many purposes. Some of these functions may be seen in the context of balancing the threads of aerobic life, others are definitely not. We will therefore here briefly compile the diversified functions of GPxs.

Since its discovery [1], GPx1 has been, and still is, considered as the prototype of an antioxidant enzyme. Knockout studies revealed that GPx1 is dispensable for the unstressed organism but pivotal for the defense against oxidative stress, as compiled in the previous edition [58] and reviewed recently [59]. In line with its role as a dispensable emergency device to fight hydroperoxide challenges, its position in the hierarchy of selenoproteins (see Chap. 11) is one of the lowest and, accordingly, symptoms of marginal selenium deficiency may largely be attributed to impaired GPx1 activity. However, transcriptional activation of GPx1 expression, involving *inter alia*, an oxygen-responsive element ORE, P53, AP-1, PU.1, and NF-κB

consensus-binding sites (reviewed in [58, 59]) point to a more complex role than simply fighting oxidative stress. With the appreciation of H_2O_2 as a physiological signaling molecule, the role of thiol peroxidases needs also to be re-viewed [60]. The "detoxification reaction" (13.1) may equally regulate redox-sensitive signaling cascades, as demonstrated by impaired signaling triggered by TNFα [61], insulin [62] (see Chap. 20), epidermal growth factor [63], and lipopolysaccharide [64] due to GPx1 overexpression. A large body of evidence, though, supports an antioxidant action of GPx1. Accordingly, a protective role in conditions associated with $O_2^{\bullet-}$/H_2O_2 formation such as viral or bacterial infection, inflammation, reperfusion injury, cardiovascular disease in general, and malignant transformation is still the hot spot of clinical GPx1 research [59].

GPx1 shares the ability to suppress oxidant-driven apoptosis with other SecGPxs. Also this potential of the GPxs is commonly subsumed under their "antioxidant" action. By reducing hydroperoxides, the enzymes might simply postpone the point of no return where saving an oxidatively damaged cell is no longer reasonable. Apoptosis, however, is often a permanent physiological event that requires regulation by a GPx. GPx2 appears to regulate apoptosis and renewal of the epithelial lining in the intestine. It is highly expressed on the grounds of the crypts where proliferation takes place and declines toward the intestinal lumen where the apoptotic epithelial cells are continuously disposed. In GPx2 knockout mice, apoptosis occurs at lower sites of the crypts. Interestingly, under this condition, GPx1 is overexpressed and largely adopts the distribution typical for GPx2, without being able to fully substitute for GPx2 in preventing apoptosis [65]. When both enzymes are knocked out, the deregulation becomes obvious: collapse of the endothelial barrier due to enforced apoptosis, followed by massive inflammation [66], and ultimately inflammation-based development of tumors (see Chap. 21). Another example of specific inhibition of apoptosis is presented by the antagonistic couple 12,15-lipoxygenase and GPx4. The 12,15-lipoxygenase is the only one that generates lipid hydroperoxides directly at biomembranes, i.e., the substrates of GPx4. Like other lipoxygenases [58], also the 12,15-lipoxygenase is product-activated, which implies that GPx4 by removing its product inactivates the lipoxygenase [67]. 12,15-lipoxygenase has long been implicated in remodeling cells and tissues [68] and has become known to do so by activating an alternative cell death pathway involving the apoptosis inducing factor, AIF, that has to be tightly controlled by GPx4 [69] (see Chap. 43). It is likely the disruption of the delicate 12,15-lipoxygenase/GPx4 balance that leads to prenatal fatality due to GPx4 knockout.

The anti-inflammatory activity of GPxs is not a simple antioxidant function either. Beyond reduction of NOX-derived H_2O_2 generated by an initiating TLR activation, GPxs interfere with inflammatory processes with a broad spectrum of distinct regulatory actions. As mentioned above, GPxs inhibit lipoxygenases and also those that produce proinflammatory mediators such as prostaglandins and leukotrienes. Lipoxygenases generally require activation by hydroperoxides and are accordingly inhibited by GPxs with appropriate specificity. The concept was first shown for the cyclooxygenase/GPx1 couple [70] and then extended to the key enzyme of leukotriene biosynthesis, 5-lipoxygenase, which, depending on the cell

type, was shown to be inhibited by GPx1 [71, 72] or GPx4 [73–75]. Via inhibition of lipid mediator synthesis, GPxs may interrupt amplifying autocrine loops such as the upregulation of COX-2 expression by PGE_2 [76, 77] (for clinical implications see Chap. 21). Further, redox-sensitive signaling cascades triggered by inflammatory cytokines are commonly inhibited by GPxs, yet with distinct efficiency. Interleukin-1-triggered NF-κB activation, for instance, proved to be more efficiently inhibited by GPx4 than by GPx1 [77, 78].

Certainly, GPxs also interfere with gene expression. A mechanistically intriguing way to activate an adaptive response has been reported for the 2-CysGPx Orp1 in yeast. This enzyme is a thioredoxin-dependent peroxidase, but its "antioxidant" activity is left unused in the interaction with the transcription factor Yap1, when the adaptive response is to be activated. Instead of catalytically reducing H_2O_2, it "uses" the oxidation equivalents of H_2O_2, stored as the sulfenic form of its C_P, to oxidize and thereby activate the transcription factor [31, 51]. In chemical terms, this action is clearly the opposite of an antioxidant one, although the final outcome of the process is an increase in the defense against oxidant challenge. Analogous H_2O_2 sensing for transcriptional activation has also been detected for thiol peroxidases of the peroxiredoxin family [79] but not yet for any of the vertebrate GPxs. The mechanism as such has, however, been discovered to occur in mammals: In the final stage of spermiogenesis GPx4 is largely deprived of its substrate GSH [57] and, therefore, selenylates an SH group of itself and likely of other proteins. Thereby GPx4 polymerizes and finally becomes cross-linked with other proteins to form the mitochondrial capsule of spermatozoa [38, 50] (see also Chaps. 14, 32, and 43). This "moonlighting for fertility" of GPx4 in mammalian sperm [80] is analogous to the activation of Yap1 by Orp1 in the adaptive response of yeast [81], and it would be surprising if such mechanisms would not be applied for metabolic regulation in higher organisms.

In short, the basic mechanistic principle of thiol peroxidases, oxidation of a reactive Sec or Cys by ROOH and its reduction by RSH, is used by nature for multiple purposes, removal of hydroperoxides being the most straightforward, but by no means the only one. Looking back at the past decades [2], we may almost be sure that the GPxs will keep entertaining us with exciting novel applications of their catalytic tricks.

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Chapter 14
Glutathione Peroxidase-4

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Abstract Within the family of glutathione peroxidases (GPxs), GPx-4 is the sole monomeric enzyme that contains Sec at the active site. Phylogenetically, it is closer to the Cys-containing homologues (CysGPx) of invertebrata and vertebrata than to the tetrameric GPxs of vertebrata containing Sec. Nonetheless, the catalytic site is fully conserved in the whole family, suggesting a similar reactivity. As the tetrameric homologues, GPx-4 accepts GSH in the reductive steps of the catalytic cycle, while a redoxin is the preferred reducing substrate of the invertebrata CysGPxs. GPx-4 is also competent for oxidizing a quite heterogeneous series of thiol substrates. Reduction of complex membrane phospholipid and cholesterol hydroperoxides in cooperation with vitamin E accounts for the inhibition of lipid peroxidation by GPx-4. By no means, however, GPx-4 can be seen as just an antioxidant enzyme. Indeed reduction of lipid hydroperoxides accounts for the anti-apoptotic and anti-inflammatory effect of GPx-4 activity, and oxidation of specific protein thiols is its peculiar function in the late phase of spermatogenesis. Whether this reaction is relevant in other biochemical pathways, where a redox switch drives a functional shift in specific proteins, remains as an open and challenging option. In this chapter, the enzymology of GPx-4 will be reviewed focusing on the two best-characterized aspects: (1) inhibition of lipid peroxidation, and (2) oxidation of specific protein motifs. We refer to other chapters in this book for insights contributed by inverse genetic studies and for the general aspects of selenium catalysis in peroxidases.

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14.1 Introduction: The Discovery and Purification of a New Enzyme

Discovery, purification, and characterization of enzymes were the landmarks of the evolution of biochemistry in the pre-genomic era of the twentieth century. Usually, enzyme discovery was the successful outcome of having identified an enzymatic activity and purified the protein that catalyzes it. GPx-4 was among the last enzymes identified by following its activity – inhibition of lipid peroxidation – and purified through chromatographic steps following an increase of the specific activity [1].

The purified protein inhibited microsomal lipid peroxidation in the presence of GSH, accounting for the effect of a "cytosolic factor" previously identified by Paul McCay and coworkers [2], whose enzymatic activity was unknown. Just on a theoretical basis, and primed by ongoing studies on the mechanism of lipid peroxidation in our laboratory [3, 4], we resorted to search for the peroxidase activity on hydroperoxide derivatives of phospholipids of the "peroxidation inhibiting protein" (PIP) that we had purified. The identification of such an activity [5] prompted us to rename PIP as a new enzyme, i.e., "phospholipid hydroperoxide glutathione peroxidase" (PHGPx) [6]. Although the peroxidatic reaction was similar to that of the already known tetrameric Glutathione Peroxidase (now GPx-1) [7], the remarkable difference was the failure of the latter to reduce hydroperoxide derivatives of complex lipid substrates [8], which was in agreement with its failure to inhibit microsomal lipid peroxidation induced by NADPH or ascorbate and an iron complex [2].

The analogy of PHGPx with the tetrameric glutathione peroxidase prompted a search for a selenium moiety, which was indeed successful, and led to the final evidence for the second mammalian selenoperoxidase (SecGPx) [6]. The possibility that PHGPx could be just a monomer of the tetrameric glutathione peroxidase was definitely ruled out when, in the laboratory of Leopold Flohé, the sequence analysis on a sample of purified PHGPx disclosed a protein poorly related to the tetrameric glutathione peroxidase, which was indeed a product of a new gene [9, 10]. Eventually, although PHGPx was the second glutathione peroxidase purified, it was systematically named as GPx-4, following the discovery of the tetrameric GPx-3 (also called plasma GPx) [11, 12] and GPx-2 (also called gastrointestinal GPx, GIGPx) [13].

14.2 Enzymology

14.2.1 Activity, Substrate Specificity, and Kinetics

The usual substrate for measuring GPx-4 activity is phosphatidylcholine hydroperoxide (PC-OOH) dispersed in Triton X-100 micelles. Soybean phosphatidylcholine, which is a mixture of phosphatidylcholines containing different fatty acids, or a specific molecular species, is routinely used to prepare the hydroperoxy substrate either by auto-oxidation or enzymatic hydroperoxidation in the presence of Soybean

Lipoxygenase IV [14]. Apparently, the enzyme lacks specificity toward the oxidizing substrate. GPx-4 indeed reduces, besides H_2O_2, all the species of lipid hydroperoxide (R-OOH) so far tested – i.e., the hydroperoxides of different free fatty acids (FFA-OOH), phospholipids, and triglycerides as well as cholesterol and cholesterol ester hydroperoxides [6, 15]. Specificity appears restricted only to the hydroperoxy group whatever it is bound to an H atom or to a large and complex lipid substrate. Notably, among the physiological oxidizing substrates, only H_2O_2 and FFA-OOH are shared with tetrameric GPx-1.

The interfacial character of the GPx-4 reaction has never been analyzed in detail, and relevant questions about interactions with specific membranes, or specific lipid rafts, are still unaddressed, although it is expected physiologically quite relevant. We currently know that the possibility of accommodating large hydroperoxide substrates results from the absence of the tetrameric interface-containing loop (see below) [16]. While considering that the acquisition of this loop is a late achievement in evolution of the family [17], a challenging question emerges about the actual relevance of the quaternary structure of GPxs that, paradoxically, does not seem to have resulted in anything else so far, but a loss of function.

GPx-1 and -4 also diverge in respect of the specificity for the reducing substrate, only GPx-1 being highly specific for GSH, while GPx-4 accepts several structurally unrelated small molecular weight thiols [18, 19]. Furthermore, GPx-4 oxidizes also specific protein thiols under the permissive condition of a low concentration of GSH, the competing substrate for the reductive step of the peroxidatic reaction (see also below) [20, 21].

In spite of the above distinct substrate specificity, the kinetic mechanism of GPx-1 and GPx-4 is apparently identical, as indeed expected, since the active site has been strictly conserved [22, 23]. The steady-state kinetic analysis fits a model of a ping-pong mechanism where the interaction of the enzyme with the substrate and the release of the products are much slower than the redox transitions. This prevents the accumulation of enzyme–substrate complexes, and thus the V_{max} and K_m are infinite. For more details on GPxs kinetics see Chap. 13.

14.2.2 Structure

The *GPx-4* gene produces three distinct mRNAs differing in their 5′ ends, encoding for the mitochondrial, the cytosolic, and the nuclear proteins [24]. While the first two mRNAs result from a longer or shorter transcript of the first exon, respectively, either including or lacking an upstream ATG, the nuclear protein is built up by an alternate promoter on a distinct transcriptional initiation site in an alternate exon located within the first intron of *GPx-4*. The N-terminal extension is cleaved off completely in the mitochondrial and partially in the nuclear product. Thus, in the rat, while the cytosolic and the mitochondrial products are identical and have an MW of 19,146 Da, the nuclear GPx-4 has a variable N-terminal extension and has a higher MW by 3,403 or 3,272 Da. All these forms coexist in nuclei [25].

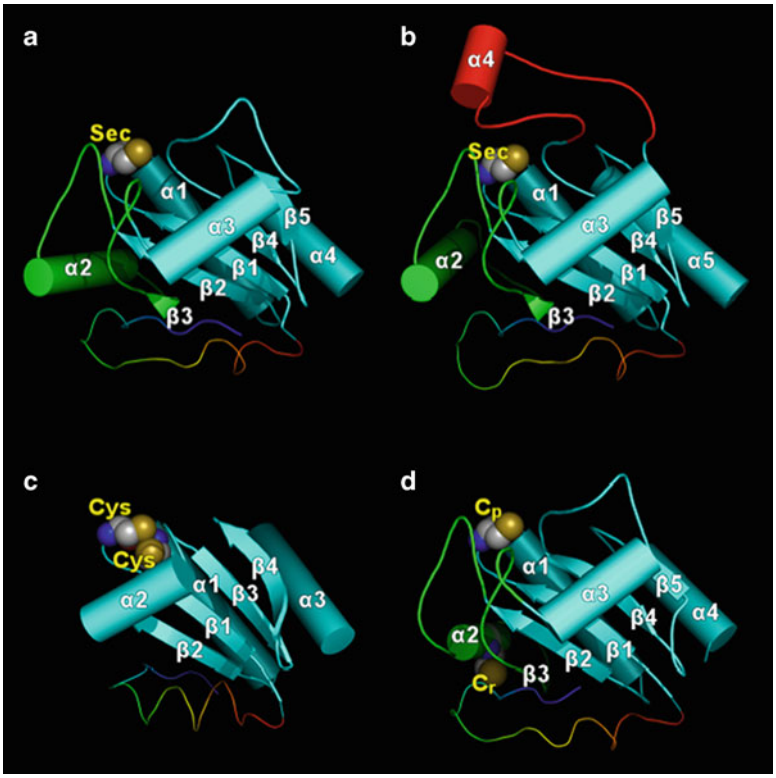

Fig. 14.1 Canonical topology and least common secondary structural elements, shown in cyan, shared by proteins belonging to the thioredoxin fold (see text). All GPxs possess an additional alpha helix (*green* α2 shown in (**a**), (**b**), and (**d**)) compared to the thioredoxin reference structure (**c**). Tetrameric GPxs shown in (**b**) have an extra alpha helix (α4 in *red*) required for oligomerization. Redox sensitive/catalytic Cys or Sec are reported as spheres. In (**a**), human monomeric GPx-4 (pdb id: 2OBI); in (**b**), single subunit of human tetrameric GPx-1 (pdb id: 2F8A); in (**c**), human thioredoxin 2 (pdb id: 1UVZ); and in (**d**), model of monomeric *Drosophila melanogaster* GPx with the peroxidatic Cys indicated as Cp and the resolving Cys as Cr within the α2-helix

Since the definition of the primary structure of cytosolic GPx-4, several hundreds of homologous sequences have been deposited in databanks. This information, integrated by crystallographic data of the U46C GPx-4 mutant [26] and homologous proteins, permitted the definition of the structural features of the whole family of GPxs [17]. GPxs are folded according to the pattern first described for thioredoxin (Trx) (Trx fold) [27, 28] and shared with several families of oxidoreductases. In the Trx fold, the typical secondary structure pattern gives rise to a conformation where the four β sheets are flanked by three α helices yielding two layers α/β/α sandwich. The minimal common motif of the Trx fold is shown in Fig. 14.1. The core pattern starting from the N-terminus is the following: β1-α1-β2-α2-β3-β4-α3. In the GPx

fold, an additional α-helix and a small β-sheet are inserted between β2 and α2 of the Trx fold consensus, whereas exclusively tetrameric GPxs, such as GPx-1, encompass another α-helix between α2 and β3.

Multiple sequence alignments and fold recognition analysis of a large number of homologous proteins revealed that the monomeric pattern is much more diffused in nature than the tetrameric pattern, the latter being apparently restricted to vertebrata and descending from the insertion of the additional α-helix generating the inter-subunit interface [17]. Notably, the large majority of non-mammalian monomeric GPxs contain a Cys substituting for Sec at the active site [17] that is associated, with few exceptions, with the presence of a second, functionally relevant, Cys residue in a variable position in the α2-helix (Fig. 14.1). This serves as resolving Cys in the peroxidatic cycle when, upon oxidation, it forms a disulfide with the peroxidatic Cys [16, 22]. This disulfide is eventually reduced by a redoxin. The formation of a disulfide within the catalytic cycle, which is eventually reduced by a Trx redox center, mirrors the catalytic mechanism of "atypical" peroxiredoxins [22].

14.2.3 Phylogeny and Homology Considerations

The GPx superfamily encompasses eight members, whose phylogeny could be reconstructed thanks to the vast amount of available sequence data in public databases (Fig. 14.2) [17]. Though a putative common ancestor may be recognized for vertebrate GPxs, the same cannot be unequivocally detected if the whole set of GPxs coming from all living organisms are taken into account. This means that complex relationships may have arisen and putative convergent evolution or lateral gene transfer, especially in bacteria, may have occurred. What unequivocally comes to light is the uneven distribution of selenium usage in GPxs during evolution. Selenium, indeed, seems a recent acquisition of the family, maybe dating back to the metazoan radiation when organisms substituted Sec for Cys as the redox-active moiety. Almost contemporary to the capacity to insert Sec, the acquisition of an α-helix favored the aggregation in tetramers (Fig. 14.1). As in the classical view of paralogy, the eight members of GPxs found in most vertebrata, and definitely in mammalia, have diversified their tissue/substrate specificity and function. The phylogenetic relationship of the vertebrata monomeric forms of GPx-4, which contain a catalytic Sec, with the majority of GPxs from invertebrata, containing a Cys residue at identical positions, is surprising: GPx-4 is indeed far closer to these Cys homologues than to the tetrameric paralogs, which carry the Sec residue at the active site. This might suggest that vertebrate GPx-4 forms have conserved the fold and scaffold features of the hypothetical monomeric Cys-based common ancestor and at the same time have undergone minimal but drastic change of acquiring the capability of inserting Sec. To some extent, the evolutionary recent vertebrate GPx-4 forms may be considered "fossil" enzymes, given that they preserve at best the features of distantly related sequences that the other members do not, and for this reason GPx-4 can be seen as a landmark peroxidase, representative of the GPx superfamily.

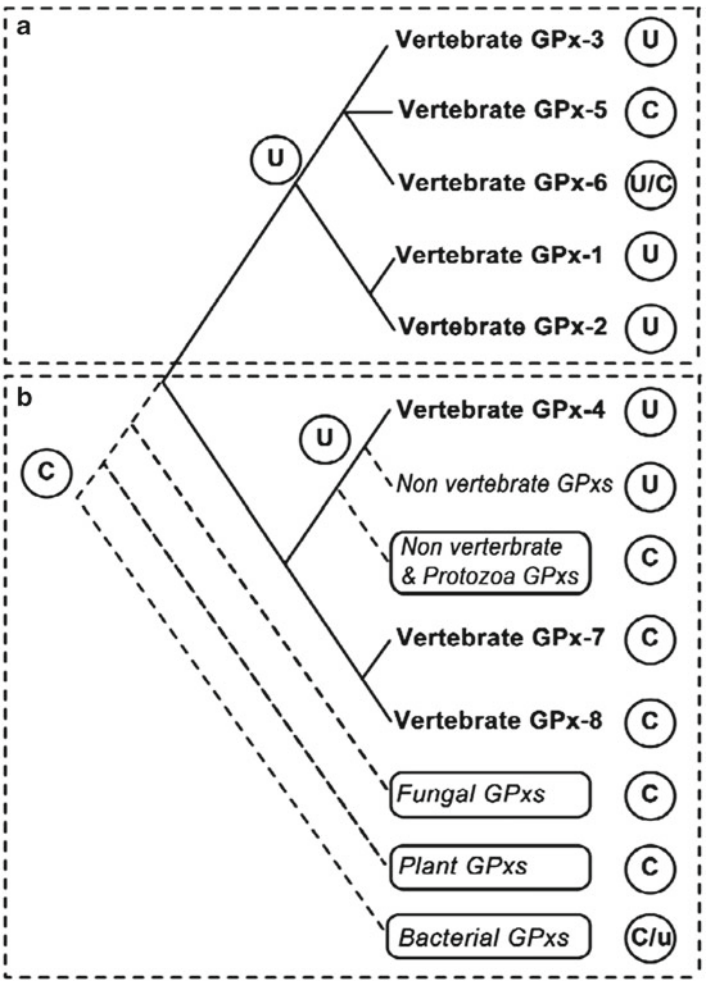

Fig. 14.2 Phylogeny of the GPx superfamily. Confirmed evolutionary reconstructions are reported in *solid lines* whereas *dashed lines* are uncertain or nonvalidated relationships (see text). Tetrameric GPxs are grouped in box a and monomeric GPxs in box b, including some reported dimeric forms in plants. Present GPxs are shown as leaves of the tree, while circles indicate either Cys (C) or Sec (U) in the catalytic center; u indicates an extremely rare, so far unique event. Internal nodes report the putative reconstruction of the original ancestor species carrying either Cys or Sec. Boxed GPxs contain the resolving cysteine (Cr) within the α2-helix (see text)

14.2.4 Active Site

The structure of the catalytic site, originally proposed from the crystal structure of bovine GPx-1, was found strictly conserved in vertebrate GPx-4, encompassing, besides the catalytic Sec, a Trp and a Gln residue, located in distant regions in the

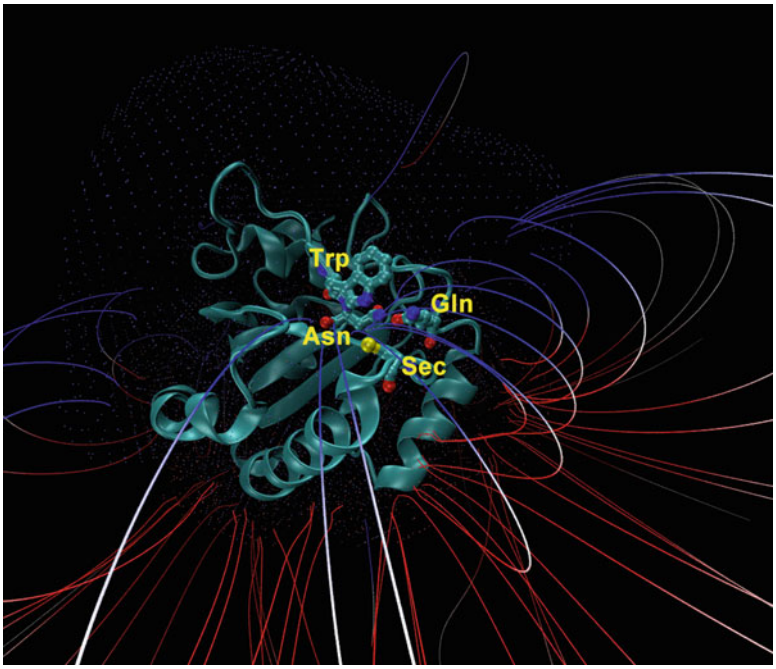

Fig. 14.3 The catalytic tetrad of GPx-4 and electric field lines in GPx-4 (PDB id: 2OBI, human enzyme). The amino acids indicated represent the experimentally validated catalytic tetrad. Electrostatic potential has been calculated on human GPx-4 by Adaptive Poisson–Boltzmann Equation and positive (*blue dots*) and negative (*red dots*) isosurfaces are shown at ±2kT/e. Electric field lines show the catalytic site surrounded by a strong cationic potential. In addition, a negative surface (*red lines* and *red dots*) is present, opposite to the positively charged catalytic pocket, creating a dipole moment as shown by the arcs connecting "*blue*" and "*red*" zones

primary structure. This catalytic triad, which was functionally validated by site-directed mutagenesis [29], has been recently revisited as a tetrad when an Asn residue was seen fully conserved in multiple sequence alignments and homology modeling and functionally validated by mutagenesis [30] (Fig. 14.3).

In GPx-4, the redox-active Se (or S in the mutant used for crystallization) lies in a flat surface that Adaptive Poisson Boltzman Solver Equation tool indicates as cationic [23] (Fig. 14.3). On the edge of this area, the oligomerization loop, found in GPx-1 but not in GPx-4, fulfills the evidence that large hydroperoxidic substrates cannot be accommodated in the tetrameric GPxs (Fig. 14.1a, b). The calculated electrostatic field also reveals that GPx-4 is highly polarized (Fig. 14.3). The cationic side overlapping the active site could be relevant for addressing the GPx-4 to specific locations in membranes where the enzyme might interact by specific electrostatic interactions instead of unspecific lipophilicity, as usually suspected for an enzyme whose action is on membranes.

14.2.5 Catalytic Mechanism

In a minimalist view, the groups surrounding the redox-active residue of GPxs (Sec or Cys), which are involved in catalysis, are the indole of Trp, the two amides of Asn and Gln, and possibly the amide in the backbone of the Gly one residue downstream the catalytic Sec/Cys residue. These are conserved at identical positions in nearly all members of the GPx family, the most remote derivatives included. For an efficient reduction of an R-OOH, the active site must fulfill the following minimal requirements: (1) ionization of the redox-active chalcogen; (2) "activation" of the O–O bond, reasonably through polarization or stretching of the bond; and (3) protonation of the leaving group $R\text{-}O^-$.

Dissociation is essential for the nucleophilic displacement reaction. This notion supported the widely diffused concept that the lower pK_a of selenol than thiol must account for the actual advantage of having Sec rather than Cys at the active site. Moreover, in *Dm*GPx, a CysGPx, the mutation of the amino acids surrounding the peroxidatic Cys affects the rate constant of the reaction much more than the pK_a of the chalcogen, clearly showing that the role of the active-site residues is broader than just lowering the pK_a of the redox-active moiety [30]. In the nucleophilic displacement reaction, where the enzyme reacts with the hydroperoxide, selenolate is expected to be a better nucleophile, but this accounts for an advantage of Se in the range of just one order of magnitude, as deduced from the comparison of kinetic analysis of a SecGPx (namely GPx-4) with a CysGPx (namely *Dm*GPx) on the same peroxidatic substrate [23].

An accurate quantum chemistry computational approach has been applied to study the steps of the catalytic mechanism in the active site of human GPx-4 or its Cys mutant. The computational protocol, rooted in advanced Density Functional Theory methodologies (DFT), has been optimized to minimize energetically the chosen set of amino acids surrounding the pocket [31]. Intriguingly, the proton of the selenol or the thiol has been observed dislocated in the catalytic cage and optimized in most of the tested locations, leading to the conclusion that it is displaced in the positively charged catalytic pocket rather than exclusively bound to the selenol or thiol [31]. In any case, the protonation of the amide of Trp seems to be somehow favored compared to the other sites, but good energies have been reported for the amides of Asn and Gln in terms of a low energy minimum and a conserved geometry of the catalytic site. In other words, the system is energetically relaxed and this "moving" proton is eventually bound to $R\text{-}O^-$ formed by R-OOH reduction. This mechanism fits two constraints of the catalysis of the reaction: ionization of the chalcogen and protonation of the leaving group. For the R-OOH substrate in the active site, the quanto-mechanical approach also indicated stretching and distortion of the O–O bond that is expected to further contribute to the catalysis. From this computational analysis, the outcome of the reaction mechanism is the instantaneous oxidation of selenium or sulfur by the hydroperoxide, without the formation of any enzyme–substrate complex. This nicely fits the non-saturation kinetics observed under steady-state conditions [19].

In GPx-4 and in the other SecGPx, the chemical form of the oxidized Se moiety has not been clarified. While the formation of a sulfenic acid derivative of the

enzyme has been experimentally observed for *Dm*GPx [16], and computationally confirmed by a well-defined energy minimum for the Cys mutant of human GPx-4 (unpublished), no analytical evidence has been so far obtained for the formation of a selenenic acid derivative of GPx-4. Furthermore, the transition state from the reduced enzyme to the oxidized form containing a selenenic acid derivative was not computationally supported by DFT calculations. This suggests the existence of a different oxidized intermediate in Cys- and in SecGPxs. In addition, a high sensitivity mass spectrometry analysis of oxidized GPx-4 and GPx-1 clearly showed a 2 a.m.u. decrease in respect to the reduced form (unpublished), thus suggesting the loss of two hydrogen atoms instead of the expected addition of one oxygen atom. This could indicate that either selenenic acid immediately extracts a hydrogen atom from suitable neighboring residue and releases water, or the oxidized form is initially different from selenenic acid.

In the reductive phase of the catalytic cycle, the oxidized intermediate is reduced by two thiol groups (e.g., 2GSH) in two steps. The formation of a mixed selenodisulfide as the first catalytic intermediate has been demonstrated for GPx-4 by MS/MS. In second step of the reductive phase, which is the last step of the peroxidatic cycle when the reduced enzyme is regenerated, the mixed disulfide is reduced by the second thiol. Very little is known about the mechanism of the catalysis of this exchange reaction. However, a faster reaction is expected for Sec than CysGPx, since selenolate is a much better leaving group than thiolate. This conclusion is supported by the NMR evidence that the rate of selenium diselenide exchange is several orders of magnitude faster than that of thiol disulfide [32]. Notably, in the last step of the peroxidatic cycle, the advantage of selenol vs. thiol is a more electrophilic character while just the opposite – more nucleophilic – is true for the first reaction of the cycle when the chalcogen is oxidized by the hydroperoxide [33].

14.3 Functions

GPx-4 is a vital enzyme. When its expression is abrogated in knockout models, the embryo dies at the gastrula stage [34]. This provides nonambiguous evidence that the maintenance of cellular homeostasis and survival strictly depends on GPx-4 activity and that the functions of this enzyme are not rescued by alternative biochemical pathways. The phenotypes obtained by deleting the different forms of GPx-4 are reviewed in Chap. 43. Here we summarize the biochemical evidence for the impact of the redox transitions catalyzed by GPx-4 on biological events.

14.3.1 Reduction of Lipid Hydroperoxides: Inhibition of Lipid Peroxidation

The notion that inhibition of lipid peroxidation by GPx-4 and GSH is due to the enzymatic reduction of R-OOH in membranes has twofold relevance. Besides leading

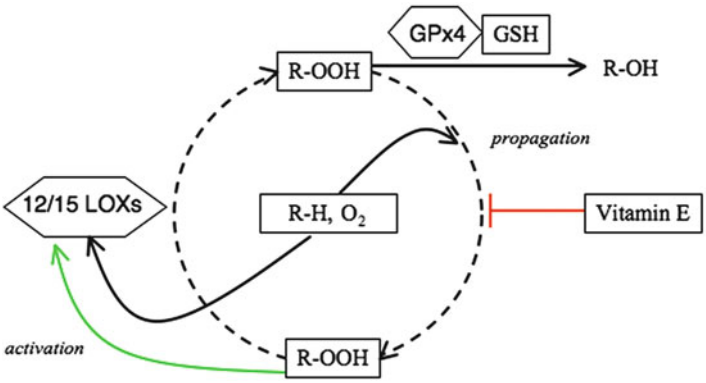

Fig. 14.4 Scheme of the synergism between GPx-4 and vitamin E in inhibiting lipid peroxidation and 12/15 lipoxygenases (LOXs). For sake of simplicity, individual specific reactions are not reported and lipid peroxidation is initiated from decomposition of preexisting phospholipid hydroperoxides (R-OOH)

to the discovery of a new enzyme, it contributed to focus the dual role of hydroperoxides that, besides being the major products of peroxidation, are also crucial initiators. Lipid peroxidation, first described more than a century ago by De Saussure, who observed the weight increase of polyunsaturated lipids exposed to air [35], is a process of oxidative degradation of lipids [36] producing, besides hydroperoxides, an array of secondary products including reactive and toxic electrophiles.

Consistently with a mechanism where, following a formation of an initiating species, lipid peroxyl radicals (R-OO$^{\bullet}$) drive the oxidative chain reaction and new chain reactions branch from radicals deriving from hydroperoxides, the protective mechanisms operating at different levels are integrated with each other [37]. While chain breaking antioxidants, such as vitamin E, slow down the R-OO$^{\bullet}$-driven propagation, the reduction of R-OOH to the corresponding alcohols (R-OH) prevents secondary initiations starting from hydroperoxide breakdown [38] (Fig. 14.4).

Rapoport and Schewe et al. first brought into light the role of enzymatically produced R-OOH in vivo. They showed that the maturational breakdown of reticulocyte mitochondria depends on a lipid peroxidation process driven by a 12/15 lipoxygenase (LOX) activity [39], thus demonstrating a physiological outcome of enzymatically produced R-OOH. The 12/15 LOX isoforms use as substrate intact membrane phospholipids and require at the same time R-OOH to become fully active [14, 40, 41] (Fig. 14.4). Consistent with its ability to reduce R-OOH in microsomal lipid peroxidation, GPx-4, in the presence of GSH, modulates the activity of reticulocyte 12/15-LOX and preserves the specificity of the oxidative process by preventing the nonenzymatic extension of lipid peroxidation [41].

The recent observation obtained by inverse genetic studies, that deletion of *GPx-4* in cells leads to apoptosis by a caspase-independent mechanism requiring a functional 12/15 LOX, and that vitamin E complements *GPx-4* deficiency [42], suggests

a molecular mechanism that is remarkably in agreement with the model of interplay between GPx-4 and a chain braking antioxidant, as summarized previously [38]. The synergistic interplay between different redox transitions working in a concerted mechanism discloses a scenario where the presence of a minute, steady-state concentration of R-OOH in membranes is physiological, and cell death descends from the unbalance of this steady state. Although further studies are required to elucidate this possibility, the control of apoptosis initiated by the activating effect of an R-OOH on LOXs seems at present sufficient to account for GPx-4 being a vital enzyme.

14.3.2 Reduction of Lipid Hydroperoxides: Regulation of Lipoxygenases and Inflammation

While the formation of oxidized phospholipids by LOXs is a relatively new emerging concept [43–45], evidence has been accumulated in the last decades about the physiological functions of enzymatically produced specific oxidation products of polyunsaturated fatty acids [46–48]. Nonconflicting evidence indicates that GPx-1 and GPx-4 are both competent for inactivating 5-LOX [49, 50], implying that the physiological activator is a common substrate for both peroxidases. In agreement with the concept of self-activation of LOXs by their reaction product, we could reasonably argue for a role of an FFA-OOH. Finally, overexpression studies show that GPx-4 prevents the activation of NF-κB much more efficiently than GPx-1 [51], thus suggesting its role in the control of inflammatory pathways where complex oxidized lipid mediators are apparently involved in regulatory redox transitions.

14.3.3 Oxidation of Protein Thiols: Spermatogenesis

In spermatids, GPx-4 is highly expressed as an active peroxidase that is transformed into an enzymatically inactive cross-linked structural protein during final sperm maturation. As such, it makes up at least 50% of the keratin-like material surrounding the helix of mitochondria in the mid-piece of spermatozoa [21]. This "moonlighting" of GPx-4 is primed by a critical GSH depletion [52], increasing its redox potential, a condition usually associated to cellular differentiation [21, 53]. Deprived of its most abundant reducing substrate, GPx-4 can react with protein thiols as alternate substrates [20, 21]. Mass spectrometric analysis revealed that during this process a selective, intermolecular reaction takes place between Sec-46 of GPx-4 and Cys-148 (porcine numbering of GPx-4 used), resulting in linear polymers representing dead-end intermediates of the peroxidatic cycle [54]. The formation of mitochondrial capsules also requires the participation of the "Sperm Mitochondrion-associated Cysteine-rich Protein" (SMCP). The involvement of SMCP has been verified by reproducing the oxidative polymerization of the capsule components

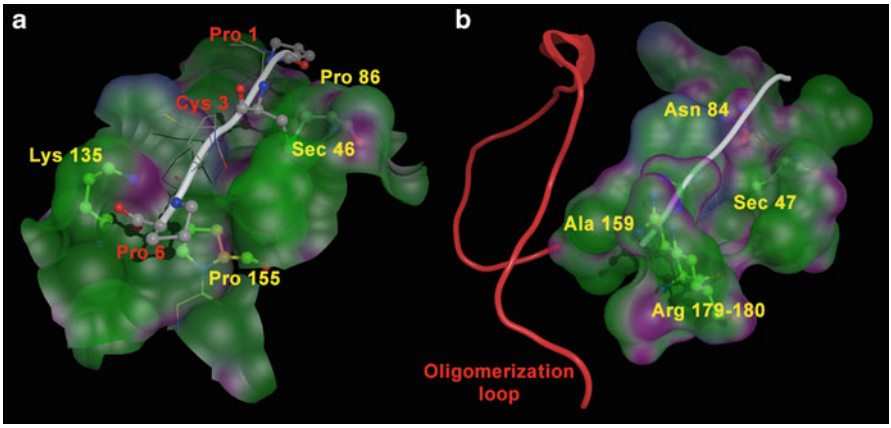

Fig. 14.5 (**a**) Molecular docking and dynamic of the PPCCPP peptide in the surface of the catalytic pocket of GPx-4 (*green* apolar, *violet*/*blue* polar residues). The Pro residues at position 155 and 86 contribute to keep flat and open the catalytic pocket of GPx-4 favoring the pose of bulky substrates. (**b**) Speculated pose of PPCCPP peptide on GPx-1. It is impossible to dock the peptide in silico due to steric clash given by Arg 179, Arg 180 (human GPx-1 numbering), and the oligomerization loop (*red ribbon*)

in vitro that is, seemingly, initiated by the oxidation of peculiar adjacent Cys motifs in SMCP [20]. Once formed, this unusual disulfide is prone to reshuffling, eventually driving cross-linking of different Cys-containing proteins [20, 55, 56]. Remarkably, similar adjacent Cys motifs, which are quite rare in the genome, are also present in protamines [57], which are the basic proteins that, by keeping compact spermatozoa chromatin, stabilize the structure and prevent transcription. The interaction of protamines with DNA is implemented by the formation of disulfides catalyzed by GPx-4 [58]. Consistently, selenium deficiency gives rise to spermatozoa with a chromatin prone to unwinding in the presence of denaturing agents [59, 60]. This role of GPx-4 in contributing to sperm chromatin stability has been validated in mice, by targeted deletion of the nuclear form of GPx-4 [61].

The ability of GPx-4 to oxidize specific peptide motifs containing adjacent Cys residues is not shared with GPx-1. This aspect has been investigated in silico using a computational approach based on molecular docking. From docking simulations, the access of large substrates to the active site in tetrameric GPx-1 is hampered by bulky amino acids, Arg and His, at position 179 and 180 (with reference to the human sequence) (Fig. 14.5). These residues, instead, are constantly replaced by Pro and light side-chain amino acids in all known GPx-4 homologues. This eases the access of bulky peptides to the catalytic pocket thanks to the flat surface of the Pro residues and the external orientation of the amino acids at position 180. Furthermore, in tetrameric GPx-1, the oligomerization loop contributes to restrict the accessibility to the catalytic site (Fig. 14.5). Intriguingly, Arg 179 has been proposed to contribute to the stabilization of GSH into the catalytic pocket of GPx-1, whereas its lack accounts for its low docking specificity in GPx-4 [22].

The small and unstructured peptide, PPCCPP, a peculiar motif in SMCP experimentally validated as substrate of GPx-4 [20], has been chosen for monitoring the docking simulation. This peptide, which does not react with GPx-1, shows instead a high binding affinity with human GPx-4. The simulation suggests that the binding zone is placed in a confined surface area located close to the catalytic Sec 46. The peptide interacts through a couple of Pro–Pro interactions (GPx-4 Pro 86 and peptide Pro 1, GPx-4 Pro 155, and peptide Pro 6) which are further stabilized by the electrostatic interaction between the peptide C-terminal and Lys 135, on one side of GPx4, and the polar interaction between the peptide N-terminal and, on the other side, the backbone carbonyl group of Gln 45. In this arrangement, the Cys residue at position 3 of the peptide is located at about 2 Å from GPx-4 catalytic Sec 46. The PPCCPP peptide, instead, cannot interact with the catalytic area of GPx-1, because Pro 86, Pro 155, and Lys 135 of GPx-4 are replaced in GPx-1 by Asn 84, Arg 179–180, and Ala 159, respectively (human enzymes numbering system used) (unpublished). This suggests that: (1) the interaction of GPx-4 with peptides and proteins involves a small surface area responsible for the correct orientation of the substrate toward the catalytic site; and (2) only the GPx-4 homologues are endowed with the correct combination of water-exposed amino acids, allowing the interaction of Sec with the substrate. In general terms, this observation is evocative of a role for GPx-4 as the catalyst of redox switches, reasonably primed by the increased redox potential of GSSG/GSH couple.

14.4 Concluding Remarks and Perspectives for Future Research

In conclusion, almost 30 years after the discovery of GPx-4 and a huge number of published studies, this enzyme is still largely uncharacterized regarding the relevance of the biological issues connected to its activity, which must be judged as still largely unresolved. Since deletion of *GPx-4* is lethal, its capability of reducing membrane hydroperoxides is not compensated by any other enzyme, including Peroxiredoxin 6, for which an activity on phospholipids hydroperoxides in vitro has been described [62]. Nevertheless, this fact also pinpoints the relevance of membrane hydroperoxides generated by specific 12/15 LOX isoforms and competent for priming a caspase independent cell death pathway [42]. This could be relevant in chronic degenerative diseases, when an imbalance between hydroperoxide production and elimination is in favor of the first. However, through the same mechanism, GPx-4 comes forth as a key player in cancer, when its activity could be relevant in controlling a physiologically useful apoptosis.

The specific capability of GPx-4 to oxidize specific protein thiols could be relevant in specific physiological functions, besides the first discovered stabilization of spermatozoa mitochondrial capsules and chromatin. This would expand the role and the functions of GPx-4 toward the area of functional redox switches [63], an emerging field in regulation of biological functions.

The final, unsolved question is about the substantial advantage of having selenium rather than sulfur at the active site. From basic chemistry, we know that selenium can be both more nucleophilic and more electrophilic than sulfur [33], and reasonably this speeds up the peroxidatic cycle. But, can this be enough? As a matter of fact, the introduction of selenium substituting for sulfur is rather limited in nature and in the same mammalian cells GPx-4 coexists with some Cys homologues. Definitely, it is hard to believe that the only difference could be limited to a not dramatic difference in the reaction rate.

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Chapter 15
Selenoprotein M

Mariclair A. Reeves and Marla J. Berry

Abstract Selenoprotein M (SelM) was discovered using computational analysis of signature sequences found in all eukaryotic selenoprotein genes. Located within the endoplasmic reticulum, SelM contains a cysteine-X-X-selenocysteine redox motif, and is most abundantly expressed in the brain. We carried out stable overexpression of SelM in two cell lines of neuronal origin, murine HT22 hippocampal cells and murine C8-D1A cerebellar astrocytes. In addition, stable knockdown was carried out in HT22 cells and transient knockdown in primary murine neuronal cultures. Our studies manipulating the expression of SelM indicate it is a neuroprotective antioxidant, and is involved in calcium regulation.

15.1 Introduction

Selenoproteins are defined by the incorporation of selenium via the 21st amino acid selenocysteine. The original selenoprotein glutathione peroxidase (GPx) was identified in 1973, and since that time 25 different selenoproteins have been identified, including SelM [1]. The selenoprotein family includes 25 members in humans and 24 in rodents [2]. Selenium is an essential trace element for humans and animals, as indicated by the detrimental consequences of dietary selenium deficiency seen in regions of China and New Zealand, and further highlighted by the embryonic lethality in mice resulting from targeted distribution of the tRNA required for selenocysteine incorporation [3, 4]. Selenium deficiency in humans can lead to Kashin–Beck disease, an osteoarthropathy occurring in regions of Tibet and China where selenium is deficient, and Keshan disease, a potentially fatal cardiomyopathy [5, 6]. In addition, myxedematous endemic cretinism, a form of mental retardation, occurs in

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regions of Africa with deficient selenium and iodine [5, 6]. In livestock, selenium deficiency leads to reduced weight gain, diarrhea, stillbirths, diminished fertility, and white muscle disease, a disease that affects both cardiac and skeletal muscle. Moreover, accumulating evidence implicates roles for selenium in physiological and pathophysiological processes including immune function, neurodegeneration, male reproduction, and cancer incidence [3, 6, 7].

Selenium is associated with antioxidant defense mechanisms. Oxidative stress is implicated in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease [8–10]. Selenoproteins with known functions include the glutathione peroxidase family (GPx), thioredoxin reductases (TRxR), and the deiodinases (DIO) [11]. In addition to these characterized families, numerous selenoproteins have been identified whose functions are not known, including a family with a common redox motif. This motif consists of CXXU, where U designates selenocysteine, and it has been identified in a subset of selenoproteins, including selenoprotein M (SelM), selenoprotein 15 (Sep15), selenoprotein W (SelW), and selenoprotein T (SelT) [12, 13].

15.2 Discovery of SelM

The discovery of Selenoprotein M was reported in 2002 [14]. Korotkov et al. reported that human SelM is a 3 kb gene with five exons and is located on chromosome 22. Characterization of SelM revealed its expression in many tissues, including brain, where SelM is most abundant, and in numerous other tissues such as heart, lung, kidney, stomach, intestine, skin, testes, uterus, and placenta [14]. This early publication indicated SelM colocalized with perinuclear structures, and its N-terminal signal peptide is necessary for protein translocation. Subsequent studies by Kryukov et al. [2] and Schweizer et al. [15] confirm Korotkov's initial findings that SelM is a selenoprotein, and that its expression is most abundant in the brain.

15.3 Antioxidant Function of SelM

SelM did not appear again in the literature until late 2005 [11]. Hwang et al. investigated effects on gene expression patterns in a transgenic mouse model of Alzheimer's disease (AD) carrying a mutant form of human presenilin-2 (PS2) [16]. This mutation causes a form of early-onset familial AD in humans [17]. A characteristic hallmark of AD is the formation of abnormal fibrous amyloid beta (Aβ) plaques. Aβ peptide, containing either 40 or 42 amino acids, is cleaved from amyloid precursor protein (APP) via β and γ secretases. The PS2 mutant consists of two missense mutations in γ-secretase resulting in an increase of Aβ42 in the plasma of AD patients [18]. The investigators found that overexpression of human mutant PS2 in transgenic mice resulted in suppression of SelM. Therefore, increased levels of

SelM via sodium selenite may contribute to neuroprotection from oxidative damage caused from AD [16]. Subsequent studies revealed the NMR structure of SelM [19]. Characterization of the NMR structures of Sep15 and SelM revealed a shared redox motif resulting in characterization of these two proteins as belonging to a new thioredoxin-like family [19]. The thioredoxin reductase (TRxR) family reduces oxidized TRxR at the expense of NADPH, which is used for regenerating cellular antioxidant systems, activating signaling molecules, reducing ribonucleotides to deoxyribonucleotides for DNA synthesis, and regulating activity of transcription factors [20–22]. Sep15 and SelM are confirmed homologs and have active-site redox motifs, which undergo conformational changes after thiol-disulfide exchange, indicating redox potential [19].

15.4 Investigation of SelM Function by Overexpression or Knockdown

Retention of selenium within the brain despite dietary deficiency attests to the importance of selenoproteins that are highly expressed in the brain [23]. These selenoproteins include GPx4, SelK, SelM, SelW, and Sep15 [23–25]. Among the previously mentioned selenoproteins, the expression profiles of SelM or SelK had yet to be characterized as of late 2007. Utilizing the Allen Brain Atlas (ABA), which provides a genome-wide gene expression database of the young adult mouse brain, a comparative analysis of selenoproteome gene expression identified neurons as key functional sites of selenium, and ultimately selenoproteins, in mammals [25]. According to the ABA, within the brain, SelM is highly abundant in the hippocampus, specifically the CA1, CA3, and dentate gyrus regions, the main olfactory bulb, and the cerebellar cortex. To confirm expression of SelM in these regions in situ hybridization was performed, and specific tags were used to manually define the gene expression. Quantification of gene expression was performed using quantitative PCR [25, 26]. Additionally, immunohistochemistry using rabbit anti-SelM antibodies revealed SelM protein expression in the rat hippocampus at embryonic, young, and adult stages, as well as in adult mouse CA neurons in hippocampus, small neurons in the granule cell layer, and large Purkinje cells. Expression of several selenoproteins including SelM, GPx1, GPx4, selenoprotein R (MsrB1), SelW, SelS, and TRxR3 were also detected differentially using Western blot analysis [25].

Manipulation of SelM expression levels has been used by several groups to investigate protein function [27–29]. The human SelM gene was successfully overexpressed at both the transcript and protein levels in CMV/GFP-hSelM transgenic rats [27]. Significant overexpression of SelM was detected in the kidney, and slight overexpression of the mRNA was seen in the brain, although increased protein levels were confirmed only in kidney. The serum in these SelM overexpressing transgenic rats had increased total antioxidants and hydrogen peroxide concentration compared to nontransgenic rats under normal conditions, as well as when challenged with 2,2′-azobiz (2-amidinopropane) dihydrochloride (AAPH) injection, which is used to

generate free radicals [27]. In addition, increased superoxide dismutase (SOD) and GPx were detected in erythrocytes in transgenic compared to nontransgenic rats. Following AAPH challenge, SOD increased in both nontransgenic and transgenic animals, with transgenic levels of SOD being higher. Contrarily, detection of GPx was significantly increased in nontransgenic, and much higher than transgenic SelM overexpressing rats following AAPH challenge [27]. Activity of GPx was significantly increased in the transgenic compared to nontransgenic rats in brain, lung, liver, and intestine, but did not change in heart and kidney. In addition, SOD activity was increased only in the brain hippocampus and intestine, but decreased in the brain cortex, heart, and kidney. Taken together, these data suggest that overexpression of human SelM differentially regulates the concentrations of antioxidants and hydrogen peroxide, and the activity of antioxidant enzymes, according to Hwang et al. [27].

The innate immune response of several infectious diseases exerts oxidative stress; however, the relationship between selenium status of the host and bacterial infection has not been well investigated [30]. Transgenic SelM overexpressing rats described above [27] had increased neutrophils when challenged with AAPH compared to the nontransgenic rat. The number of lymphocytes decreased by fivefold in these animals causing an increase in the neutrophil to lymphocyte ratio. Corticosterone is known to play the role of a key hormone regulating the neutrophil to lymphocyte ratio by destruction of lymphocytes or extending the half-life of neutrophils [31]. Corticosterone levels were assessed in transgenic verses nontransgenic rats and no difference was observed. The authors concluded that increased neutrophils due to SelM overexpression has the potential to provide defense against bacterial infections and other inflammatory processes, and this process is independent of corticosterone [27].

In a subsequent study, utilizing selenium supplementation of SelM overexpressing transgenic rats, protein phosphorylation was investigated, specifically the extracellular signal-regulated kinases (ERK) signaling pathway, α, β, and γ secretase activity, and Tau phosphorylation [28]. Sodium selenite is known to be anti-apoptotic and to activate the mitogen-activated protein kinase (MAPK) pathway, also called ERK, to prevent damage to brain tissues and cells [32, 33]. In response to selenium treatment, the ERK signaling pathway was significantly increased in SelM overexpressing transgenic rats, and unchanged in nontransgenic rats [28]. Additionally, SelM overexpression induced the inhibition of the α/γ-secretase activity, decreasing Aβ42 production. Furthermore, previous studies suggest that a high level of phosphorylation of Tau paired helical filaments is tightly associated with microtubule disorganization and generation of neurofibrillary lesions [34, 35]. SelM overexpression inhibited Tau phosphorylation in several sites [28].

Based on the reports of high SelM expression in brain and the presence of a redox motif, we carried out stable overexpression of SelM in two cell lines of neuronal origin, murine HT22 hippocampal cells and murine C8-D1A cerebellar astrocytes. In addition, stable knockdown was carried out in HT22 cells and transient knockdown in primary murine neuronal cultures [29]. Overexpression of SelM resulted in a reduction in reactive oxygen species and apoptotic cell death in response to oxidative challenge with hydrogen peroxide. By contrast, knockdown of SelM

using shRNA in HT22 cells and primary neuronal cultures caused decreased cell viability and apoptotic cell death comparable to levels resulting from addition of hydrogen peroxide [29]. These studies further demonstrated the functional importance of SelM in preventing oxidative stress.

15.5 SelM: An ER Protein Involved in the Regulation of Calcium

Seven selenoproteins reside in the endoplasmic reticulum (ER); these are type 2 iodothyronine deiodinase (DIO2), Sep15, SelK, SelM, SelN, SelS, and SelT [36]. The characterization of these proteins highlights specific ER functions involving the control of protein folding in the ER (Sep15), retrotranslocation of misfolded proteins from the ER to the cytosol (SelS), metabolism of thyroid hormone (DIO2), regulation of glucose metabolism and inflammation (Sep15 and SelS), and regulation of calcium homeostasis (SelM, SelN, and SelT) [29, 37–41]. The importance of calcium regulation for neuronal viability and responses to oxidative stress is well documented, highlighting the potential importance of SelM in this capacity [42]. In addition, disruption of neuronal calcium homeostasis is implicated in the mechanism of neuron degeneration in several diseases including Alzheimer's disease and Huntington's disease [43]. Overexpression of SelM in HT22 cells reduced changes in cytosolic calcium evoked by oxidative stress without affecting basal calcium levels [29]. In primary cortical cultures, knockdown of SelM increased baseline levels of calcium. This indicates that SelM influences the process through which cells release calcium from their internal stores and, ultimately, may alter apoptotic pathways [44]. Whether SelM directly or indirectly regulates calcium has yet to be elucidated.

15.6 Concluding Remarks

Selenoproteins are dependent on a dietary trace element, have a unique high energy cost for biosynthesis, are quite diverse in tissue and subcellular localization, and exert specific functions within their subcompartments [6, 45]. The specific trademarks of SelM indicate its importance among selenoproteins, including the high levels of SelM within the brain, especially given that selenium is better retained in the brain than most other organs under conditions of dietary selenium deficiency [11, 14, 23, 24]. The presence of the CXXU redox motif and location in endoplasmic reticulum correlates with the antioxidant properties and regulation of calcium by SelM. Neuroprotection is observed when SelM is overexpressed, and contrarily, when SelM is knocked down, cell viability is decreased. Furthermore, suppression of SelM in the mouse model for Alzheimer's disease that overexpresses a mutated

form of human presenilin-2 caused an increase in γ-secretase activity, which is reversed in the SelM overexpressing transgenic rat model [16, 28]. Further investigation of this protein will be necessary to determine the pathways through which SelM functions as a neuroprotective antioxidant.

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Chapter 16
Selenium Transport in Mammals: Selenoprotein P and Its Receptors

Josef Köhrle, Ulrich Schweizer, and Lutz Schomburg

Abstract Selenoprotein P (SePP) is a unique selenoprotein in many respects. It carries up to ten selenocysteine moieties, which have been inserted cotranslationally with the help of two separate SECIS elements in its mRNA. The majority of serum SePP is secreted by the liver where hepatocytes convert nutritional selenocompounds into SePP for transport and distribution. Therefore, serum concentration of SePP is a useful biomarker for the selenium status of an individual. Recently, two endocytic receptors, i.e., Lrp2/megalin and Lrp8/ApoE receptor 2, have been identified which participate in target cell-specific SePP uptake and retention. A SePP-cycle has been proposed based on a tissue-specific sequence of reversible biosynthesis, secretion, and reuptake. Brain, testes, and kidney appear to use the SePP-cycle in order to preserve tissue selenium in times of poor nutritional supply. In how far individual genotype differences and common disease signals impair this pathway and disturb normal selenium metabolism and its hierarchical distribution by affecting SePP biosynthesis, secretion of isoforms and reuptake is a central research issue in basic science and biomedicine.

16.1 Selenoprotein P (SePP): A Unique Member of the Selenoprotein Family

Among all mammalian selenoproteins, Selenoprotein P (SePP) is unique in several ways; SePP transcripts contain ten selenocysteine (Sec) codons within the open reading frame in both rodents and humans, two separate Sec-insertion sequence (SECIS) elements in the 3′-untranslated region of the transcripts direct cotranslational

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Sec insertion, and, in addition to extracellular GPx3, SePP is the only selenoprotein secreted into blood accounting for about 50% of selenium in plasma of healthy well-nourished individuals [1–6]. Accordingly, it has been speculated since the first description of SePP that its high selenium content is indicative of a transport function elicited by this unique selenoprotein [7] reaching its cellular targets via specific receptor(s) [8].

This interpretation was corroborated in subsequent studies by a number of complementary findings: e.g., (1) dietary selenium intake controls SePP in parallel to selenium concentrations in plasma [9]; (2) molecular cloning of the rodent cDNA identified a selenium-rich stretch of amino acids comprising an impressive segment of nine selenocysteine (Sec) residues within the C-terminal domain [10] which is well conserved throughout evolution [11]; (3) metabolic labeling studies indicated that dietary selenium is taken up fast by the liver and incorporated into circulating SePP which, slowly declining, gives rise to increasing kidney-derived GPx3 concentrations [12]; (4) SePP constitutes the essential Se-containing component in serum-based cell culture medium supporting growth and survival of primary cells [13, 14]; (5) transgenic mice with genetically disrupted SePP biosynthesis (*Sepp*-knockout, $Sepp^{-/-}$) develop a number of Se-dependent phenotypes including growth defect, male infertility, and neuronal abnormalities [15, 16]; and (6) the identification of specific SePP binding and uptake by members of the lipoprotein receptor-related protein (Lrp) family confirms transport by SePP as a regulated and targeted process of selenium supply to specific target tissues [17–19].

Together, these important findings and a number of additional very insightful experiments have contributed to our current understanding of SePP serving as an endogenous selenium transport protein essential for regular selenium distribution, selenium homeostasis, and overall health (Fig. 16.1). In how far SePP fulfills additional important enzymatic, protective, and metabolic functions in vivo is currently subject to intensive research. In the following sections, we review and highlight mainly its selenium transport and supply function.

16.2 Lipoprotein Receptor-Related Protein-2 (Lrp2, Megalin) and Lrp8 (ApoER2): Two Multifunctional Endocytic Receptors Involved in SePP Metabolism

Circulating lipoproteins are recognized by glycosylated cell-membrane receptors (lipoprotein receptor-related proteins, Lrp) which serve as recognition, entry, and signaling devices in lipid metabolism [20]. In general, these receptors appear to not only control lipid homeostasis, delivery, and turnover, but are also involved in targeted transport processes and renal reuptake mechanisms of hormone- and vitamin-binding proteins and their ligands [21].

Among these proteins, Lrp2 (also known as megalin or glycoprotein-330) has been shown to be essential for growth, CNS development, and endocrine regulation. It is expressed in epithelial cells of the lung airway, epididymis, mammary gland,

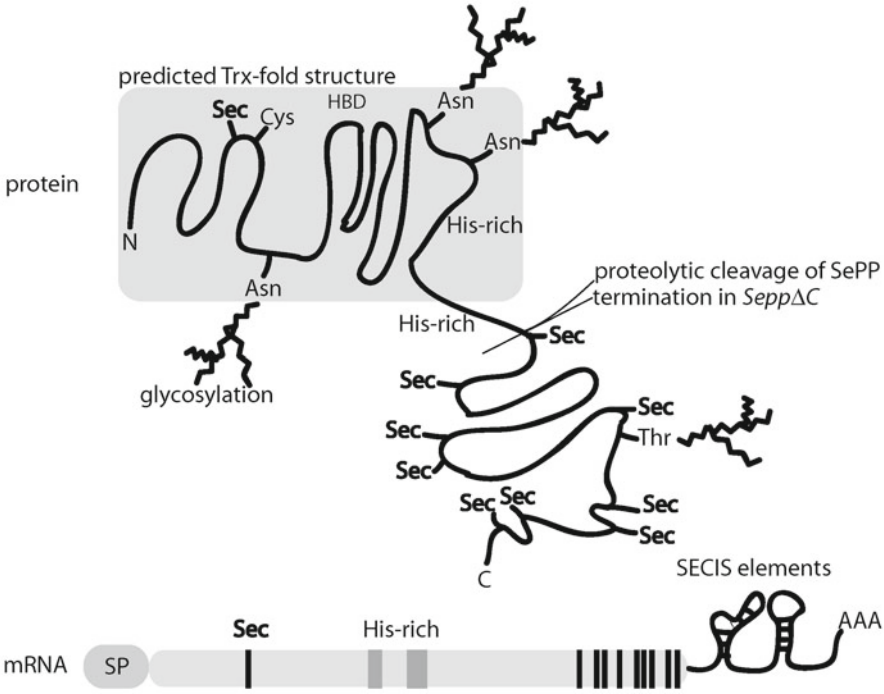

Fig. 16.1 Structural features of selenoprotein P (SePP). SePP comprises two domains. The N-terminal domain, which is predicted to adopt a thioredoxin-fold, contains a Sec-X-X-Cys motif within the turn between a beta-sheet and alpha-helix, showing weak peroxidase activity. The Sec-rich C-terminal domain is without homology to any known protein and likely serves a transport function. A classical N-terminal signal sequence (SP) directs SePP biosynthesis into the ER lumen. The secreted protein carries three N-glycosylation sites and one O-linked glycosylation site. A heparin-binding domain (HBD) has been identified along with two His-rich domains, which potentially mediate association to the extracellular matrix. SePP isoforms may result from differential glycosylation, proteolytic cleavage, or premature translational termination

inner ear, neural tube, and renal proximal tubules. Importantly, Lrp2 participates in binding and internalization of a whole variety of circulating carrier proteins for specific hydrophobic ligands. Accordingly, *Lrp2* knockout mice lacking the receptor in the kidney lose a number of essential hormones and vitamins in their urine by failing to reabsorb, e.g., vitamin D3/vitamin D-binding globulin, progesterone/clara cell secretory protein, vitamin A/retinol-binding protein, androgens and estrogens/sex hormone-binding globulin, thyroid hormones/transthyretin, and vitamin B12/transcobalamin [21, 22]. By now, the importance of Lrp2-mediated (re-)uptake and internalization of hydrophobic ligands along with their high molecular weight carrier proteins from serum or the primary glomerular filtrate has been demonstrated in a number of animal models and in patients with inherited diseases in specific components controlling the uptake or lysosomal dissociation and release process [23, 24].

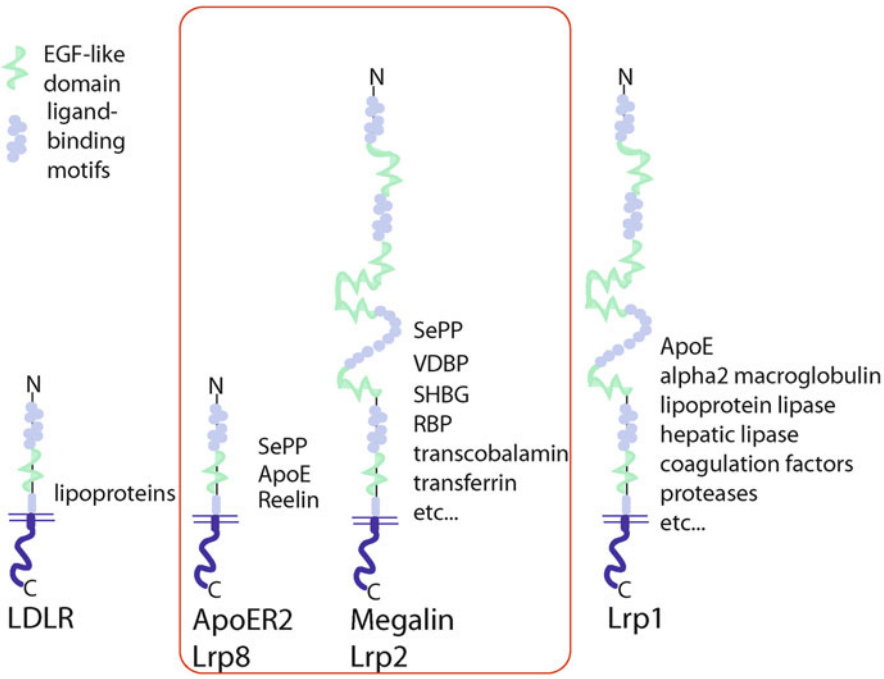

Fig. 16.2 Schematic structure of endocytic SePP receptors of the lipoprotein receptor-related protein (LRP) family. LRPs, a large number of different serum proteins, in particular Lrp1 and megalin, are multifunctional proteins capable of binding a large number of serum proteins. ApoER2 and megalin have been demonstrated to bind SePP and mediate SePP uptake into target cells in vivo. ApoER2 mediates selenium uptake into Sertoli cells and into neurons. Megalin is highly expressed in the kidney tubule epithelium. Lack of either receptor in mice leads to decreased Se/SePP uptake into respective tissues

These findings have challenged the free hormone hypothesis that lipophilic hormones are bound to carrier proteins for reservoir and transport functions only, and that just the free unbound hormones are of endocrine importance. Instead, the respective carrier proteins and bound ligands, in combination with Lrp2, now seem to actively control hormone homeostasis, targeted delivery, and turnover by specific serum protein–endocytic receptor interactions [25]. This mechanism is reminiscent of the active transport principle used by Odysseus et al. in form of the "Trojan Horse" [26]. The additional importance of Lrp2 for selenium homeostasis by specific binding and reuptake of SePP came initially as a surprise to the field of selenium biology [17, 27], but appears highly plausible as a logical addition to the list of functions for this internalizing system controlling the uptake of essential circulating serum factors such as nutrients, vitamins, and hormones.

Lrp8 (Apolipoprotein E receptor 2, ApoER2) contains five functional domains resembling those of the LDL-R (Fig. 16.2). It was originally isolated based on its sequence homology to the LDL-R and very low density lipoprotein receptor

(VLDL-R). Lrp8 is one of the cell surface receptors being involved in reelin signaling. Reelin is a large neuronal signaling molecule guiding neuronal cell migration during central nervous system development [28]. Reelin interacts with both Lrp8 and VLDL-R. Genetic inactivation of both receptors leads to neuronal migration deficits, tremor, and ataxia [29]. These findings are in line with the classical *reeler* phenotype observed in a spontaneously occurring mouse strain characterized by impaired motor coordination, tremor, and ataxia [30].

Interestingly, a similar neurological phenotype is observed in *Sepp*$^{-/-}$ mice on diets with low selenium content [31]. This phenotypic resemblance was resolved when Lrp8 was identified as a second receptor capable of SePP recognition, binding, and uptake in testes [18] and brain [19]. Together, a Se-delivery system consisting of SePP as the transport device along with Lrp2 and Lrp8 as tissue-specific endocytic uptake receptors can be envisaged, which appears to constitute the physiological pathway controlling the hierarchical supply of selenium to brain and testes. No evidence has been presented on a potential role of VLDL-R being involved in SePP transport.

16.3 Mouse Models of Modified SePP, Lrp2, or Lrp8 Expression

16.3.1 Classical Gene Targeting

Global genetic inactivation of *Sepp* in mice was simultaneously reported by two independent groups observing a similar phenotype of grossly disturbed selenium metabolism [15, 16]. Interestingly, not only circulating selenium concentrations were decreased in *Sepp*$^{-/-}$ mice, but also the known preferential targets of selenium supply, i.e., brain and testes, displayed strongly reduced selenium contents. Accordingly, the well-known importance of regular testes selenium for reproduction was corroborated in the male *Sepp*$^{-/-}$ mice as they were infertile [32, 33].

A neurological phenotype became apparent in *Sepp*$^{-/-}$ mice which was strictly dependent on nutritional selenium supply [31, 34]. This finding opened a new area of selenium research and is reviewed in Chap. 18 in detail. SePP is now viewed as a local selenium organification, retention, and reversible storage device in brain, safeguarding neuronal selenium content even against an unfavorable gradient of low serum selenium concentrations in times of poor supply [35]. Under regular conditions, the so-called “SePP-cycle” (Fig. 16.3) including local SePP biosynthesis, extracellular deposition, and reuptake appears extremely efficient in order to protect the vulnerable neuronal structures from loss of the essentially needed trace element [4].

Besides providing selenium systemically from liver to the other organs, hepatic SePP biosynthesis plays an important role in preserving whole body selenium status [36]. In comparison to wild-type mice, *Sepp*$^{-/-}$ mice show a higher selenium loss via

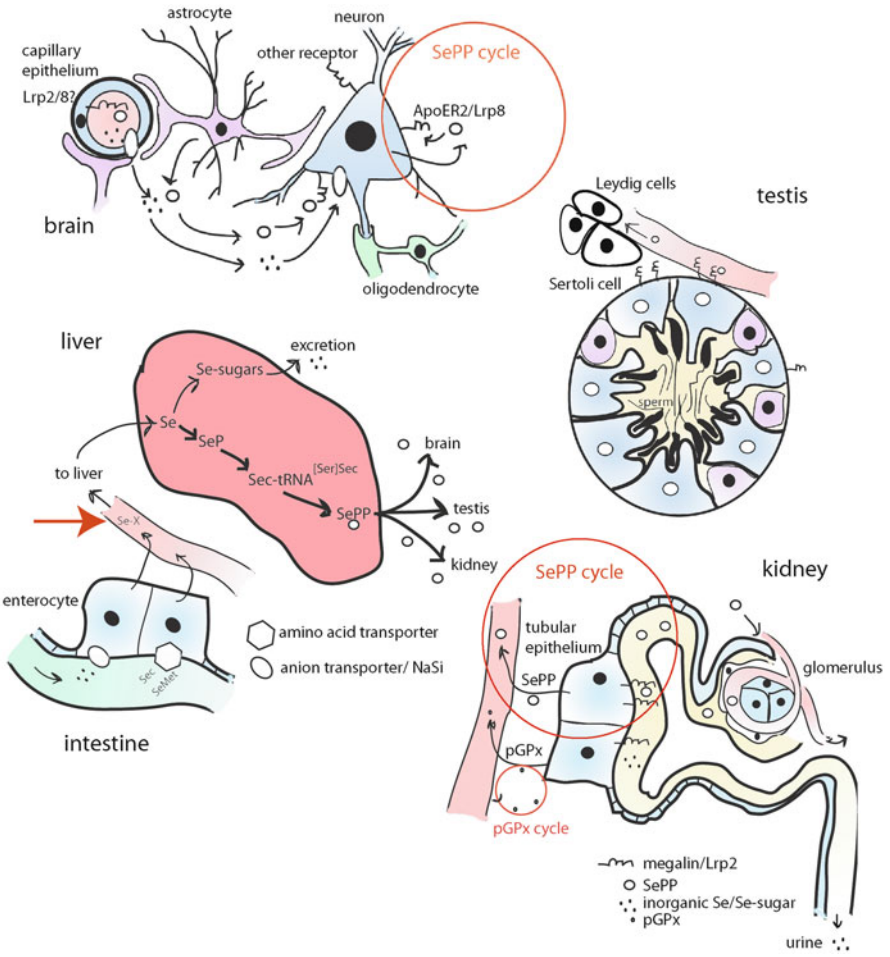

Fig. 16.3 Summary of selenium transport processes described in vivo. Dietary selenium is primarily taken up along the absorptive epithelium of the small bowel by transporters specific for inorganic divalent anions (e.g., the sodium-dependent sulfate transporter, NaSi-1) or transporters for amino acids and small peptides. The molecular identity of the different selenocompounds traveling through the portal circulation to the liver has not been fully elucidated. Within the liver, the selenocompounds are converted into Sec-tRNA$^{[Ser]Sec}$ and translationally inserted into selenoproteins. GPx1 has been proposed as a hepatic storage form for excess selenium, which may also be converted into selenosugars or selenonium ions and excreted. Free Sec concentrations in the tissue are very low. The liver is central to Se metabolism as the major source of plasma SePP, which transports selenium to privileged target tissues, e.g., brain, testis, and kidney. Brain selenium supply is complicated, since several cellular membranes must be crossed to finally reach the neurons. Megalin may be involved in SePP uptake along the choroid plexus and ependymal epithelium, while ApoER2 is expressed by neurons. Astrocytes synthesize SePP in vitro and may thus contribute to neuronal Se supply. Neurons express SePP and may store excess selenium extracellularly in the form of SePP. Brain retains its privileged selenium status during dietary restriction via reversible SePP expression, extracellular deposition, and reuptake. We have termed neuronal SePP synthesis and ApoER2-mediated SePP reuptake in brain as "SePP-cycle." Testis function likewise

the urine. This effect can be ascribed to a better availability of selenium in liver for the production of small selenocompounds which become filtered in the kidneys for secretion, e.g., trimethylselenonium and selenosugars [37]. Hepatic SePP thus fulfills more roles than merely transporting and distributing the trace element by ensuring its efficient biotransformation and organification into a readily usable circulating form. SePP apparently also enables efficient selenium retention in circulation as well as prevention of its renal excretion, a principle well known from homeostatic mechanisms which have evolved for preserving low molecular mass hormones and vitamins.

Lrp2$^{-/-}$ mice usually die perinatally [38]. A second mutant mouse strain on a different genetic background was independently characterized of which a fraction of mutant mice survive into adulthood [39]. We have used these mice as a model system for the analysis of the physiological functions of Lrp2 in adult mice as discussed later with respect to renal SePP metabolism [27].

In contrast, *Lrp8*$^{-/-}$ mice are born at the expected Mendelian ratio and appear grossly normal. Reduced male fertility of these mice was associated with a reduced expression of the selenoenzyme glutathione-peroxidase 4 (GPx4) in the initial segments of the epididymis [40]. The molecular role of GPx4 during spermiogenesis had been elucidated earlier and was shown to depend on a specific "moonlighting" process converting the active selenoenzyme into a component of the intracellular cytoskeleton essential for sperm structure and motility [41]. Therefore, male infertility of *Lrp8*$^{-/-}$ mice appears secondary to decreased selenoprotein expression in sperm which in turn depends on SePP-mediated selenium supply [42].

Interestingly, a similar selenium deficiency develops not only in testes but also in brain of *Lrp8*$^{-/-}$ mice [19]. Again, the developing phenotype was strictly dependent on the selenium supply leading to severe neurological impairment on experimental diets with reduced selenium content. The phenotypic similarity of the neurological phenotypes of *Lrp8*$^{-/-}$ and *Sepp*$^{-/-}$ mice fed a low selenium diet was again striking and corroborated a function of brain Lrp8 in SePP uptake.

Fig. 16.3 (continued) depends on ApoER2-mediated SePP uptake. Inactivation of either protein leads to decreased GPx4 expression in maturing spermatozoa and infertility. In contrast to brain, selenite appears not to cross the blood-testis barrier. Megalin expressed along the kidney tubular epithelium is involved in reuptake of SePP from the primary glomerular filtrate. Accordingly, inactivation of megalin leads to urinary loss of SePP. Megalin-positive cells express the highest levels of GPx1, GPx3, and SePP within the kidney, and inactivation of megalin decreases expression of all three proteins. Plasma GPx3 originates from the kidney epithelium, but most GPx3 is deposited locally within the kidney. SePP secretion by the kidney is a novel notion and instructive hypothesis. Another "SePP-cycle" can thus be proposed involving glomerular filtration, reuptake, and renal resynthesis of SePP. Kidney insufficiency in patients is associated with low Se status. Tissues expressing selenoproteins, but not ApoER2 and megalin, likely operate a still elusive Se uptake mechanism which might rely on the poorly characterized selenocompounds from the gastrointestinal tract

16.3.2 Isoforms of SePP

The two-domain structure of SePP implies two separate functions for the circulating selenoprotein, i.e., a presumable enzymatic activity linked to the first Sec residue within the N-terminal thioredoxin-like domain and a selenium supply function mainly mediated by the Sec-rich C-terminus [43]. Indeed, respective experiments have indicated that purified SePP elicits phospholipid hydroperoxide peroxidase activity with thioredoxin as a preferred cofactor in vitro [44]. In order to delineate the significance of the two domains in a physiological model, transgenic mice with a shortened SePP isoform lacking the C-terminal Sec-rich domain were generated and compared to wild-type and classical *Sepp*$^{-/-}$ mice [45]. Again, testis and brain selenium concentrations were strongly reduced in this mouse model, only slightly higher than in *Sepp*$^{-/-}$ mice. These findings corroborate that the C-terminus is important for selenium supply to the hierarchically preferred target tissues. Selenium transport limited to the single N-terminal Sec residue is thus not sufficient to maintain normal selenium homeostasis. Shorter SePP isoforms, which might prematurely terminate at the initial Sec residues, have been described in rat and mouse serum, but their physiological function and regulation remain to be studied (1).

16.3.3 SePP in the Liver

Liver is the central organ for selenium organification and selenium metabolism. Several studies with labeled selenocompounds have indicated that liver is the organ converting dietary selenium into circulating SePP for supply of other tissues [12, 46]. This interpretation is in line with clinical data, e.g., the reduced serum selenium and SePP concentrations in patients with liver disease [47]. But SePP is expressed in most tissues [48]. In order to define the importance of hepatic SePP for regular selenium metabolism, mice carrying a conditional allele of tRNA$^{[Ser]Sec}$ (*Trsp*$^{fl/fl}$) were crossed with a cell-specific Albumin-Cre strain abrogating selenoprotein biosynthesis specifically in hepatocytes [49]. The mice were viable and showed almost complete loss of hepatic *Trsp* at 3 weeks of age. As expected, residual expression of hepatic selenoproteins was minimal, including SePP, causing strongly reduced circulating selenium and SePP concentrations [49, 50].

When different tissues were compared with respect to their dependence on hepatically derived SePP, it appeared that kidney selenium and kidney-derived Gpx3 concentrations were strongly reduced in *Alb-Cre*; *Trsp*$^{fl/fl}$ mice, while brain selenium remained unaffected [50]. These findings lent further support to the importance of liver for regular selenium metabolism and suggested cerebral SePP to contribute to the local "SePP-cycle." In how far hepatic SePP was sufficient to support kidney, brain, testes, and other organs with the essential trace element was studied in a complementary mouse model. Here, hepatocyte-specific expression of a human *SEPP1* transgene was studied on a *Sepp*$^{-/-}$ background generating mice with SePP

expression in liver only [33]. Compared to *Sepp*$^{-/-}$ mice, those with liver-specific expression of *SEPP1* had increased selenium concentrations in most tissues, were less sensitive to selenium restriction in terms of neurological dysfunction, and had restored male fertility [33].

Collectively, these mouse models highlighted the importance of liver-derived SePP for regular selenium metabolism and tissue supply. Nevertheless, without locally expressed SePP, the brain was, e.g., still more sensitive to selenium restriction despite circulating SePP availability. This observation indicates that hepatic SePP was essential for regular selenium transport into brain but not fully sufficient to sustain the regular selenium homeostasis in all tissues, especially in the preferentially supplied central nervous system.

16.3.4 Selenoprotein Metabolism in the Brain

SePP is locally expressed in the human brain [51, 52]. In our current model, SePP is an endogenous device both to transport selenium into and within the brain. Lrp8, a neuronal SePP receptor, is important for selenium uptake by neurons [19], although Lrp8 may not represent the only SePP receptor in the brain (see Chap. 18 for a detailed discussion). Megalin likely contributes to selenium uptake along the blood–brain barrier [27]. The roles of various selenoproteins in brain development, function, and degeneration are treated in Chap. 18.

16.3.5 SePP in the Kidney

The importance of SePP for kidney selenium status and metabolism has not been addressed by tissue-specific knockout studies of SePP. Instead, the physiological role of SePP for kidney selenium can be deduced from a number of findings in the aforementioned SePP-specific mouse models. *Sepp*$^{-/-}$ mice showed strongly decreased kidney selenium concentrations. This finding implies that either hepatically derived circulating SePP transports selenium to the kidneys or renal SePP biosynthesis itself is crucially important for controlling local tissue selenium content [15, 16]. In order to solve this conundrum, hepatic or renal SePP biosynthesis needs to be specifically disrupted.

The former was achieved by hepatocyte-specific inactivation of *Trsp* impairing biosynthesis of all selenoproteins in hepatocytes [50]. Strongly reduced SePP levels in plasma were determined in parallel to reduced kidney selenium concentrations. Reduced renal selenoprotein expression in *Sepp*$^{-/-}$ mice was rescued by hepatic expression of the human *SEPP1* transgene [33]. Yet, wild-type levels of kidney selenium were not completely restored, either because of the lower general selenium status of the animals or because of the missing renal SePP expression. Together, these data indicate that circulating SePP contributes to renal selenium levels, but do

not completely rule out a role for local SePP biosynthesis controlling renal selenium concentrations.

Lrp2 is abundantly expressed in the kidneys [53]. Lrp2/megalin has been implicated as a renal SePP receptor [17], but *Lrp2*$^{-/-}$ mice analyzed in this pioneering study did not survive birth, and thus a role of Lrp2 in renal SePP uptake could not be directly demonstrated. A tissue-specific mouse model has been generated by crossing *Lrp2*$^{fl/fl}$ mice with renal tubule-specific *ApoE-Cre* mice. These mice were fertile and viable, but suffered from symptoms of osteomalacia secondary to vitamin D loss via the urine and hypocalcemia [54]. Urinary Se-loss and renal selenoprotein expression have not been analyzed in these mice.

We have taken advantage of another *Lrp2* mutant mouse strain of which a significant fraction of *megalin*-deficient mice survive into adulthood. These mice carry a missense mutation in the extracellular domain of Lrp2 developing a less severe phenotype as compared to classical *Lrp2*$^{-/-}$ mice [39]. When selenium metabolism was analyzed in these *Lrp2*-mutant mice, selenium status was low, GPx activities were decreased in kidney and serum, and SePP concentrations were reduced in serum [27]. Movement coordination deteriorated in *Lrp2*-mutant mice when challenged by feeding a low selenium diet. Interestingly, full-length SePP was detected in the urine of these mice indicating, on the one hand, that SePP is partially filtrated by the glomeruli into the primary urine and, on the other hand, that renal Lrp2/megalin recognizes, binds, and removes SePP from the primary urinary filtrate and prevents SePP loss in wild-type mice [27]. These findings provided the physiological support to the initial demonstration of specific SePP binding to the renal proximal tubule epithelium as seen in ligand blotting assays [17].

A reduction of immature SePP fragments in renal lysates prepared from *Lrp2*-mutant mice was also observed [27]. This finding suggests that SePP is synthesized in the kidney and may contribute to circulating SePP levels. However, to firmly make this point, kidney-specific *Sepp*-deficient mice need to be generated and analyzed. Activities of plasma GPx3 and renal GPx1 are reduced in *Lrp2*-mutant mice, suggesting that renal selenoprotein synthesis depends in part on selenium internalization from the primary urine via Lrp2. Whether patients with mutations in *LRP2* also suffer urinary selenium loss has not been tested.

16.4 Integrated View on Selenium Metabolism via SePP–Lrp Interaction: The SePP-Cycle

Based on available data, the sequence of SePP biosynthesis, secretion, and reuptake can be envisaged as a SePP-cycle. It may underlie some aspects of the hierarchical retention of selenium in certain tissues (Fig. 16.3). This so-called SePP-cycle appears to protect brain from selenium loss during selenium deficiency, might be of central importance for kidney selenium metabolism protecting the organism from excess selenium loss by renal SePP biosynthesis, and is likely involved in testes selenium uptake and use.

16.5 Regulation of SePP Expression

SePP gene expression has been studied in several cell types and experimental models. Proinflammatory cytokines such as interleukin-1α, TNFα, and interferon-γ suppress gene expression in cell lines involving activation of nitric oxide synthase-2 [55, 56], and TGFβ represses SePP transcription by a SMAD-binding element in the proximal human promoter [57].

Recently, also positive effects on the human SePP promoter were demonstrated by the forkhead box transcription factor FoxO1a, and this effect was enhanced by overexpression of peroxisomal proliferator-activated receptor-γ coactivator 1α (PGC-1α) [58]. These observations link SePP expression in human hepatoma and neuroblastoma cells to repression by insulin, and can explain SePP stimulation by the glucocorticoid dexamethasone. In contrast, high SePP levels have been associated with insulin resistance/type 2 diabetes. However, high glucose levels augmented SePP expression and secretion in cultured hepatocytes [59]. Moreover, SePP is proposed to serve as an insulin-antagonistic hepatokine [60]. This study, unfortunately, has not addressed the established role of SePP as a selenium transport protein, and thus failed to consider SePP-dependent changes in selenoprotein expression in insulin target tissues. This would have been important, since enhanced expression of GPx1 is known to cause insulin resistance in mice [61].

16.6 Comparison of Experimental Concepts with Clinical Data

A systematic comparison of potential biomarkers of selenium status in humans has recently been compiled and published [62]. Plasma or red blood cell selenium concentrations or GPx activities in serum, plasma, or blood cells are often used as markers of selenium status. In comparison, SePP turned out to be another versatile endpoint in populations with low to moderate selenium supply reaching a plateau at higher intake levels than cell GPx1 or circulating GPx3 activities. Several groups have concluded that SePP serum concentration represents the best diagnostic indicator of adequate selenium status in humans [63–65], provided that underlying liver, kidney, or inflammatory diseases have been excluded as confounding factors.

Recent population-based studies on the associations between various cancer forms and SNPs of genes encoding for selenoproteins and enzymes involved in metabolism of ROS provided some evidence that SePP variants (Ala234Thr, rs3877899; G>A in 3′ UTR, rs7579) affect selenium availability to target tissues such as prostate or colon thereby modulating cancer risk in the context of other gene–gene (e.g., SOD2), gene–nutrient and life style interactions [66–69]. These associations and the postulated underlying SePP-dependent selenium uptake and supply mechanisms need to be confirmed by independent studies. Decreased expression

of SePP mRNA and SePP protein has been reported for several preneoplastic and cancerous lesions, e.g., of the respiratory and the GI tracts (gastric, colorectal, and hepatic cancers) as well as for prostate [70–73]. Unfortunately, SePP status has not been monitored in several other relevant and large cancer, cardiovascular, and metabolic trials (e.g., SELECT, NPC, SU.VI.MAX, SETCAP, etc.) analyzing potential protective or therapeutic effects of supplementation with selenocompounds alone or in combination with other antioxidative compounds such as vitamin E [74–76].

The recent availability of several specific antibodies recognizing human SePP helped to clarify the picture of systemic SePP distribution vs. local production indicated by cellular expression of SePP transcripts. These studies confirmed previous hints that SePP reaches various tissues and cells via circulation, but also provided evidence for local SePP production and secretion, e.g., within the CSF and in ependymal cells in the brain [51, 52]. SePP expression and immunostaining showed specific spatial and temporal patterns during brain development and pathological alterations in brains from patients suffering from neurodegenerative diseases such as Alzheimer's [1, 77]. Together, these observations suggest a strategic location of SePP in brain potentially protecting cell types of high activity and functional relevance from selenium deficiency thereby ensuring regular development, differentiation, and expression of Se-dependent antioxidative defense systems.

Patients on chronic hemodialysis and apheresis are known to have an impaired selenium status and frequently receive selenium supplements [78]. Currently, it is not clear, whether they lose SePP or its (shorter) isoforms during the filtration process or whether their damaged renal tissue expresses insufficient Lrp2/megalin for adequate reabsorption of filtrated SePP. Since a low selenium status is a negative prognostic factor for long-term survival of chronically ill patients, it appears mandatory to control the trace element status of hemodialysis patients in order to avoid severe selenium deficiency [79].

Besides cancer, chronic and degenerative diseases, the selenium status and SePP are implicated in male fertility, which is reviewed in detail in Chap. 32. A genetic inherited defect causing an impaired SePP biosynthesis and low circulating SePP levels has recently been described in humans. Individuals with certain mutations in *SECISBP2* display very low or undetectable levels of SePP [80]. Interestingly, some of these subjects are reported with mental retardation and abnormal gait [81] or delayed neurological and motor skill milestones [82]. However, apparent SePP deficiency in patients carrying *SECISBP2* mutations does not lead to a phenotype as severe as in *Sepp*$^{-/-}$ mice, indicating that brain selenium metabolism is not completely impaired in these individuals. In contrast, severe neurological symptoms involving brain atrophy and epilepsy were recently reported in patients carrying a mutation in another rate-limiting factor of selenoprotein biosynthesis, i.e., the selenocysteine synthase gene, *SEPSECS* [83]. However, selenium and SePP status in the circulation or the CSF have not been determined in *SEPSECS*-deficient patients, but are likely reduced and involved in the clinical phenotype.

16.7 Summary

There is a functional interaction of hepatically derived or tissue-derived SePP and SePP-receptors from the Lrp family, namely Lrp2 and Lrp8. This interaction ensures the hierarchical supply and retention of selenium in testes and brain, and the general retention of SePP in mammals by preventing its loss via the urine. Pharmacological intervention of these endocytic receptor/SePP interactions might become of importance in cases of certain forms of male infertility, neurodegenerative disorders, or in patients exposed to excess oxidative stress, e.g., during various forms of chemotherapy, systemic inflammation, and bacterial or viral infections.

One of the important experimental tasks in the future will thus be the molecular characterization of the different SePP isoforms, their physiological functions, regulation, and interaction with the different SePP-receptors. Some evidence has been provided that Lrp8/apoER2 may be selective for full-length SePP, while Lrp2/megalin also accepts shorter SePP isoforms [1].

Of particular interest is also the relation between SePP-dependent delivery of selenium to the kidneys in comparison to the local recycling, biosynthesis, and secretion of renal-derived GPx3, which constitutes the second selenoprotein significantly contributing to the circulating blood selenium content. Our current knowledge is limited with regard to expression and regulation of SePP during development, selenium compartmentalization, and supply to tissues and cells not depending on SePP and devoid of the Lrp receptors. The molecular aspects of SePP downregulation in various cancer cells are not yet understood. Whether impaired expression and secretion of SePP during hepatic acute phase response and in various cancer cells can be overcome to improve the health status and better recovery from these diseases remains one prime challenge for future studies. Nevertheless, with the identification of SePP as the major transport, distribution, and storage protein for Se, and the characterization of specific receptor-mediated tissue-specific uptake processes, a number of previous enigmatic findings are now understood by a plausible molecular pathway. But given the promiscuity of both Lrp2 and Lrp8 for the alleged substrates to be recognized, bound, and internalized, future studies will also need to address specificity of the SePP/Lrp interactions in more detail.

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Chapter 17
Selenoproteins in the Endoplasmic Reticulum

Dmitri E. Fomenko

Abstract The endoplasmic reticulum (ER) is an organelle which is present in all eukaryotic cells. The ER is a major compartment where protein folding, glycosylation, and disulfide bond formation occur. Selenocysteine containing proteins (selenoproteins) are a subgroup of thiol oxidoreductases. Recent studies demonstrated that 7 of 25 known human selenoproteins, Sep15, SelM, SelT, D2, SelS, SelK, and SelN, reside in the ER. Thus, ER-associated redox processes are dependent on selenoproteins, and consequently, on selenocysteine biosynthesis and overall selenium status of the cell. At the same time, ER selenoproteins include the least characterized human selenoproteins. In this chapter, we summarize recent results on the ER selenoproteome.

17.1 Introduction

The endoplasmic reticulum (ER) is a branched network of tubules and flattened sacs that extends throughout the cytoplasm in eukaryotic cells and connects to other compartments. The ER is the major cellular compartment responsible for protein folding, protein modification, lipid and carbohydrate metabolism, calcium homeostasis, membrane trafficking, and other essential processes in the cell. In the last decade, there has been a growing interest in the biological processes in the ER, especially, in the understanding of ER redox homeostasis. This compartment is characterized by very intense and diverged redox processes that are catalyzed by numerous thiol oxidoreductases [1]. Thiol oxidoreductases are structurally distinct, but mechanistically similar families of enzymes, which have catalytic cysteine (Cys) in their active sites. They regulate a variety of biological functions, such as

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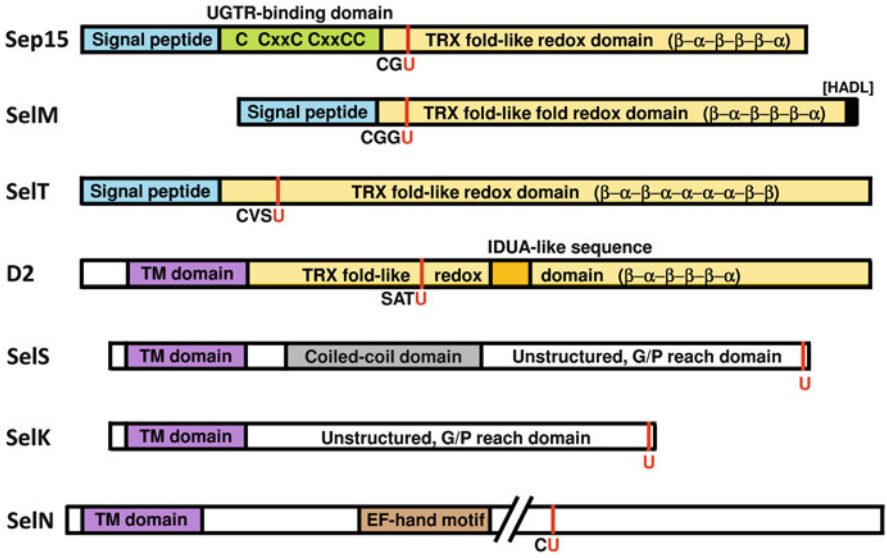

Fig. 17.1 Domain structures of human ER-resident selenoproteins. Cleavable signal peptides are shown in *blue* for Sep15, SelM, SelT, and SelN. A single-spanning transmembrane domain is shown in *violet* for D2, SelS, SelK, and SelN. A UGTR-binding domain of Sep15 is shown in *green*. Thioredoxin-like fold domains of Sep15, SelM, SelT, and D2 are shown in *yellow*, and the coiled-coil domain of SelS in *gray*. The ER retention signal of SelM is shown in *black*, the EF-hand motif of SelN in *brown* and the IDUA-like motif of D2 in *orange*. Location of Sec (U) in all selenoproteins is shown in *red*

protection against oxidative stress, signal transduction, protein folding, modification, and regulation. In rare cases, the catalytic Cys could be replaced by selenocysteine (Sec), which is a Cys analog with selenium in place of sulfur. All Cys/Sec replacements have thus far been observed only in active sites of various thiol oxidoreductases [2]. The reason such a replacement evolved is most likely related to the more pronounced nucleophilic properties of selenium, making Sec a more efficient catalyst compared to Cys. Sec is regarded as the major biologically active form of selenium.

The synthesis of selenoproteins depends on dietary selenium, making this element essential for mammals and many other organisms. The human selenoproteome was characterized recently and 7 of 25 identified selenoproteins were localized to the ER [3]. These selenoproteins are Sep15, SelM, SelT, D2, SelS, SelK, and SelN (Fig. 17.1). ER selenoproteins can be divided into two major groups; Sep15, SelM, SelT, and DI2 form the first group. They possess a thioredoxin fold, which is most common among thiol oxidoreductases and appears to be an optimal fold for thiol-dependent redox reactions [4] (Fig. 17.2). There are hundreds of thousands of thioredoxin fold proteins in protein databases. Considering the large number of known thioredoxin fold proteins and the low number of such selenoproteins, we suggest that thioredoxin fold selenoproteins have evolved from the corresponding

Selenoprotein	Redox motif	Protein secondary structure
Sep15	CxU	
SelM	CxxU	
SelT	CxxU	
D2	SxxU	
SelS	U	
SelK	U	
SelN	CU	

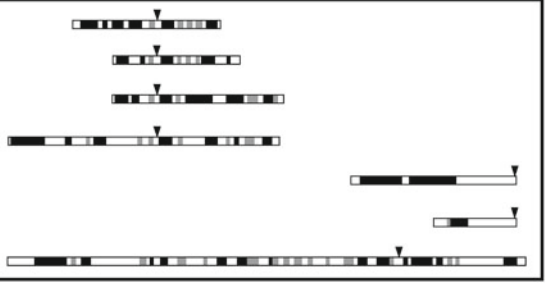

Fig. 17.2 Redox motifs and predicted secondary structures of human ER selenoproteins. X in the redox motif corresponds to any amino acid. α-helixes are shown in *black* and β-strands in *gray*. Sec is indicated with a *black arrow*. Thioredoxin-like fold proteins are highlighted in *gray*

Cys-containing analogs by mutation of a Cys-codon (either TGT or TGC) to the Sec-encoding TGA codon [2]. Such a mutation must occur in a gene encoding a Sec insertion (SECIS) element that occurs in the 3′ untranslated region (3′-UTR) in eukaryotic selenoprotein genes. The SECIS element is required for insertion of Sec into protein during translation of the corresponding mRNA.

SelS, SelK, and SelN belong to the second group of ER selenoproteins. These proteins contain Sec in the C-terminal part and, most probably, evolved by other mechanisms, for example, C-terminal extension of an ancestor protein (Fig. 17.2). Such extension must have occurred after formation of a SECIS-like structure in the 3′ UTR of the corresponding gene resulting in recoding of a TGA-stop codon as Sec. As a result, the newly formed protein obtains an additional, redox function, determined by selenocysteine. Surprisingly, ER selenoproteins are among the most poorly studied within the human selenoproteome. Only deiodinase 2 (D2) is a well-characterized ER selenoprotein with an assigned function. Preliminary data showed an association of other ER selenoproteins with quality control of glycoprotein folding, redox control of retrotranslocation of the terminally misfolded proteins from the ER to the cytosol, calcium homeostasis, and regulation of glucose metabolism and inflammatory processes. In this chapter, we summarize available information on each ER selenoprotein.

17.2 15 kDa Selenoprotein (Sep15) and Selenoprotein M (SelM)

The human 15 kDa selenoprotein (Sep15) is a 165 amino acid thioredoxin-like fold protein [5] with Sec in the active site. Sep15 was identified 13 years ago as a protein of unknown function which may mediate the cancer chemopreventive effects of selenium [6, 7]. Sep15 has a cleavable N-terminal signal peptide, a Cys-rich UDP-glucose:glycoprotein glucosyltransferase (UGTR) binding domain, and a thioredoxin-like

fold domain with Sec in context of a CGU redox motif (Figs. 17.1 and 17.2). Selenoprotein M (SelM) was identified several years later [8]. Similar to Sep15, SelM is a 145 amino acid thioredoxin-like fold protein with Sec in the active site [5]. It has a cleavable N-terminal signal peptide, a thioredoxin-like fold domain with a Sec residue in the context of a CGGU redox motif, and an ER retention signal sequence (HADL) in the C-terminal portion of the protein (Figs. 17.1 and 17.2). The redox domains of Sep15 and SelM have 26% identity and 39% similarity suggesting that they evolved from a common ancestor.

17.2.1 Sep15

The human Sep15 gene is localized on chromosome 1p31 and consists of five exons and four introns. It is highly expressed in prostate, thyroid, liver, and kidney. ER targeting of Sep15 is determined by its N-terminal signal peptide, which is cleaved after the protein is translocated to the ER. The signal peptide sequence is followed by a Cys-rich domain (C CxxC CxxCC) with no similarity to any known protein sequence (Fig. 17.1). This domain is not involved in metal coordination and was found to be responsible for Sep15 interaction with UGTR, an essential glycoprotein that plays a role as a folding quality control sensor in the ER [9, 10]. Mutation of any Cys in the UGTR binding motif affects UGTR/Sep15 complex formation.

Mammalian genomes encode two forms of UGTR. UGTR1 is a part of the calnexin/calreticulin N-linked glycoprotein folding quality control pathway in the ER. It recognizes partially unfolded glycoproteins and returns them to the calnexin/calreticulin refolding cycle by adding the glucose moiety from UDP-glucose to the mannose in an asparagine-linked $Man_9GlcNAc_2$ oligosaccharide. UGTR is not active with properly folded or completely misfolded glycoproteins [11]. Sep15 copurified with UGTR1 as well as coimmunoprecipitated with both UGTR forms. The binding of Sep15 did not change UGTR1 activity in the thyroglobulin assay. However, a 1:1 stoichiometry of UGTR/Sep15 interaction with a dissociation constant of 20 nM suggests a possible role of Sep15 in modulating UGTR activity, or a role of Sep15 in disulfide bond assembly on a specific set of UGTR targets [10].

Another interesting observation is related to the lack of ER retention sequence in the C-terminal region of mammalian Sep15. Thus, Sep15 cannot exist freely in the ER and the association with UGTRs is the only way for Sep15 to stay in this compartment. In addition, a role of Sep15 in protein folding quality control is supported by an increased Sep15 expression under ER stress, especially in an adaptive ER stress that activates the unfolded protein response (UPR) [12]. Accumulation of unfolded proteins in the ER causes UPR, which is one of the major cellular pathways mediating signaling from the ER to the nucleus resulting in expression of genes that inhibit translation and lead to degradation of unfolded proteins. Activation of UPR leads to induction of genes responsible for protein folding and results in refolding or elimination of misfolded proteins from the ER. Sep15 expression is

upregulated by ER stress caused by tunicamycin and brefeldin A treatments, but is rapidly downregulated by acute ER stress caused by DTT and thapsigargin [12].

Sep15 deficiency did not cause detectable ER stress, suggesting that Sep15 controls folding of a specific group of glycoproteins, or that Sep15 function could be compensated by other proteins or mechanisms. The 3D structure of *Drosophila melanogaster* Sep15 thiol oxidoreductase domain was solved (PDB accession code 2A4H) and a thioredoxin-like fold was demonstrated for this protein [5]. This protein consists of two α/β layers with a central β-strand surrounded by α-helices. Typical for thioredoxin fold thiol oxidoreductases, the active-site redox motif of Sep15 is located in the coil between β1 and α1; however, this protein contains an unusual, but an absolutely conserved CxU redox motif instead of a typical CxxC-derived motif. These observations suggest that Sep15 is a typical thioredoxin-like fold thiol oxidoreductase. To characterize the role of Sep15 in disulfide bond formation in the ER, the equilibrium redox potential was determined for Cys-containing *D. melanogaster* Sep15. A −225 mV redox potential is between those of disulfide isomerase (−175 mV) and thioredoxin (−270 mV). Thus, Sep15 may serve as a weak protein disulfide isomerase or a weak disulfide reductase [5, 13].

An interesting observation with vascular plant Sep15 is the natural replacement of the redox Sec with arginine, which occurs within a CxR motif. The active site arginine is encoded with different codons in vascular plants that exclude the possibility of arginine codon recoding. Thus, the function and reaction mechanism of plant Sep15 may differ from those in other organisms. Sep15 expression can be regulated by the selenium status of the cell. The human Sep15 gene has two polymorphic sites at nucleotide positions 811 (C/T) and 1125 (A/G) in the 3′-UTR. The 1125 (A/G) polymorphic site occurs within the SECIS element and it has been shown to be involved in determining the efficiency of Sec incorporation into protein depending on the level of selenium [14]. The frequency of this polymorphism varies among different ethnic groups manifesting different frequencies in breast and in head and neck tumors. More than 50% downregulation of Sep15 expression was observed in malignant mesothelioma cells, and these cells were less responsive to selenium supplementation. In addition, the 1125 (A/G) polymorphic variant of mesothelioma cells was less responsive to changes in selenium levels compared to the same cells expressing the other allele [15]. Several other studies reported an effect of Sep15 polymorphism on colorectal, prostate, and lung cancer development and progression [16–18]. Furthermore, Sep15 is located in a chromosomal locus which is often deleted or mutated in human cancers. Taken together, these observations suggested an important role of Sep15 in cancer protection. However, a recent study has shown an opposite tissue-specific effect wherein colon cancer protection was found to be mediated by Sep15 downregulation [19].

A new member of Sep15 protein family, Fep15, was identified by computational analysis of fish genomes. This selenoprotein exclusively occurs in fishes and may have a specialized function in these organisms. Fep15 contains an N-terminal cleavable signal peptide sequence, a thioredoxin-like fold redox domain with a single Sec in its active site, and an ER retention sequence. In contrast to Sep15, Fep15 lacks the Cys-rich UGTR-binding domain [20].

17.2.2 SelM

Human SelM is a 16.2 kDa selenoprotein containing 145 amino acids. SelM gene is located on chromosome 22q12.2 and consists of five exons and four introns. The highest level of SelM expression was observed in the brain [8]. A cleavable N-terminal signal peptide is responsible for ER targeting of SelM [3, 5]. In contrast to Sep15, SelM does not have a Cys-rich UGTR-binding domain and UGTR interaction with this selenoprotein has not been observed (Fig. 17.1). The structure of the mouse SelM Cys mutant was solved using NMR and, similar to the Sep15 structure, a thioredoxin-like fold was observed [10]. As is typical for thiol oxidoreductases, SelM has a CxxC motif-derived active site (CGGU) that is located in the coil region between β1 and α1 (Fig. 17.2). SelM exists as a Sec-containing protein in most organisms, but is poorly characterized compared to Sep15. However, Sep15 and SelM sequence and 3D structure similarity [5], as well as the presence of a pronounced redox motif in the active site, suggest that SelM is an ER resident thiol oxidoreductase. Localization of this protein in the ER is determined by a C-terminal ER retention sequence. SelM has an atypical SECIS element in the 3′-UTR in that the unpaired AA motif in the apical bulge of other mammalian selenoproteins is substituted with a CC motif [8]. This unusual SECIS element is conserved among human, mouse, and rat SelMs.

A transgenic rat overexpressing human SelM was generated and an antioxidant role of this protein was demonstrated [21]. Transgenic rats showed a decreased level of H_2O_2 and elevated activity of antioxidant enzymes. Increased glutathione peroxidase (GPx) activity was observed in brain, lung, liver, and intestine. Superoxide dismutase (SOD) activity was increased in hippocampus and intestine, but decreased in the brain cortex, heart, and kidney. These results suggested that SelM may regulate GPx and SOD functions. The neuroprotective effect of SelM was demonstrated in hippocampal HT22 cells which are cerebellar astrocyte C8-D1A cells. SelM overexpression was associated with decreased reactive oxygen species and apoptotic rate, mediated by H_2O_2 treatment. SelM knockdown in primary neuronal cultures resulted in a strong apoptotic cell death rate. In addition, SelM overexpression decreased calcium influx in response to hydrogen peroxide, and SelM knockdown was associated with high level of cytosolic calcium [22]. Taken together, these results suggest that SelM may have an oxidoreductase function protecting brain from oxidative damage, and it may also be involved in calcium homeostasis. A possible protective role of SelM in Alzheimer's disease was also demonstrated [23].

17.2.3 Differences in Sep15 and SelM

Being similar to each other, Sep15 or SelM show very weak similarity to other thioredoxin fold proteins [5]. This observation suggests a common evolutionary origin for Sep15 and SelM that comprise a distinct thioredoxin-like fold protein family. As assessed by genome sequence analysis data, both proteins evolved in the

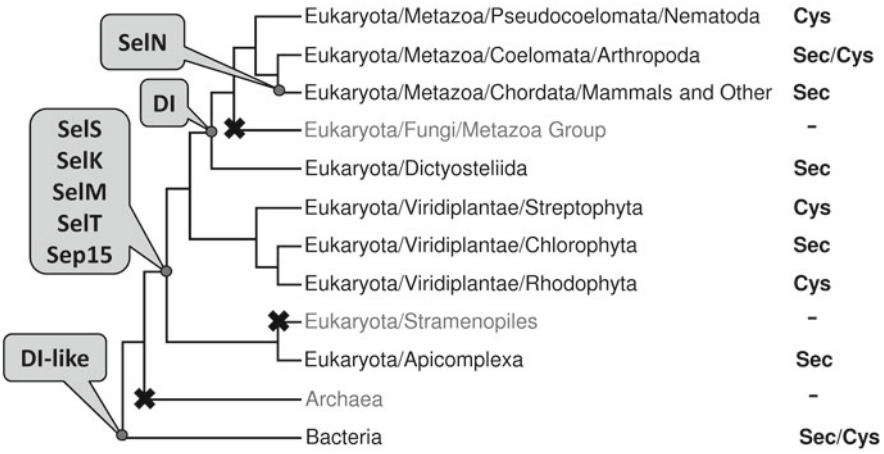

Fig. 17.3 Distribution of ER-resident selenoproteins and their homologs in different phyletic groups. Phyletic groups lacking ER selenoproteins or Cys-variants of these proteins are shown in *gray*. A possible evolutional origin of indicated selenoproteins is marked with *gray dot*. The events of loss of ER selenoproteins are shown with *crossed black lines*

early ancestor of eukaryotes (Fig. 17.3) and have a common pattern of occurrence from alga to mammals with the exception of vascular plants that lack SelM. The redox domains of Sep15 and SelM have a high sequence homology to each other, and have similar 3D structures [5]. Sep15 and SelM also have significant differences: (1) domain organization; (2) redox motifs; (3) mechanisms for retention in the ER; and (4) distinct, but partially overlapping, tissue expression patterns. These differences suggest that SelM and Sep15 diverged early in evolution of eukaryotes and may have different thiol oxidoreductase functions within the ER. However, their functions may overlap and they possibly compensate for each other in the event one becomes deficient.

17.3 Selenoprotein T (SelT)

Human SelT is a 22.3 kDa and 195 amino acid selenoprotein identified in a computational study as a protein of unknown function [24]. SelT gene is located on chromosome 3q25.1 and consists of six exons and five introns. This protein is evolved in an early eukaryote (Fig. 17.3). The highest levels of SelT expression were observed in kidney, brain, heart, thymus, and testes [25]. In another study, high SelT mRNA levels were observed in mouse testes and in the anterior lobe of the pituitary [26]. Widespread expression of SelT was observed during embryogenesis and in adult animals [27]. A recent study has shown that SelT is a thioredoxin-like protein belonging to the new Rdx protein family, together with SelW, SelH, SelV, and an additional fish selenoprotein, Rdx12 [25].

The redox active site (CVSU) of this protein is located in a typical region for thiol oxidoreductases, which is a position between β1 and α1. Taken together, these observations suggest that SelT is a thioredoxin-like fold thiol oxidoreductase. SelT is likely an ER resident protein, although the N-terminal GFP-fused SelT was found mostly in the Golgi with low occurrence in the ER and cytosol [25, 27]. SelT N-terminal signal sequence was predicted by SignalP with 100% probability using Hidden Markov Model and Neural Network methods. However, this sequence is not functional in vivo and does not influence SelT localization in the ER. About a 70 amino acid, four α-helical insert divides the redox domain into two parts and was found to be responsible for SelT targeting to the ER [27]. The exact mechanism of this targeting is not known, but the hydrophobic nature of the four α-helical insert suggests the possibility that this protein associates with the ER membrane.

The thioredoxin fold domain of SelT is exposed to the ER or cytosol. Another possibility is that the interaction of the SelT α-helical insertion region with an unidentified protein in the ER occurs similar to the interaction between Sep15 and UGTR. SelT does not have a pronounced ER retention signal in the C-terminal region. The membrane association of SelT as well as its binding to ER resident protein(s) may explain the ER localization of SelT. SelT knockdown in mouse fibroblast cells affected cell adhesion and activated the expression of another member of Rdx12 family – SelW [28]. This observation suggests roles of SelT in redox regulation and cell anchorage. The role of SelT in regulation of calcium homeostasis and neuroendocrine secretion in response to a pituitary adenylate cyclase-activating polypeptide (PACAP) was demonstrated in a recent study [27]. PACAP is a 38 amino acid neuropeptide that mediates cAMP-dependent regulation and trophic effects in the central nervous system through two types of G protein-coupled receptors. Rapid and long-lasting induction of SelT was mediated by PACAP and cAMP in a PC12 cell line. Further study showed an increased intracellular calcium concentration in cells overexpressing SelT. Mutation of the SelT Sec to alanine diminished this effect suggesting a redox mechanism of calcium regulation. Knockdown of SelT expression inhibited the PACAP-mediated increase in the intracellular calcium concentration [27].

17.4 Deiodinase 2 (D2)

The human genome encodes three deiodinases D1, D2, and D3, which are thioredoxin-like fold selenoproteins sharing an SxxU redox motif. The redox motif is located in a coil region between β1 and α1 (Fig. 17.2). D1 and D3 are plasma membrane proteins and D2 is localized in the ER. D1 and D2 are involved in activation of the thyroid hormone by deiodination of an outer (phenolic) ring in T4, while D3 is involved in irreversible inactivation of T3 by deiodination of an inner, tyrosyl ring [29–33]. The human D2 gene is located on chromosome 14q24.2 and encodes a protein of 272 amino acids. D2 is the only deiodinase that is present in the ER with an assigned function and is one of the most studied selenoproteins. This protein is

responsible for activation of the pro-hormone thyroxin (T4) by deiodination resulting in the formation of the short-lived active hormone form, T3. T3 in turn regulates gene expression in most tissues by binding to the nuclear thyroid hormone receptor, activating T3-dependent transcription factors [30].

The deiodinase activity regulates thyroid hormones homeostasis, resulting in general adaptation to the changes in iodine ingestion, temperature, starvation, and energy homeostasis. The D2 domain structure is similar to that of D1 and D3 and consists of a 20–25 amino acid N-terminal uncleavable hydrophobic sequence followed by the thioredoxin-like fold domain, divided by α-L-iduronidase (IDUA)-like sequence (Fig. 17.1) [29, 32, 34]. Occurrence of the IDUA-like sequence is critical for the catalytic activity. It is located in structural vicinity to the active site Sec and is responsible for T4 binding [34]. The N-terminal hydrophobic sequence is responsible for ER targeting and attachment of D2 to the ER membrane. The catalytic thioredoxin fold domain is exposed to the cytosol and the catalytically active form of D2 is represented by homodimer complex [29, 31, 32].

The reaction mechanism of deiodinases is not fully understood; however, one hypothesis suggests formation of a selenoiodine intermediate with subsequent reductive iodine elimination by an unknown reductase [32]. The human D2 gene has an unusual feature: the presence of two in-frame TGA codons in the open reading frame [35]. Both TGA codons function as Sec insertion sites; however, the second Sec and the remaining seven C-terminal amino acids are not critical for D2 catalytic activity. The function of the C-terminal extension is not known. The activity of D2 is regulated by selective ubiquitinilation, mediated by WSB-1 with subsequent proteosomal degradation. However, D2 ubiquitinilation is reversible. The enzyme activity can be recovered by the VDU1/2 deubiquitinilating system [29–31].

A D2 knockout mouse model was generated recently, and only a mild phenotype was demonstrated [36]. D2 KO animals are unable to control normal body temperature following cold exposure and have bone development defects [37, 38]. Obesity, glucose intolerance, and exacerbated hepatic steatosis phenotypes were detected in a thermoneutral condition [39]. Thus, D2 is not an essential selenoprotein. Perhaps, its function can be compensated by D1. Deiodinases are primarily found in vertebrates; however, a Sec-containing deiodinase was also detected in the *Dictyostelium discoideum* genome. Several Sec-containing deiodinase homologs were detected in prokaryotic genomes [40] (Fig. 17.3).

17.5 Selenoprotein N (SelN)

SelN is 65.8 kDa, 590 amino acid long integral ER-membrane selenoprotein [3, 41, 42]. The human SelN gene is located on chromosome 1p36.13 and consists of 13 exons and 12 introns. It has a predicted N-terminal cleavable ER-targeting sequence followed by a hydrophobic transmembrane domain, making SelN a single-spanning membrane protein [42] (Fig. 17.1). A major part of SelN is exposed to the

ER. It lacks an ER retention signal and stays in the ER through association with the ER membrane [43]. SelN, lacking the N-terminal hydrophobic domain, was detected mostly in the nucleus.

The predicted redox active site of SelN is located at position 462 and is represented by a single Sec residue in the context of CUGS motif. SelN is ubiquitously expressed with the highest level in skeletal muscles [42]. Its expression level is elevated in mouse fetal tissues suggesting a role of SelN in early development and cell proliferation. A predicted EF-hand calcium-binding motif is present in the middle part of SelN (Fig. 17.1); however, this motif was proposed to serve only a structural function [42, 43]. SelN appears to be heavily glycosylated. There are five potential N-glycosylation sites at positions 126, 189, 482, 504, and 530; however, the role on this modification is not clear.

Certain polymorphisms and SelN mutations were linked to several muscular disorders; this attracted much attention of the research scientific community in recent years. The loss of SelN function is associated with congenital muscular dystrophy, rigid-spine muscular dystrophy [41], multiminicore disease [44], desmin-related myopathy [45], and congenital fiber-type disproportion [46]. These disorders share a common clinical phenotype and are characterized by scoliosis, neck weakness, neck wasting, trunk muscles, spinal rigidity, severe respiratory insufficiency, and poor axial muscle strength [44–47]. Muscle biopsy of SelN knockout mice showed protein aggregation of muscle fiber and sarcomere disorganization [45].

The role of SelN in satellite cell function and muscle regeneration was suggested [47]. A recent study demonstrated a significant role of SelN for regulation of ryanodine receptor calcium release activity in human and zebrafish muscles [47]. RyR1 is a major component of the ryanodine receptor and its malfunction showed a phenotype similar to that of the SelN knockout. The functions of both proteins are required for calcium efflux [48]. Physical interactions of SelN and RyR were demonstrated in vivo by immunoprecipitation and colocalization experiments. Finally, the role of SelN as a reductase in the regulation of RyR calcium channel activity was shown. This observation suggests that SelN is a redox regulator of calcium homeostasis. Another study demonstrated a significant role of SelN in oxidative stress defense [49]. SelN deficiency was associated with oxidative and nitrosylative stress. SelN has the narrowest pattern of occurrence and is found in vertebrates only (Fig. 17.3).

17.6 Selenoprotein S (SelS)

Human SelS is 21.2 kDa, 189 amino acid long ER membrane selenoprotein. It was identified 10 years ago in a computational study as a protein with an unassigned function [3]. The human SelS gene is located on chromosome 15q26.3 and consists of seven exons and six introns. The N-terminal part of SelS has a transmembrane domain responsible for SelS attachment to the ER and plasma membranes [3, 50]. The transmembrane domain is followed by a coiled-coil region and an unstructured redox domain with the C-terminal Sec in position 188. The coiled-coil 3D structure

was solved recently (amino acids 50–125; PDB accession code 2Q2F) and this domain was hypothesized to be involved in protein binding or SelS homodimer formation [50]. The cytosolic region of SelS has no pronounced secondary structure and is rich in proline (10.9%), glycine (21.9%), serine (15.6%), arginine (9.4%), and lysine (10.9%). A high content of positively charged lysines and arginines suggest that the C-terminal part of SelS binds negatively charged regions of other proteins.

A recent study showed that SelS is a component of the ER-associated protein degradation (ERAD) system [50, 51]. ERAD is a pathway which protects cells from accumulation of misfolded proteins by transferring these proteins from the ER to cytosol for subsequent ubiquitination and proteasomal degradation [52, 53]. An interaction of the C-terminal part of SelS with the cytosolic ERAD component ATPase p97 was demonstrated by coprecipitation [50]. SelS was proposed as a mediator in the interaction of p97 ATPase and the ER membrane integral protein Derlin-1 forming a new type of retrotranslocation channel [50, 51]. A direct interaction of SelS and Derlin-1 is weak. In addition, p97 ATPase-binding E3 ubiquitin ligase did not directly interact with SelS [53]. Newly identified Derlin-2 and Derlin-3 are involved in alternative ER retrotranslocation channels and SelS was also found as a Derlin-2 complex component [53, 54]. The SelS gene was shown to be upregulated by ER stress, consistent with the role of SelS in the unfolding protein response [55, 56].

The specific function of SelS in the ERAD machinery in not known. Various ERAD components contain conserved Cys residues; however, it is not clear whether SelS is involved in the redox control of certain Cys within the ERAD components or it is involved in redox control of unfolded proteins. In addition, another study showed that the interaction of Derlin-1 and p97 ATPase was not affected by SelS knockdown [53]. This observation suggests that only the identification of additional SelS interacting partners may clarify the role of this protein in ERAD. The roles of SelS in protection of Min6 pancreatic cells from oxidative stress [56] and protection of RAW264.7 murine macrophages from ER-induced apoptosis [57] were described; however, the mechanisms of these effects are not clear.

An association of SelS expression and type II diabetes in an animal model *Psammomys obesus* was reported [58]. SelS was downregulated in liver, adipose tissue, and skeletal muscle in the fed state of diabetic *P. obesus* in comparison with healthy animals. A yeast two-hybrid screen for binding partners of the cytosolic part of SelS yielded serum amyloid A1b (SAA1b) [58]. SAA1 is secreted by liver and adipose tissue during the acute phase of inflammation and is thought to be involved in diseases associated with diabetes. Furthermore, SelS was found to be insulin regulated [59]. Its expression was increased after insulin stimulation of human adipocytes. Finally, SelS was suggested as a factor in the development of metabolic disease, especially in the context of insulin resistance [59]. The SelS promoter region polymorphism was associated with elevated proinflammatory cytokine expression considering possible roles of SelS in inflammatory responses [60]. Other studies have demonstrated an association of SelS promoter polymorphism with preeclampsia [61], gastric cancer [62], coronary heart disease, ischemic stroke [63], and colorectal cancer [16]. In addition, neuroprotective role of SelS was suggested

[64]. Taken together, the biological function of SelS in ERAD and its role in plasma membrane remains unknown. SelS is evolved in an early eukaryote (Fig. 17.3).

17.7 Selenoprotein K (SelK)

Human SelK is 10.6 kDa, 94 amino acid long selenoprotein identified in selenoproteome computational studies about 10 years ago [3]. The human SelK gene is located on chromosome 3p21.31 and consists of five exons and four introns. The domain structure of SelK is represented by an N-terminal transmembrane domain followed by the cytosolic Sec-containing region [65]. Sequence analysis did not reveal ER targeting and ER retention signals. Thus, SelK is a single-spanning ER membrane selenoprotein with its redox domain exposed to the cytosol. Its cytosolic part lacks a pronounced secondary structure and is extremely rich in proline (11.4%), glycine (14.3%), and arginine (12.9%). The presence of the positively charged, arginine-rich unstructured domain suggested a possibility that SelK interacts with negatively charged segments of other proteins. SelK is ubiquitously expressed [26, 66].

A wide tissue pattern of SelK expression suggested a general biological function of this protein. The presence of Sec in the C-terminal part of human SelK (position 92) suggested a redox function for this protein. Overexpression of SelK protected neonatal rat cardiomyocytes from oxidative stress induced by hydrogen peroxide [66]. Another study found an opposite effect on *Drosophila* embryos and in cultured *Drosophila* Schneider S2 cells [67]. SelK knockdown did not affect redox homeostasis in this model system. A recent study revealed relatively high levels of SelK in lymphoid tissues and various immune system cells. A SelK knockout mouse model was generated to address the biological function of SelK and its role in immune response [68]. SelK KO animals were viable and showed no phenotypes; however, specific defects in immune cells were observed. SelK deletion affected receptor-mediated Ca flux and other Ca-dependent functions, such as proliferation and chemotaxis in T-cell, neutrophils and macrophages, and Fcγ receptor-mediated oxidative burst in macrophages. In addition, SelK KO animals exhibited impaired immune responses to West Nile virus (WNV) infection. This infection was characterized by an increased viral titer in the brain and increased mortality [68].

SelK is upregulated under ER stress condition and protects HepG2 cells from ER stress agent-induced apoptosis [69]. Taken together, current research data on SelK are not sufficient to predict its function and additional work in this area is required, however, a significant role of SelK in ER-associated redox processes can be suggested. SelK is evolved in early eukaryotes (Fig. 17.3) and homologs with Cys or Sec are widely distributed among eukaryotes.

An interesting observation is related to the similarity between SelS and SelK. Both proteins are single-spanning ER membrane selenoproteins with unstructured and positively charged sequences exposed to the cytosol. Both proteins have Sec-containing sequences near the C-termini. In addition, ubiquitous expression patterns and similar phyletic distribution support the possibility of a functional similarity between SelS and SelK.

17.8 Concluding Remarks

A thorough examination of what is known about the seven selenoproteins that reside in the ER reveals that, with few exceptions, there is still much to be done in elucidating their functions. The selenium field is moving at such a rapid pace that we can envision many exciting, new developments relating to the function of this subclass of selenoproteins in the next few years.

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Chapter 18
Selenoproteins in Nervous System Development, Function, and Degeneration

Ulrich Schweizer

Abstract The discovery of spontaneous neurological phenotypes in selenoprotein P-deficient (*Sepp*$^{-/-}$) mice marks a turning point in our appreciation of selenium (Se) and selenoproteins within the nervous system. Before, Se was viewed mainly as a cofactor of glutathione peroxidase 1 (GPx1), and feeding animals low Se-containing diets or targeted inactivation of *Gpx1* have merely exacerbated neurological damage caused by experimental brain ischemia or exposure to neurotoxins. Case reports on a possible relationship between Se and neurological disease in patients were inspiring and often visionary, but initially failed to provide a solid mechanistic framework to explain the observed phenotypes. *Sepp* inactivation for the first time provided a tool to experimentally modulate brain Se content and brain selenoprotein expression. Since then a large and still growing number of transgenic mouse models affecting cerebral selenoprotein expression have been analyzed with respect to possible neurological defects. These studies revealed that, apart from more general protective roles during neurodegeneration, many specific developmental processes depend on selenoproteins. Recently, the essential roles of selenoproteins in human neurobiology were supported by the identification of patients carrying mutations in genes involved in selenoprotein biosynthesis. The phenotypic similarities between these patients and transgenic mouse models proved that mice represent a valid model for the study of many aspects of the neurobiology of Se. This chapter will summarize the topic from the perspective of molecular genetics.

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18.1 Introduction

Brain selenium (Se) levels remain rather stable in experimental animals during periods of severe dietary Se restriction [1–3]. Only one report demonstrated spontaneous neurological symptoms in Se-deficient mammals, i.e. "leg-crossing," in Balb/c mice maintained on a Se-deficient diet [4]. Therefore, a role of Se in brain function could not be established, since good experimental models were not available. First hints towards some function of Se in the brain came from experimental models of neurodegenerative disease where Se-deficiency exacerbated neurological damage. It was later shown that some of these effects are likely mediated by glutathione peroxidase 1 (GPx1), an enzyme capable of hydrogen peroxide degradation. According to the oxidative stress hypothesis of neurodegenerative disease, many researchers assumed that the main role of Se in neurobiology was related to the degradation of peroxides. This role seemed entirely related to the stress response during acute illness, because *Gpx1*$^{-/-}$ mice did not show any spontaneous neurological phenotype.

The first animal model demonstrating a direct link between brain Se levels and neurological symptoms was established with mice genetically deficient for selenoprotein P (*Sepp*$^{-/-}$) [5, 6]. As detailed in Chap. 16, SePP is involved in the molecular mechanism directed to preferentially supply Se to the brain. Disruption of this mechanism renders the brain dependant on dietary Se intake and lowers brain Se levels during times of Se restriction. Accordingly, selenoprotein expression in brain is reduced and complex neurological phenotypes develop. The brain expresses almost all selenoproteins [7] and within the brain neurons are the primary site of selenoprotein expression [7, 8]. Thus, it appears likely that different aspects of cerebral selenoprotein deficiency are mediated by the lack of different selenoproteins in affected structures.

Initial case reports have suggested a link between intractable childhood epilepsy and low Se levels [9, 10]. Recently, syndromes of congenital selenoprotein biosynthetic deficiency have been discovered, one associated with severe childhood epilepsy and brain atrophy [11] and one associated with milder developmental delay and impaired movement coordination reminiscent of *Sepp*$^{-/-}$ mice [12].

18.2 Selenoprotein-Transgenic and -Deficient Mouse Models

18.2.1 Gpx1 in Stroke Models

A role for GPx1 in degradation of cerebral reactive oxygen species and protection from stroke seems likely: In a mouse model of cerebral hypoxia/ischemia-reperfusion (HI), *Gpx1*-overexpressing mice are protected [13, 14]. In contrast, *Gpx1*$^{-/-}$ mice suffer increased infarct volume, more pronounced neuronal cell death, and increased neurological deficits [15]. Together, these studies strongly suggest that reactive oxygen species are indeed causally involved in the pathogenesis of HI-related tissue

damage – and GPx1 is a protective selenoenzyme in this condition. What remains unclear is where GPx1 exerts is beneficial role. It is known that GPx1 is induced in astrocytes surrounding the ischemic core [16]. However, GPx1 is a ubiquitous enzyme. It may act within neurons, in microglia, along the endothelial cells of the blood-brain barrier, or in infiltrating immune cells including neutrophils. Hence, it is not at all clear which cell types – and which processes – are affected by selenoprotein activity [17, 18].

18.2.2 Neurotoxic Disease Models

Exposure of animals to metamphetamine (MA), 6-hydroxydopamine (6-OHDA), methyl-phenyl-tetrahydropyridine (MPTP), or diiminopropionitrile (DIPN) leads to specific loss of dopaminergic neurons and of Parkinson's disease (PD)-like movement phenotypes in rodents. Cellular lesions in 6-OHDA, MA, and MPTP models of PD are increased in Se-deficient rats and can be reduced by Se-administration [19, 20]. Pretreatment of mice with Se in the DIPN model resulted in reduced lipid peroxidation products and inhibited neurobehavioral alterations in a dose-dependent manner [21]. Striatal dopamine depletion was prevented by Se-supplementation in the MA model [22]. Induction of Se-dependent enzymes like GPx1 via Se-administration dose-dependently attenuated neurodegeneration in 6-OHDA treated rats [23] and MA-treated rats and mice [20, 24]. These Se-dependent protective effects are likely mediated by modulation of GPx1 activity, because mice that overexpress *Gpx1* are protected against 6-OHDA induced nigral degeneration [25]. In the MPTP model, *Gpx1*$^{-/-}$ mice showed increased sensitivity [26] providing further support for a role of both hydrogen peroxide in nigral cell loss and protection afforded by the endogenous selenoenzyme GPx1.

18.2.3 Transgenic Mouse Models with Reduced Cerebral Se Levels: SePP and SePP Receptors

SePP contains multiple selenocysteine residues in one polypeptide chain. As described in detail in Chap. 16, SePP is a plasma Se transport protein produced mainly in liver and taken up for Se supply by target tissues including brain, testis, and kidney. *Sepp*$^{-/-}$ mice represent the first reliable model to study Se effects in the brain. Our group and the group of R. Burk and K. Hill succeeded at the same time at genetic inactivation of *Sepp* in mice [5, 6]. These mice exhibited reduced plasma Se levels and reduced Se content in many organs and tissues including the brain [5, 6]. Both strains of mice show a neurological phenotype which depends on dietary Se intake [27, 28]. When fed a low Se diet to their lactating mothers, *Sepp*$^{-/-}$ mice develop a severe runting phenotype and die. When dietary Se restriction starts from weaning,

the offspring develop a severe neurodegenerative phenotype leading to death within 2 weeks. Brainstem axonal damage and gliosis was reported in Se-deficient *Sepp*$^{-/-}$ mice [29]. Even when fed a diet containing the mouse equivalent of the human recommended dietary allowance (RDA) of Se, (0.15 mg Se/kg), *Sepp*$^{-/-}$ mice develop a neurological phenotype including a movement disorder with a wide waddling gait [27, 28, 30]. Delayed cerebellar development is a hallmark of hypothyroidism. Cerebral deiodinase (Dio2) activity and thyroid hormone levels were unaltered, leading to normal cerebellar development in *Sepp*$^{-/-}$ mice [31]. Seizures are frequent from 5 weeks of age, but can be controlled by increasing dietary Se intake [27]. Synaptic transmission is altered in *Sepp*$^{-/-}$ mice, even if fed a high Se diet, but the mechanism remains unclear [32]. The Se-rich C-terminus is likely important for efficient Se transport, since mice expressing a truncated SePP protein containing only the single N-terminal selenocysteine residue (*Sepp*$^{\Delta C/\Delta C}$) also exhibit diminished Se content in the brain and brain pathology [29, 33].

Expression of the human *SEPP1* transgene under the control of a hepatocyte-specific promoter in *Sepp*$^{-/-}$ mice, fully complemented the *Sepp*$^{-/-}$ phenotype under Se-sufficient (RDA diet) conditions, although brain selenoenzyme levels were not fully restored to those of control litter mates [34]. When these mice were fed a Se-restricted diet, brain Se levels and cerebral selenoprotein expression decreased, and ultimately the mice succumbed to Se-deficiency. In our interpretation, lowering of dietary Se intake failed to sustain hepatic SePP expression and hence Se supply to the brain diminished. In the absence of cerebral SePP expression, *Sepp*$^{-/-;\ SEPP1}$ mice were not able to maintain brain Se levels unlike Se-deficient wild type mice [34]. *Sepp* expression is not only found in mouse brain [5, 35], but also in human brain [36] where SePP was even easily detectable in cerebrospinal fluid. In support of a dual role of *Sepp* expression in brain and liver, we have observed that hepatic inactivation of SePP expression lowered plasma Se and kidney Se content, but not brain Se content and selenoprotein expression [30].

A major breakthrough in the field was the identification of ApoER2/*Lrp8* as specific SePP receptor in testis and brain [37, 38]. As detailed in Chap. 16, ApoER2 belongs to the lipoprotein receptor-related protein family of endocytic receptors. Burk suggested a SePP::ApoER2 ligand::receptor couple, because *Apoer2*$^{-/-}$ mice fed a Se-deficient diet replicated the *Sepp*$^{-/-}$ phenotype [39]. ApoER2 is not only an endocytic receptor, but can initiate intracellular signaling events upon ligand binding. To explore a potential signaling role of the SePP::ApoER2 complex, *Apoer2 signaling* mutants were studied, but apparently did not impair brain Se content [40]. We have compared *Apoer2*$^{-/-}$ mice with *Sepp*$^{-/-}$ fed a Se-sufficient (RDA) diet. As shown in Fig. 18.1, *Apoer2*-deficient mice do not exhibit the characteristic movement phenotype known from *Sepp*-deficient mice fed an identical diet for the same time. We conclude from this finding that a simple ligand–receptor couple cannot explain these data and hypothesized that more than one SePP receptors are active in the brain.

Megalin/*Lrp2* was identified as another SePP-binding protein in vitro, in histological sections [41], and in vivo [42]. Brain Se content and selenoprotein expression are reduced in *Lrp2*$^{-/-}$ mice and the effect is enhanced when the animals were fed a

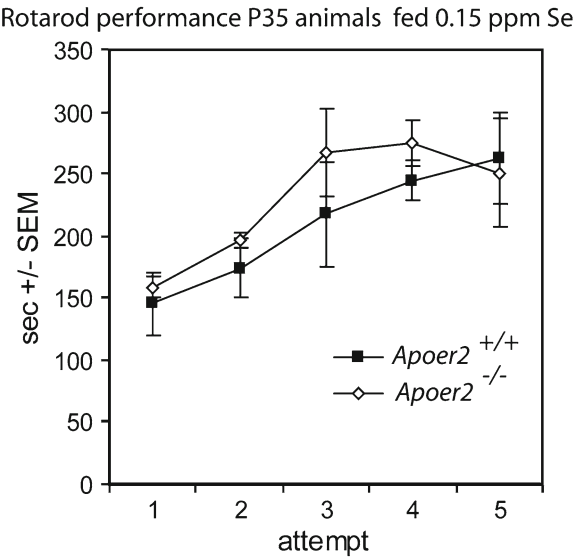

Fig. 18.1 Rotarod performance of *Apoer2*$^{-/-}$ mice. Mice were fed a diet containing 0.15 ppm Se (the RDA, for mice) from weaning. Under the same conditions, *Sepp*$^{-/-}$ mice developed a movement phenotype [34]. Four to five mice per genotype were analyzed on postnatal day 35 (unpublished data)

low Se diet [42]. After several weeks of feeding low Se diet to adult *Lrp2*$^{-/-}$ mice, movement coordination deteriorated, reminiscent of *Sepp*$^{-/-}$ mice [42]. Since megalin is expressed along the blood-brain barrier, but not on neurons or astrocytes, these data support a role of megalin in cerebral Se uptake.

Variation of phenotype depending on the exact Se content of the diet and its chemical form is a common observation in the field, at least when working with *Sepp*, *Apoer2*, and *megalin*-deficient mice [28]. It is therefore mandatory to strictly define these variables in the future.

18.2.4 Transgenic Mouse Models with Global Selenoprotein Deficiency: Mutations in tRNA$^{[Ser]Sec}$

Transfer RNA$^{[Ser]Sec}$ (gene symbol *Trsp*) is absolutely essential for functional selenoprotein expression. Knockout of *Trsp* thus completely abrogates cellular selenoprotein expression. A comprehensive discussion of *Trsp* genetic models can be found in Chap. 44.

We have conditionally inactivated *Trsp* in neurons [8]. This experiment represented a *proof-of-principle* whether selenoproteins play any important role in brain function, because the alternative hypothesis, that the complex phenotypes of *Sepp*$^{-/-}$ or *Apoer2*$^{-/-}$ mice lead indirectly to neurological deficits, could not be refuted at the beginning. In neuron-specific *Trsp*-deficient mice, cerebral selenoprotein expression was significantly reduced (Fig. 18.2). Mutant mice showed growth retardation, loss of balance, and increased excitability. Hippocampal slices from mutants exhibited

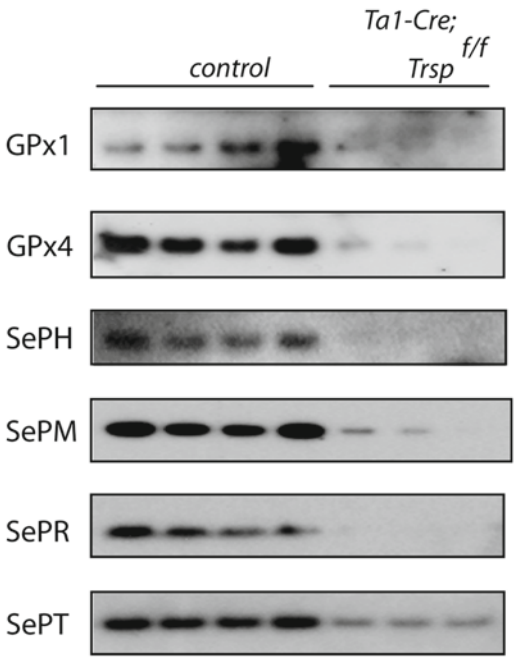

Fig. 18.2 Expression of neuronal selenoproteins is abrogated in *Trsp*-deficient mice. Western Blot of brain samples from 3 to 4 individual mice per genotype. Residual expression of SePT and SePM indicate significant non-neuronal expression of these selenoproteins

spontaneous epileptiform activity in vitro. A few days later, massive neurodegeneration followed with signs of apoptosis, enlarged ventricles, and mircocephaly [8]. When a Cre-transgenic mouse line was used that directed *Trsp* deletion to the cerebellum, massive Purkinje cell death and cerebellar hypoplasia were observed (*unpublished*). Purkinje cell loss is also observed upon *Gpx4*-inactivation, but to define the roles of selenoproteins during development in the various cerebellar cell types requires more study. A novel finding was the specific lack of parvalbumin-expressing (PV-) cortical and hippocampal interneurons in *Trsp*-mutant animals. PV-interneurons represent the largest fraction of cortical interneurons. Interestingly, neuropeptide Y-expressing interneurons and the third major subtype, calretinin (CR)-expressing interneurons, developed normally [8]. We were able to show that PV-cells are similarly affected by inactivation of *Gpx4* [8, 43]. Thus, it seems as if PV-cells are particularly sensitive towards *Gpx4* inactivation or lipid peroxides. In fact, when dissociated cortical neurons are cultured in vitro using a chemically defined medium, combined deprivation of Se and vitamin E leads to a reduction in the number of PV-expressing GABAergic neurons [44].

Expression of a hypomorphic *Trsp* allele, $Trsp^{\Delta AE}$, leads to neurological phenotypes similar to $Sepp^{-/-}$ mice fed an RDA diet from weaning [45]. In these mice, endogenous *Trsp* is deleted and genetically complemented by a *Trsp*-transgene lacking the *activation element* (ΔAE) in the promoter (see Chap. 44). Brain selenoprotein expression is significantly reduced in $Trsp^{\Delta AE/\Delta AE}$ mice, including GPx4. The mice show signs of progressive neuronal loss, but the most striking neuroanatomical

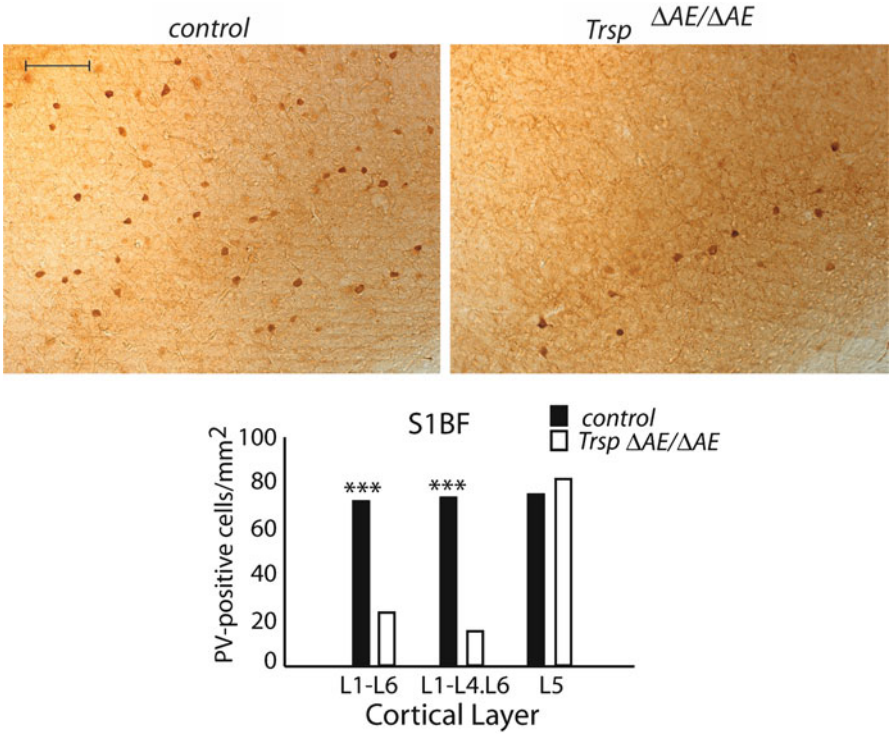

Fig. 18.3 Reduced number of parvalbumin(PV)-expressing cortical interneurons in the primary somatosensory cortex is reduced in mice expressing a hypomorphic *Trsp* allele. Neuronal layer 5 appears spared, while all other layers contain significantly less PV + interneurons. Student's *t*-test, ***$p < 0.001$ (unpublished data)

phenotype is a significant reduction in cortical PV-interneurons (Fig. 18.3). Thus, it appears as if the number of PV-interneurons correlates with cerebral *Gpx4* expression.

18.2.5 Transgenic Mouse Models with Specific Selenoprotein Deficiency

Gpx4 knockout mice have been generated in several laboratories [43, 46, 47]. Inactivation of *Gpx4* is embryonic lethal and leads to cell death in several cell types, including embryonic fibroblasts [43]. We have therefore analyzed neuron-specific conditional *Gpx4*-deficient mice [43]. These mice displayed a phenotype somewhat milder than neuron-specific *Trsp*-deficient mice, suggesting that GPx4 is essential, but not the only essential selenoprotein in neurons [8]. Neuron-specific *Gpx4*-knockout

mice suffer general neurodegeneration, but do not lose balance, although they seem hyperexcitable, similar to *Trsp*-knockouts [43]. *Gpx4*-mutant neurons progressively degenerate in culture, unless they are rescued with α-tocopherol [43]. An important neuropathological finding is the massive reduction of PV-interneurons in cortex and hippocampus of *Gpx4* mutants, while CR-expressing cells remained normal [8]. Thus, selective loss of PV-interneurons is a common phenotype in mice with a global reduction of cortical selenoprotein expression (*Sepp*$^{-/-}$, *Trsp*$^{\Delta AE}$, neuron-specific *Trsp*-knockout) and neuron-specific *Gpx4*-knockout mice. A reduction of hippocampal PV-interneuron number and schizophrenia-associated behavioral phenotypes were reported in mice deficient for the glutathione biosynthesis enzyme subunit, glutamyl-cysteine ligase modifier (*Gclm*$^{-/-}$ [48]). Consistent with an enhanced susceptibility towards pro-oxidative conditions, PV-interneuron number is reduced in a rodent model of schizophrenia (perinatal buthionine sulfoximine intoxication; a GSH biosynthesis inhibitor), and interneurons lose their GAD67- and PV-expression upon exposure to NMDA-receptor antagonists, ketamine or MK-801 [49]. Reduced number of PV-neurons in the prefrontal and auditory cortices is a neuropathological hallmark of schizophrenia and psychosis induced by ketamine intoxication. Accordingly, it has been shown that *GCLM*-polymorphisms and low cerebral GSH-levels correlate with schizophrenia in human patients [50]. A comprehensive account on Se and the glutathione system can be found in Chap. 13.

Hyperexcitability and epilepsy presumably result from a more complete loss of PV-interneuron function. Whether *Gpx4*-overexpression can protect from PV-neuron loss in models of psychiatric disorders has not been tested, although *Gpx4*-transgenic mice have been generated and *Gpx4* seems to mediate neuronal protection from damage [51].

Gpx4 is also essential in very early neural development. Ufer et al. have reported that *G-rich binding factor*-deficient mice exhibit neuronal cell death, because Gpx4 expression was impaired [52]. More details of *Gpx4* are found in Chaps. 14 and 43.

18.2.5.1 Thioredoxin Reductase (Txnrd1 and 2; TrxR1 and 2)

Txnrd genetic inactivation is embryonic or perinatal lethal [53, 54]. Neural-specific inactivation of *Txnrd2* did not lead to any apparent neurological phenotype [55]. In contrast, neural-specific inactivation of *Txnrd1* caused cerebellar hypoplasia associated with a movement phenotype in mice [55]. The layering of the cerebellar cortex was disrupted, in particular in the more anterior lobules of the vermis, and Purkinje cells appeared scattered within granule cells [55] (Fig. 18.4). Interestingly, when we inactivated *Txnrd1* using the same neuron-specific Cre lines as before for *Trsp* and *Gpx4*, no neurological or neuroanatomical phenotype was noted, including cerebellar cortex layering and cerebral interneuron expression [55]. However, we noted a compensatory upregulation of cellular GPx activity in *Txnrd1*-deficient brains (Fig. 18.5a, b). Moreover, in aged animals, movement coordination was

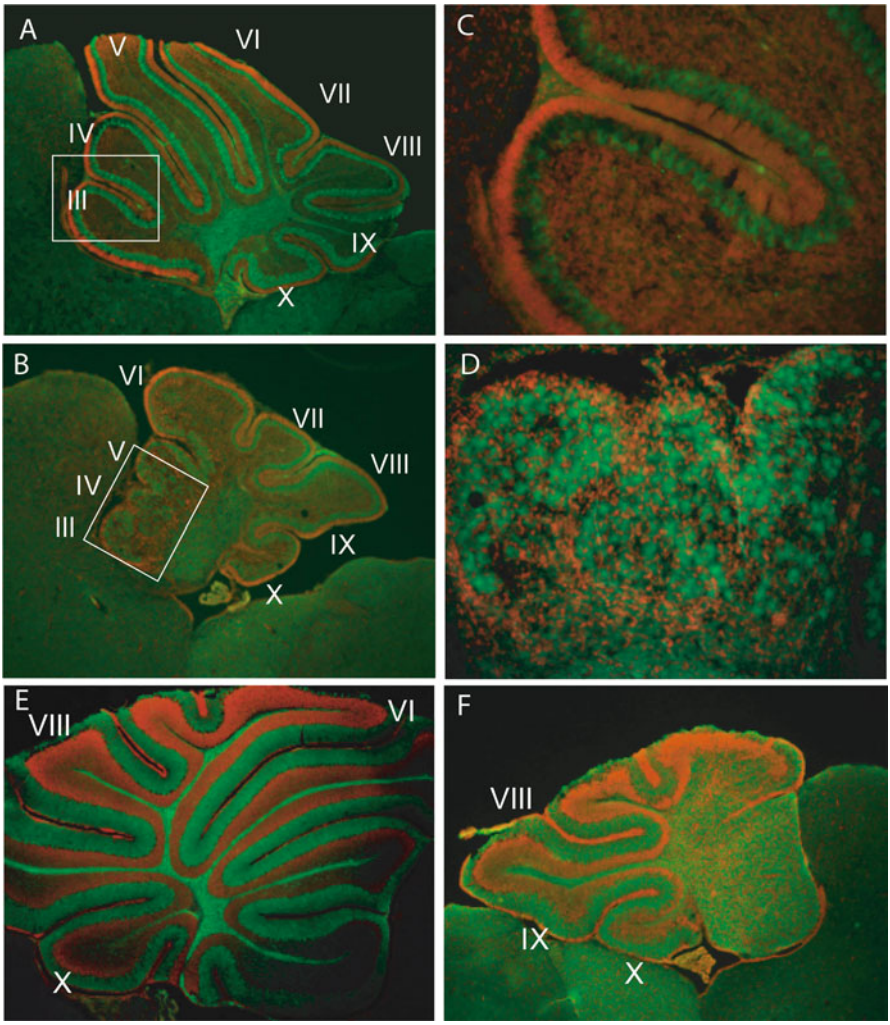

Fig. 18.4 Cerebellar hypoplasia in *nestin-Cre*; *Txnrd1*$^{fl/fl}$ mice. (**a**, **b**) Hypoplasia is apparent on postnatal day 7. Sections are stained for parvalbumin (Purkinje cell marker, *green*) and DAPI (nuclear marker, *red*). (**a**, **c**) Wildtype control. (**b**, **d**) *Nes-Cre*; *Txnrd*$^{fl/fl}$. (**c**, **d**) Magnified view (*boxes* indicated in (**a**, **b**)) demonstrates the irregular distribution of Purkinje cells in the *Txnrd1*-mutant. (**e**, **f**) Cerebellar hypoplasia on postnatal day 14 demonstrates that the phenotype is more prominent in rostral foliae

decreased in the *Txnrd1*-mutants (Fig. 18.5c). Finally, loss of rotarod performance was accelerated by feeding a low Se diet (Fig. 18.5d). Thus, Txnrd1 and Txnrd2 are not essential for neurons, albeit *Txnrd1* plays some role in radial glia biology during development and may be important for long-term maintenance of neurons. More data on the thioredoxin system can be found in Chap. 12.

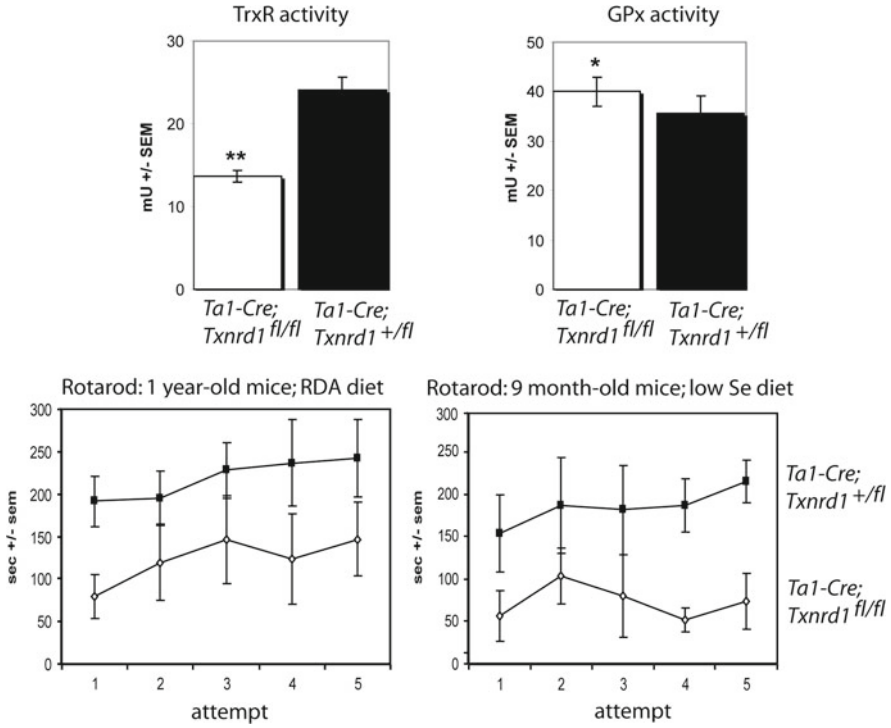

Fig. 18.5 Loss of neuronal *Txnrd1* expression leads to neurodegeneration in old mice. While cerebral Txnrd1 activity is reduced, GPx1 activity is increased in *Ta1-Cre*; *Txnrd1*$^{fl/fl}$ mice. Performance on the rotarod is decreased in 1 year-old *Ta1-Cre*; *Txnrd1*$^{fl/fl}$ mice was fed RDA diet, while development of the phenotype is accelerated by feeding low Se diet. $n = 5–6$ mice per genotype (unpublished data)

18.2.5.2 Deiodinases (Gene Symbols *Dio1–3*)

Deiodinases are a class of selenoproteins not involved in protection from reactive oxygen species or lipid hydroperoxides. These enzymes catalyze the elimination of iodide from iodothyronines, i.e. metabolites related to and including thyroid hormones. While *Dio* are treated comprehensively in Chap. 29, we will focus here on the possible roles of *Dio1–3* in neurobiology. Since Dio are involved in local activation and inactivation of thyroid hormones, they may modulate developmental processes which depend on thyroid hormone. All Dio genes have been inactivated in transgenic mice. *Dio1*$^{-/-}$ mice do not exhibit any neurological phenotype [56]. *Dio2*$^{-/-}$ mice exhibit pituitary resistance to thyroid hormone, while cerebellar development, is reportedly normal [57]. Moreover, developmental cortical PV-expression

depends on thyroid hormone [58], but is apparently not changed. *Dio2*$^{-/-}$ mice exhibit surprisingly mild phenotypes [59]. The most striking developmental phenotype of *Dio2*$^{-/-}$ mice is failure of cochlear development and subsequent sensorineural hearing loss [60]. *Dio3*$^{-/-}$ mice exhibit a complex phenotype involving central hypothyroidism [61]. These mice also suffer from failed cochlear development [62]. Moreover, retinal photoreceptor development is deranged in *Dio3*$^{-/-}$ mice [63]. Taken together, the auditory and visual systems are obviously affected by *Dio* deficiency, while much less data is available on other neuronal systems. *Sepp*$^{-/-}$ mice have no hearing impairments (US and German Mouse Clinic, unpublished).

18.3 Syndromes of Impaired Selenoprotein Expression in Human Patients

Two case reports initially suggested that impairment of Se metabolism may underlie intractable childhood epilepsy [9, 10]. At the time it was not possible to identify a molecular cause, although subsequently the phenotypic similarity between patients [10] and *Sepp*$^{-/-}$ mice became apparent. Se-responsive epilepsy during childhood or adolescence, brain atrophy, movement disorder with spasticity, low circulating Se levels, low plasma GPx activity, and elevated liver enzymes suggested a common mechanism.

18.3.1 Progressive Cerebellar-Cerebral Atrophy (SEPSECS-Mutations)

The novel syndrome called progressive cerebellar-cerebral atrophy (PCCA) was described as an autosomal-recessive phenotype affecting several non-consanguineous Jewish Sephardic families of Moroccan or Iraqi ancestry [11]. The syndrome involves profound mental retardation, progressive microcephaly, and severe spasticity. Myoclonic or generalized tonic–clonic seizures are often observed and cerebral and cerebellar atrophy involved gray and white matter. Affected individuals were found homozygous or compound heterozygous for missense mutations in the selenocysteine synthase gene (*SEPSECS*) [11]. Human *SEPSECS* mutants expressed in *Escherichia coli* were unable to complement the deletion of bacterial *SelA* and sustain bacterial selenoprotein biosynthesis [11]. However, a complete loss of function in humans would be surprising in light of the phenotypes of *Trsp*-, *Txnrd1*-, *Txnrd2*-, *and Gpx4*-deficient mice. It is thus important to investigate residual selenoprotein expression in serum or cells derived from affected individuals. The phenotypic similarities to *Sepp*$^{-/-}$ (except cerebellar involvement) and neuron-specific *Trsp*-knockouts (including cerebellar atrophy) are nevertheless striking. PCCA represents the first clinical syndrome related to selenoprotein biosynthesis that is definitely associated with a neuro-developmental and neurodegenerative phenotype in humans.

18.3.2 SECISBP2-Syndrome

Mutations in *SECISBP2* were initially identified in patients with a rare form of resistance to thyroid hormone ([64] and Chap. 4). Patients showed delayed growth and retarded bone age likely associated with abnormal thyroid hormone signaling. Accordingly, patients exhibit high T4 levels, low/normal T3, slightly elevated TSH and clearly elevated rT3 (see Chap. 29). Expression of Dio2 in patient-derived fibroblasts was very low. Moreover, plasma SePP and GPx activity were low or undetectable leading to the general defect in selenoprotein biosynthesis associated with a homozygous missense mutation in one kindred and compound heterozygosity in another patient. In the meantime, more patients have been identified and some carrying apparently more severe mutations also exhibit neurological impairments [12, 65]. One girl was described with mental retardation, sensorineural hearing loss, and waddling gait [65]. These phenotypes are consistent with cochlear Dio2-deficiency and severe SePP-deficiency in brain. Two other patients with apparently lower SECISBP2 bioactivity also exhibit bilateral sensorineural hearing loss and undetectable plasma SePP [12] as well as many more phenotypes (see Chaps. 4, 5, 22, and 29). Overt neurodegeneration/brain atrophy, epilepsy, or spasticity were not reported in SECISBP2-syndrome suggesting that residual selenoprotein expression is higher than in PCCA.

18.4 Conclusions

A role for selenoproteins in neurobiology is no longer hypothetical. Mainly by stringent application of molecular genetics in mice and more recently by molecular genetics in carefully defined clinical syndromes, clear and essential roles and function of selenoproteins in many facets of neural development, function, and degeneration have been defined. The models and syndromes teach us that many shades of gray exist between mild impairment of selenoprotein expression and severely compromised selenoprotein biosynthesis. Some of the biosynthetic deficiencies may be responsive to pharmacological treatment with therapeutic doses of Se. Whether selenoprotein deficiency plays a major role in common neurological diseases remains to be seen.

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Chapter 19
Selenoproteins in Cardiovascular Redox Pathology

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Abstract Redox-active selenoproteins, such as the mammalian glutathione peroxidases (GPxs), are essential components of the antioxidant defense systems that serve to limit the damaging accumulation of intracellular and extracellular oxidants. Accumulating evidence from epidemiological and experimental studies indicates that deficiencies in these key antioxidant proteins promote cardiovascular disease and that their excess is often protective against injury and stress. In this chapter, we will examine the role of GPxs in cardiovascular diseases, highlighting their role in modulating vascular function, thrombosis, and atherogenesis.

19.1 Introduction

A role for selenium in heart disease has been known for some time. In human populations, a cardiomyopathy, Keshan disease, is endemic in provinces of China with low selenium in the soil [1]. Decreased expression of selenoproteins is characteristic of this disease, and replacement of selenium in the diet increases selenoprotein expression and is a successful preventive treatment. Experimental evidence confirms a role for individual selenoproteins in complex cardiovascular diseases, such as atherosclerosis and stroke, primarily through modulating the damaging effects of reactive oxygen species (ROS). Here, we focus on the role of the glutathione peroxidases (GPxs) in modulating vascular function and cardiovascular disease risk.

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19.2 Mammalian Glutathione Peroxidase 1 (GPx-1)

GPx-1 is a ubiquitously expressed member of the GPx-family that contains a selenocysteine (Sec) at its catalytic site [2] and exists as a tetramer. It was the first Sec-containing protein to be identified [3]. GPx-1 is found intracellularly, primarily in cytosolic and mitochondrial compartments [4], where it reduces intracellular hydrogen and lipid hydroperoxides using glutathione as an obligate cofactor. Although knockout of GPx-1 in mouse models is not fatal, GPx-1 is necessary for optimal protection following direct exposure to oxidants [5, 6], or to stress-induced oxidants, such as those produced during ischemia-reperfusion or inflammation [7–11]. Thus, decreased expression of GPx-1 leads to enhanced susceptibility to injury in many disease models. Similarly, in individuals with coronary artery disease red blood cell GPx-1 activity was found to be a strong predictor of future cardiovascular events with individuals in the lowest tertile having over a threefold increase in cardiovascular disease risk compared with those in the highest tertile of activity [12]. Consistent with these findings in human populations, excess GPx-1 has been found to protect the cardiovascular system from oxidative stress-induced injury in transgenic mice overexpressing this selenoprotein.

19.2.1 Ischemia-Reperfusion Injury and GPx-1

GPx-1 has been found to modulate ischemia-reperfusion injury in both cerebral (stroke) and cardiac models of injury. In brain, GPx-1 deficient neurons were found to be more susceptible to apoptosis following middle cerebral artery (MCA) occlusion [7]. In addition, in this model, lack of GPx-1 results in increased production of oxidative stress markers and enhanced activation of NFκB, a process dependent, in part, on oxidant production [7, 13]. Furthermore, in GPx-1 deficient mice, cerebral injury was exacerbated by vascular dysfunction that limited microvascular blood flow following ischemia [14]. Injury following MCA occlusion could be limited by treatment with ebselen, a GPx-mimetic, which attenuated infarct size and improved vascular function in GPx-1 deficient mice [14]. (As discussed further in Sect. 19.2.3, endothelial dysfunction is a hallmark of endothelial redox imbalance that is caused by GPx-1 deficiency.) Although ebselen has a broader substrate specificity than GPx-1 and may have additional antioxidant effects on cells, similar protective effects of excess GPx-1 were found in transgenic GPx-1 overexpressing mice, which had significantly less cerebral injury following MCA ischemia-reperfusion injury than mice with normal levels of GPx-1 [15]. Overall these findings suggest a critical role for redox-balance in modulating neuronal protection in response to cerebral ischemia-reperfusion. Interestingly, it has been reported that ebselen may also improve neurological outcomes following stroke in human subjects [16]; however, these early studies have not been replicated.

In mouse cardiac ischemia-reperfusion injury models, GPx-1 has also been found to preserve cardiac function, as hearts from transgenic mice overexpressing GPx-1

are more resistant to myocardial ischemia-reperfusion injury than those from non-transgenic controls. Consistent with this finding, we found that aged GPx-1 deficient mice with 50% of the normal GPx-1 (heterozygous knockout mice) have structural abnormalities in the myocardial vasculature and diastolic dysfunction following myocardial ischemia-reperfusion [8]. Subsequent studies found increased susceptibility to myocardial injury following ischemia-reperfusion in hearts from male, but not female, GPx-1-deficient mice [17]. These findings may be attributed, in part, to other compensatory antioxidant mechanisms in female GPx-1 deficient mice that preserve pools of reduced ascorbate and augment the conversion of nitrate to nitrite, a possible cardio-protective species. Although the underlying basis for additional redox-protection in female mice is not known for certain, additional evidence suggests that estrogen may offer some protection in females. In addition to enhancing GPx-1 expression [18], estrogen may also control the expression of other redox-active selenoproteins, including GPx-3, that are upregulated in females compared to males [19, 20]. Most studies examining the role of GPx-1 in mice have been limited to male mice, where compensatory mechanisms are not sufficient to offer protection against oxidant stress in the context of GPx-1 deficiency. In a separate study, excess reactive oxygen generation in male GPx-1-deficient hearts subjected to ischemia-reperfusion injury correlated with diminished mitochondrial function, characterized by increased damage to mitochondrial DNA, decreased levels of mitochondrial protein expression, and reduced NADH and ATP generation [21]. GPx-1 is one of many antioxidant enzymes found in mitochondria, possibly to limit damage due to ROS normally generated in this organelle during respiration and following stress, such as during reperfusion following ischemia. Thus, ischemia-reperfusion injury generates excess ROS, in part, via increased mitochondrial output of oxidants and the damaging effects of mitochondria oxidants may be augmented by lack of GPx-1. In support of a crucial role of GPx-1 in mitochondrial ROS-flux, absence of GPx-1 has been shown to be accompanied by increased mitochondrial production of hydrogen peroxide [21, 22].

19.2.2 Cardiac Hypertrophy

Angiotensin II (AII) is a vasoactive peptide that promotes hypertension, vascular remodeling, and cardiac hypertrophy, in part, via AII-receptor 1 mediated activation of NADPH oxidases to increase superoxide generation. In studies by Ardanaz et al. [23], GPx-1 deficiency was found to augment specifically AII-induced left ventricular hypertrophy, increase myocyte cross-sectional area and intraventricular septal thickness, and lower cardiac shortening fraction after only 7 days of administration. Under this short-term AII treatment, there was a similar increase in blood pressure between the control and GPx-1-deficient mice, with no structural changes in aorta and no differences in cardiac fibrosis. Although the mechanistic basis for the increased cardiac hypertrophy and dysfunction is not fully known, it is likely that it is related to excess ROS caused by GPx-1 deficiency.

19.2.3 Endothelial Dysfunction and Vascular Tone

Endothelial dysfunction is in part characterized by a decrease in bioavailable nitric oxide (NO) and a subsequent loss in normal endothelium-dependent vasorelaxation responses to flow or to NO-dependent agonists, such as acetylcholine or bradykinin [24]. Excess ROS promotes endothelial dysfunction by reducing bioavailable NO directly (for instance by the reaction of NO with superoxide to form peroxynitrite) or by activating the production of superoxide from NADPH oxidase. In addition, ROS can decrease the availability of cofactors necessary for the activation of endothelial NO synthase (eNOS), which produces NO. Importantly, loss of bioavailable NO contributes to platelet activation, proliferation of vascular smooth muscle cells, and pro-inflammatory activation of the endothelium. We have found that GPx-1 plays an essential role in modulating endothelial function by preserving bioavailable NO. In mouse knockout models, both heterozygous and homozygous GPx-1 deficient mice [8, 25, 26] have endothelial dysfunction, consistent with a crucial role of normal levels of GPx-1 for vascular homeostasis. In fact, we found that GPx-1 deficiency resulted in a (paradoxical) vasoconstrictor response to the vasoactive agonists that cause vasodilation in normal vessels. Furthermore, endothelium-independent responses to NO generators, such as sodium nitroprusside, are preserved in GPx-1-deficient mice, indicating that the smooth muscle responses to NO are intact in these mice. Consistent with a role for GPx-1 in reducing oxidants, we found that plasma and aortic levels of the isoprostane, $iPF_{2\alpha}$-III, were increased by GPx-1 deficiency [8, 25]. Compensatory treatment with L-2-oxothiazolidine-4-carboxylic acid to increase intracellular thiol pools restored vasorelaxation responses in GPx-1-deficient mice [25], and lowered $iPF_{2\alpha}$-III levels.

Other studies indicate that excess GPx-1 can compensate for the negative effects of the vasoactive peptide AII [26]. Thus, carotid arteries from heterozygous GPx-1 knockout mice showed diminished vasodilatory responses to acetylcholine at low doses of AII that had no effect on endothelium-dependent vasodilation in wild type vessels [26], whereas carotid arteries from GPx-1 overexpressing mice were resistant to dysfunction caused by higher doses of AII that compromised vascular function in wild type vessels. Suppression of GPx-1 can also lead to endothelial dysfunction. Thus, in a mouse model of hyperhomocysteinemia caused by partial deficiency of the cystathionine-beta-synthase gene, we found that GPx-1 expression is suppressed [27], in part, by mechanisms that reduce the translation of GPx-1 [28]. As in the genetic knockout models, diminished GPx-1 expression in hyperhomocysteinemia also resulted in endothelial dysfunction [29] characterized by a reduction in bioavailable NO. Other studies have reported diminished endothelial function caused by a combination of GPx-1 deficiency and hyperhomocysteinemia [30]. Overexpression of GPx-1 in the context of hyperhomocysteinemia prevented the loss of bioavailable NO and restored normal endothelial vasodilatory responses [27]. Although studies in human populations clearly show an effect of homocysteine on cardiovascular risk [31], there is a growing controversy regarding the importance of homocysteine in human cardiovascular disease as simple B-vitamin therapies

that lower homocysteine levels fail to reduce disease risk [32, 33]. Nonetheless, in coronary artery disease patients, homocysteine and GPx-1 activity are predictors of cardiovascular disease, with the combination of lowest GPx-1 activity and highest plasma homocysteine conferring the greatest risk [31]. Furthermore, in human hypertensive patients, recent studies suggest that GPx-1 activity is inversely correlated with endothelium-dependent vasodilation responses, illustrating the importance of GPx-1 in modulating vascular function in humans as well as in animal models [34] and indicating that modest alterations in GPx-1 levels may significantly diminish endothelial function. Paradoxically, in some vascular beds, hydrogen peroxide may modulate arachidonic acid-mediated vasodilation [35], suggesting excess GPx-1 may limit these responses by reducing hydrogen peroxide essential for vessel relaxation. In support of this concept, excess GPx-1 has been shown to decrease vasodilatory responses to low micromolar concentrations of hydrogen peroxide in isolated cerebral vessels [36]. The specific consequence of decreased GPx-1 on these pathways is unclear; however, these and other studies suggest that there are complex effects of ROS on vascular function that may depend on many factors, including the amount of ROS, the type of ROS, and the time course of its production.

19.2.4 Inflammation and Atherogenesis

Endothelial dysfunction and oxidative stress are thought to promote atherogenesis; yet in the context of a high fat diet, GPx-1 deficiency on a C57Bl/6 background did not promote atherogenesis. Rather, GPx-1 deficient mice had decreased severity of aortic sinus lesions [37] possibly due, in part, to compensatory upregulation of glutaredoxin-2, a redox-active enzyme that can preserve protein thiol redox state [38]. In the context of ApoE deficiency, however, lack of GPx-1 was found to increase atherogenesis in response to a Western diet [39] and in combination with streptozotocin-induced diabetes mellitus [40]. In each of these models, GPx-1 deficiency was found to augment inflammatory changes associated with the development of atherosclerotic lesions. Notably, compared to ApoE-deficient mice, ApoE/GPx-1 double knockout mice showed excess aortic ROS production, enhanced NADPH-stimulated ROS production, and enhanced mitochondrial ROS generation, indicating increased vascular oxidant stress caused by lack of GPx-1 [39]. Other studies have found that ebselen, a GPx-mimic, decreases aortic lesion formation in ApoE-deficient diabetic mice, illustrating a role of oxidant stress in atherogenesis in ApoE-deficient mice. As mentioned above, ebselen has a broader substrate specificity than GPx-1 and can effectively reduce membrane phospholipids that are normally reduced intracellularly by GPx-4. As discussed further below, GPx-4 overexpression was also found to slow atherosclerotic lesion development in ApoE-deficient mice [41].

Other studies in endothelial cells suggest that GPx-1 modulates pro-atherogenic gene expression in response to intracellular oxidants generated during cyclic stress [42] or following endotoxin exposure [11]. In fact, GPx-1 deficiency alone promotes upregulation of adhesion molecules in human microvascular endothelial cells [11],

resulting in a pro-inflammatory state. In human subjects, the greatest risk of cardiovascular [43] events was found in individuals with a combination of the lowest levels of GPx-1 activity and most extensive atherosclerosis [44], suggesting that deficiencies in GPx-1 can potentiate human atherogenesis. These findings are consistent with the initial Athero*Gene* studies of coronary heart disease patients that reported a significant protective effect of increased levels of red blood cell GPx-1 activity against future cardiovascular events [12]. Furthermore, in humans, there is a genetic polymorphism of GPx-1 involving a T for a C substitution that results in an amino acid difference (Leu substitution for Pro) at position 198 (Pro198Leu) in the GPx-1 protein. It has been suggested that the Leu variant may be associated with decreased expression of GPx-1 under conditions where selenium is limited [45, 46]; studies indicate that the Leu variant may contribute to risk in Keshan disease [46], which is caused, in part, by selenium insufficiency. Similarly, in a case-control study of coronary artery disease patients in China, the presence of the Leu allele was associated with increased disease risk [47]. Other studies from Japan found an association of the Leu allele with increased risk of restonosis following stenting [48], and in other studies, enhanced vascular disease in Japanese type 2 diabetic subjects [43, 49]. Further analysis, however, is necessary to understand the significance of these GPx-1 polymorphisms and to determine if these variant proteins alter in vivo GPx-1 activity to modulate cardiovascular risk.

19.3 Glutathione Peroxidase-3 (GPx-3)

GPx-3 is a secreted glycoprotein, often referred to as plasma GPx. The major source of human plasma GPx-3 is renal proximal tubules [50], although recent findings suggest that adipose tissue may also contribute to circulating levels of GPx-3, at least in the mouse [51]. In both mice and humans, GPx-3 has been found in many other tissues including lung, heart, liver, brain, breast, placenta, skeletal muscle, and spleen. Similar to GPx-1, GPx-3 contains Sec at the active site, exists as a tetramer, and reduces hydrogen and lipid hydroperoxides. Unlike GPx-1, GPx-3 may utilize thioredoxin and glutaredoxin, as well as glutathione, as reducing cofactors [52]. Recent findings suggest that lack of GPx-3 in knockout mice is not fatal [53], although, functionally, lack of GPx-3 has been shown to cause NO insufficiency and promote thrombosis [54, 55], as discussed in the following section.

19.3.1 GPx-3, Stroke, and Thrombosis

In 1996, we found a causal relationship between a deficiency of GPx-3 and thrombotic stroke in two brothers [54] with childhood cerebrovascular thrombotic disease. Mechanistically, excess peroxides caused by the deficiency of GPx-3 promoted platelet activation by inactivating NO, a known inhibitor of platelet activation. Subsequent studies by our group found evidence for GPx-3 insufficiency in other

families predisposed to childhood stroke [55]. Although the underlying genetic defects in these families are unknown, the deficiency appears to be due to a dominantly inherited defect that reduces plasma GPx-3 activity approximately 50% in affected patients. Concurrent with a decrease in plasma GPx-3, NO fails to block platelet P-selectin expression and platelet aggregation in studies with normal gel-filtered platelets mixed with GPx-3 deficient plasma. These findings suggest that modest alterations in circulating GPx-3 can alter platelet homeostasis, thereby contributing to platelet-dependent thrombosis and stroke. Additional studies in our laboratory found that GPx-3 expression was transcriptionally upregulated by hypoxia [56], suggesting that increased expression of GPx-3 in response to lower oxygen tension, as in ischemic stroke, may guard against ROS-induced damage during reoxygenation. In our subsequent analysis of human thrombotic disorders, we have identified a variant haplotype (H_2) in the GPx-3 gene promoter that correlated with reduced transcriptional activity under normoxic and hypoxic conditions [57]. Furthermore, we found that this haplotype is a strong, independent risk factor for cerebral venous thrombosis [58] and that it is associated with increased risk of arterial ischemic stoke in young individuals [57]. Independent studies by Nowak-Gottl et al. [59] in a German cohort confirmed that the H_2-haplotype was a risk factor in arterial ischemic stroke in children. This latter study found no association between GPx-3 genotypes and thromboembolic or cerebral sinovenous thrombosis in children.

To study further the role of GPx-3 in maintaining the balance between hemostasis and thrombosis, we developed a GPx-3 knockout model. Consistent with altered platelet function in GPx-3 deficient patients, we found attenuated bleeding times, elevated soluble P-selectin (a marker of platelet and endothelial activation), and increased platelet aggregation in response to ADP infusion in an in vivo model of platelet activation as well as increased ADP-activation of platelets in in vitro platelet assays [60]. Several observations suggest the presence of NO insufficiency in these mice: circulating levels of cGMP are decreased, and vascular beds have endothelial dysfunction. To determine whether alterations in platelet function would result in stroke injury, we used the cerebral MCA ischemia-reperfusion model. We found that GPx-3-deficient mice were more sensitive to cerebral injury following MCA ischemia-reperfusion, with increased infarct size and greater neurological impairment. Clopidogrel, a platelet inhibitor, significantly reduced stroke volume and improved neurological function, suggesting that platelet activation contributed to the extensive injury caused by GPx-3 deficiency in this model. Furthermore, use of MnTBAP, an antioxidant, was similarly able to reduce brain injury following MCA ischemia-reperfusion, indicating the importance of oxidative mechanisms in the underlying dysfunction caused by GPx-3 deficiency.

19.4 Glutathione Peroxidase-4 (GPx-4)

GPx-4 is a widely expressed, intracellular selenoprotein that exists as a monomer rather than a tetramer. This enzyme is often referred to as the phospholipid GPx, as it can effectively reduce oxidized membrane phospholipids. GPx-4 exists in several

forms in the cell, including a long form with a mitochondrial targeting sequence that is found in mitochondria, and a short form that is found outside of the mitochondria. Although enzymatically, GPx-4 can reduce hydrogen and lipid hydroperoxides, intracellularly, it primarily reduces oxidized membrane phospholipids and has little effect on hydrogen peroxide levels. In mice, knockout of GPx-4 was found to be lethal, and its deficiency in cells grown in culture has been shown to promote apoptosis [61]. Thus, in order to study its in vivo protective function, studies have examined the consequences of overexpression of this essential selenoprotein, rather than its deficiency. Overexpression of GPx-4 has been found to increase survival to oxidants in cells grown in culture as well as in a transgenic mouse model [62, 63].

19.4.1 GPx-4 and Cardiac Ischemia-Reperfusion

To study the role of mitochondrial oxidants on ischemia-reperfusion injury, a transgenic mouse was engineered to overexpress specifically a rat mitochondrial form of GPx-4 [64] and used in the Langendorff model of global no-flow ischemia-reperfusion injury. In these studies, overexpression of GPx-4 resulted in improved contractile function characterized by improved rates of contraction, developed pressure, and peak-systolic pressure compared to non-transgenic hearts. These functional improvements may be the result of decreased mitochondrial damage, as there was less overall lipid peroxidation in mitochondria and electron transport complexes had preserved function in hearts from GPx-4 transgenic mice. Overall, these findings suggest that excess GPx-4 in mitochondria effectively removes harmful oxidants during ischemia-reperfusion to lessen cardiac contractile dysfunction.

19.4.2 GPx-4 and Atherogenesis

Excess GPx-4 was also found to lessen atherogenesis in ApoE-deficient mice [41]. This protective effect correlated with a reduction in lipid peroxidation in aorta without any change in overall plasma lipid levels. In isolated mouse aortic endothelial cells, overexpression of GPx-4 reduced endothelial production of hydroperoxides and decreased adhesion molecule expression in response to oxidized phospholipids, suggesting that GPx-4 overexpression reduces lipid oxidation and inflammatory responses to lessen atherogenesis. These studies also found that treatment of isolated cells with exogenous catalase could reduce hydroperoxide release more effectively, suggesting that in these cultured mouse endothelial cells, hydrogen peroxide is the major hydroperoxide generated in response to exposure to oxidized lipids.

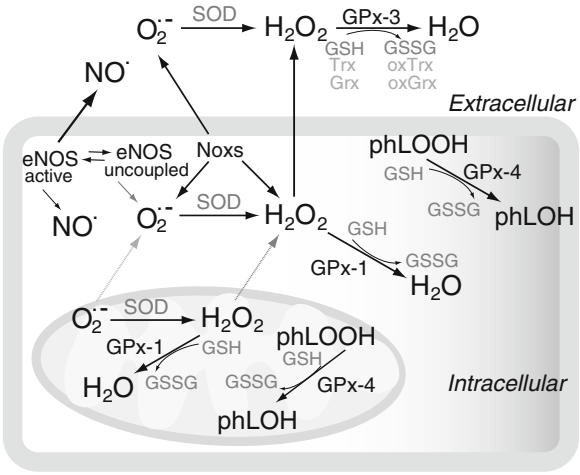

Fig. 19.1 Role of GPxs in modulating ROS-flux in endothelial cells. Superoxide is generated intracellularly from enzymatic sources as well as from mitochondrial respiration. Various NADPH oxidases (Noxs) may contribute to extracellular or intracellular superoxide pools and may also directly produce hydrogen peroxide. Other enzymatic sources, not represented in the figure, also contribute to superoxide and/or hydrogen peroxide production. Endothelial nitric oxide synthase (eNOS) is an essential source of nitric oxide (NO); however, in the absence of other reductive cofactors, this enzyme can become uncoupled leading to the production of superoxide. Superoxide is converted to hydrogen peroxide spontaneously or by a family of superoxide dismutases (SOD), one of which is in the mitochondria, one is cytoplasmic, and one is extracellular. Superoxide and other reactive oxygen species (ROS) contribute to phospholipid oxidation (phLOOH). GPx-1 and GPx-4 are both found in the cytoplasm and mitochondria where they reduce hydrogen and phospholipid hydroperoxides, respectively, using glutathione (GSH) as a reducing cofactor. GPx-3 is an extracellular glycoprotein that may utilize GSH, thioredoxin (Trx), or glutaredoxin (Grx) as cofactors in the enzymatic reduction of hydrogen peroxide. Reduction of cellular ROS maintains bioavailable nitric oxide. NO can readily combine with superoxide to produce peroxynitrite. In addition, excess ROS can diminish eNOS activity, leading to its uncoupling and further production of superoxide

19.5 Concluding Remarks

Clinical and experimental models suggest a crucial role for redox-active selenoproteins in modulating endothelial function to preserve bioavailable nitric oxide, regulate platelet homeostasis, and lessen atherogenesis. In addition, through their regulation of cellular oxidant accumulation, these enzymes also serve to moderate damage during ischemia/reperfusion, as is found following stroke or myocardial infarction. This chapter has focused on the role of the intracellular GPxs, GPx-1 and GPx-4, and the extracellular GPx-3, that together function to maintain optimal protection against soluble and membrane hydroperoxides (Fig. 19.1). Genetic and epidemiological studies indicate that GPx-1 and GPx-3 may both modulate cardiovascular disease

risk in human subjects, suggesting the importance of understanding the mechanisms by which these selenoproteins regulate the underlying disease processes.

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Chapter 20
Glutathione Peroxidase 1 and Diabetes

Xin Gen Lei and Xiaodan Wang

Abstract Discovery of development of type 2 diabetes-like phenotypes in glutathione peroxidase-1 (GPx1) overexpressing mice reveals a novel function of this "oldest" and most abundant selenoprotein in the body. The finding signifies an exciting progress in Se biology, and helps understand metabolic impacts of Se supplementation on human health. While its dual role in coping with reactive oxygen and nitrogen species has received broad recognition, unique functions and mechanisms of GPx1 in β cell physiology, insulin synthesis and secretion, and body glucose homeostasis are just being unveiled. By modulating intracellular redox status, the GPx1 overproduction or knockout is able to regulate functional expressions of key transcriptional factors or protein in pancreatic islet and insulin-responsive tissues.

20.1 Introduction

Six forms of glutathione peroxidase (GPx) enzymes have been found in mammals. Among them, GPx1 [1] is an 84 kDa tetrameric protein that was the first identified and the most abundant Se-dependent enzyme. Mainly located in the cytoplasm, GPx1 is able to catalyze the reduction of H_2O_2 and organic hydroperoxides using GSH as the cofactor [2]. Due to this property, GPx1 has been widely considered to be among the major intracellular antioxidant enzymes in vivo. In fact, physiological importance of GPx1 activity in antioxidant defense was clarified using the GPx1

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knockout mice [3]. Knockout of GPx1 leads to increased susceptibility of liver and lung to toxicities of paraquat and diquat that induce the generation of reactive oxygen species (ROS) [4, 5]. While supplementation of high levels of dietary vitamin E in GPx1 knockout mice did not provide the same protection as in wild-type mice [6], GPx1 overproduction conferred extra protection against acute oxidative stress induced by ROS generators [7]. In contrast, knockout of GPx1 actually protected mouse primary hepatocytes against authentic peroxynitrite toxicity [8, 9] or mice against overdose of drugs such as acetaminophen that induces formation of reactive nitrogen species (RNS) [10]. Apparently, GPx1 exerts a dual role in coping with oxidative stress initiated by RNS vs. ROS [3, 11].

20.2 Association of Glutathione Peroxidase 1 with Diabetes

Although alteration of GPx1 expression is implicated in pathogeneses of several chronic diseases [12–15], its link to diabetes and the clinical significance have attracted serious attention only very recently. Diabetes mellitus is a group of metabolic diseases that will affect more than 10% of the American population in the coming decade [16]. Major types of diabetes include: type 1 diabetes (referred to as insulin-dependent diabetes, IDDM), type 2 diabetes (referred to as non-insulin-dependent diabetes, NIDDM), gestational diabetes, and maturity onset diabetes of the young (MODY). The ultimate pathogenesis of diabetes, regardless of type, is insufficient functional insulin in the circulation to maintain body glucose homeostasis, resulting from either defective insulin production or insulin insensitivity.

20.2.1 Islet Physiology and Free Radical Biology

Pancreatic islets of Langerhans constitute approximately 1–2% of the mass of the pancreas that represents a crucial endocrine structure for regulating body glucose metabolism and homeostasis. There are five main types of cells in islets that produce and secrete various hormones: α cells for glucagon, β cells for insulin and amylin, δ cells for somatostatin, PP cells for pancreatic polypeptide, and ε cells for ghrelin. As a major portion of islets, the β cells are highly efficient in glucose uptake upon exposure to rising glucose supply. Thus, extracellular hyperglycemia readily causes intracellular hyperglycemia in β cells. Consequently, auto-oxidation of glucose in these cells may elevate ROS production [17] and cause upward changes in oxidative stress markers such as 8-hydroxy-2-deoxyguanosine and 4-hydroxy-2,3-nonenal [18]. Intriguingly, pancreatic islet cells produce a relatively low amount of antioxidant enzymes including GPx1, superoxide dismutase (SOD), and catalase. Compared with the liver, islets contain only 1% of catalase, 2% of GPx1, and 29% of SOD1 activities, respectively [19–21]. Thus, β cells are considered to be susceptible to oxidative stress that can be induced by hyperglycemia. This perception was supported by the fact that β cells are a primary target of the diabetogenic agents, streptozotocin and alloxan, that generate ROS including H_2O_2 [22, 23].

20.2.2 Earlier Evidence and Perception on the Link of Antioxidants to Diabetes

The predicated susceptibility of β cells to oxidative injury has given a good reason to link antioxidants to diabetes. In addition, there are at least two more mechanisms for free radicals and antioxidants to be implicated in diabetes and insulin resistance [16, 24–26]. One is the responsiveness to ROS by key regulators of β cells and insulin, such as the transcription factors, pancreatic duodenal homebox 1 (PDX1) and forkhead box A2 (FOXA2), and mitochondrial protein uncoupling protein 2 (UCP2) (see below). The other is the oxidative modification of insulin signal proteins by ROS in insulin target tissues. As activation of serine/theronine in protein kinase B (AKT) leads to the translocation of glucose transporter 4 to the cell membrane for glucose uptake [27], impaired AKT activation is associated with insulin resistance [21]. Acting as an oxidative inhibitor of protein tyrosine phosphatase [28], ROS including H_2O_2 modulate the insulin-induced phosphorylation of the insulin receptor β-subunit [25] and AKT on Ser^{473} [29]. Because protein tyrosine phosphatases function as negative regulators of insulin signaling [28], normal physiological levels of ROS are required for sensitizing insulin signaling [30].

Nevertheless, the common perception, until very recently, was that ROS and RNS were detrimental to β cells or insulin action and that upregulating antioxidant defense in islets or whole body was beneficial to prevent and treat insulin resistance and diabetes [26]. Seemingly, there were circumstantial data from experimental and clinical studies to justify this "prevailing" notion. From the Se biology standpoint, inorganic Se was found to act as an insulin-mimic [31, 32]. Dietary Se deficiency was correlated with abnormal glucose and lipid metabolism [33], whereas decreased plasma Se concentrations or selenoperoxidase activity were detected in diabetic subjects [34]. Likewise, levels of the superoxide-scavenging enzyme extracellular-SOD were shown to be inversely related to fasting plasma glucose, insulin resistance, and incidence of diabetes [35–37]. A functional polymorphism of MnSOD was also associated with the incidence of diabetes [38]. Supplemental antioxidants delayed diabetic nephropathy [39]. A comprehensive analysis of this topic can be found in a recent review [26].

20.2.3 Recent Findings on Pro-Diabetic Roles of GPx1

It was striking for us to find that GPx1 overexpressing mice became obese at 6 months of age in the course of determining their increased resistance to various oxidant exposures [40]. Subsequent characterization indicated that these mice developed hyperglycemia, hyperinsulinemia, hyperlipidemia, and insulin resistance, along with elevated pancreatic β cell mass, islet insulin secretion, plasma leptin concentration, and hepatic lipogenesis [40–42]. In contrast, knockout of GPx1 and SOD1 alone or together resulted in decreases in pancreatic β cell mass, plasma insulin concentration, and glucose-stimulated insulin secretion [42]. But, body insulin

sensitivity was improved in these knockout models. Meanwhile, there was a strongly positive correlation between erythrocyte GPx1 activity and insulin resistance in pregnant women with gestational diabetes [43]. A β cell-specific overexpression of catalase or metallothionein in nonobese diabetic mice accelerated onset of diabetes [44]. High glucose led to increased selenoprotein P mRNA expression and protein secretion in rat hepatocytes [45]. Treating these cells with the anti-hyperglycemic drug metformin produced a dose-depended decrease in selenoprotein P mRNA and protein, suppressed glucocorticoid-stimulated production of selenoprotein P, and downregulated mRNA expression of selenophosphate synthetase 2 (an enzyme essential for selenoprotein biosynthesis). Because selenoprotein P is the major transport form of Se, diminishing Se supply to extrahepatic tissues may be one of the mechanisms for the antidiabetic action of metformin [45].

20.2.4 Clinical Relevance of the Pro-Diabetic Role of GPx1 in Human Health

The scientific significance and clinical implication of the type 2 diabetes-like phenotypes induced by GPx1 overproduction have been recognized after a post-hoc analysis of the Nutrition Prevention Cancer (NPC) trial revealed a more than two-fold increase in type 2 diabetes incidence in the Se supplemented compared to the placebo group [46]. A similar trend was also seen in the prematurely terminated Selenium and Vitamin E Cancer Prevention Trial (SELECT) [47]. Most recently, multivariate logistic regression analyses of the ORDET cohort study [48] in Northern Italy and large cross-sectional analyses within the US Third National Health and Nutritional Examination Survey (NHANES 1988–1994; 2003–2004) revealed a strong positive correlation between Se intake or serum Se concentration and the prevalence of type 2 diabetes [49, 50]. Moreover, high body Se status was associated with adverse plasma lipid profiles in adults of the USA, UK, and Taiwan [51–54], although mixed effects of Se on diabetic risk or blood glucose were shown in two French studies [55–57], two small case-control European studies [58, 59], and the US Health Professionals Follow-up study [60]. While more basic and clinical research will be needed to elucidate the full metabolic spectrum of Se in glucose homeostasis and diabetes, illustrating the pro-diabetic role of GPx1 overproduction in mice provides a plausible mechanism to explain the adverse effects of Se super-nutrition on glucose metabolism in humans.

20.3 Mechanisms of Glutathione Peroxidase 1 on Diabetes

As discussed above, ROS are able to interact with key regulators of islet β cell mass and insulin synthesis, secretion, and sensitivity. Thus, the metabolic phenotypes of the GPx1 overexpression and knockout mice were presumably mediated by the

redox regulation of those key factors, because altering GPx1 expression resulted in detectable changes in intracellular ROS status in islets and presumably other tissues as well [42, 61].

20.3.1 Regulation of Islet β Cell Mass and Insulin Synthesis

Maintaining pancreatic islet β cell mass is recognized as a pivotal prevention from pathogenesis of both types 1 and 2 diabetes [62]. Regulation of the islet β cell mass takes place at neogenesis, replication, and survival. Transcriptional factor PDX1 is the best known and probably the most important regulator for β cell differentiation and survival as well as expression of the insulin gene and many other genes related to glucose metabolism [63, 64]. Importantly, expression and function of PDX1 are affected by intracellular ROS via a posttranscriptional defect in PDX1 mRNA splicing [65], nucleo-cytoplasmic translocation of the protein [66], and phosphorylation of Ser^{61} and/or Ser^{66} on the protein [67]. In fact, GPx1 overproduction resulted in an upregulation of PDX1 mRNA and protein in islets, along with an attenuated degradation (phosphorylation) of PDX1 protein. The decrease in phosphorylated PDX1 protein in GPx1 overexpressing mice was likely due to a less oxidative environment in islets, as shown by the lower intracellular ROS levels and attenuated phosphorylation of c-jun terminal kinase (JNK) protein. The reduced phosphorylation of Thr^{308} at AKT could partially account for the decreased phosphorylation of PDX1 protein [67–69]. Consequently, an elevated functional PDX1 protein in islets led to hypertrophy of β cell mass and increased pancreatic and plasma insulin concentrations [63, 70–72]. In contrast, the reverse was induced by the GPx1 knockout [42].

Demonstrating hyperacetylation of histone 3 and 4 (H3 and H4) in the PDX1 gene promoter of the GPx1 overexpressing mice [61] unveiled a novel epigenetic regulation of this key transcriptional factor in vivo. Hyperacetylation of H3 and H4 has been suggested to precede transcriptional activation [73, 74], which may help explain the increased islet PDX1 mRNA levels in the GPx1 overexpressing mice. Seemingly, the overproduced GPx1 activity was able to remodel chromatin at the PDX1 promoter to form a more accessible structure for transcription [75]. This remodeling was likely mediated by modulating intracellular ROS status, because the genotype difference in H3 and H4 acetylation was correlated well with that of intracellular ROS levels [61]. Moreover, GPx1 overproduction protected the PDX1 promoter from the H_2O_2-induced H3 and H4 deacetylation [61].

Another important transcriptional factor for β cell differentiation and survival is NeuroD/Beta2 that was also upregulated by the GPx1 overproduction [41]. However, effects of GPx1 overproduction on islet FOX2 mRNA levels were not statistically significant [41]. In vivo, FOXA2 binds the PDX1 gene promoter/enhancer to activate the gene transcription [76]. It is very intriguing that while both GPx1 and SOD1 knockouts decreased pancreatic PDX1 protein levels, only the SOD1 knockout decreased islet FOXA2 mRNA and protein levels and the binding of FOXA2 protein to the PDX1 promoter [42]. Apparently, the regulation of FOXA2 was more

superoxide-dependent [26]. Unlike the GPx1 overproduction [61] or the SOD1 knockout, the GPx1 knockout did not affect islet PDX1 mRNA and H3 and H4 acetylation [42]. Possibly, the extremely low baseline of GPx1 activity in pancreatic islets precluded a detectable response to the gene knockout.

An increased activation of p53 protein (phosphorylation on Ser^{15}) in islets of the GPx1 knockout mice, similar to that by the SOD1 knockout, might also contribute to their decreased islet β cell mass [42]. In diabetic subjects, the β cell apoptosis seems to be a more deciding factor than replication compared with control subjects [77]. This event can be triggered by high glucose [78] and cytokines that induce ROS and RNS formation [79]. However, it is hard to explain why double knockout of GPx1 and SOD1 did not elevate islet p53 activation [42] and why overproduction of GPx1 actually upregulated islet p53 mRNA [41]. It is also fascinating to notice that the hypertrophy of islet β cell mass and upregulation of insulin production seems to be a unique feature of GPx1 overproduction, because insulin content or insulin gene expression in islets was not altered by overexpressing catalase up to 50-fold [44, 80], two forms of metallothionein up to 30-fold [44, 81], or three forms of SOD enzymes up to tenfold [82, 83].

20.3.2 Regulation of Islet Insulin Secretion

Mitochondrial membrane potential is considered to be a driving force for insulin secretion by β cells [84]. As the only uncoupling protein present in rodent and human β cells, UCP2 negatively regulates mitochondrial membrane potential and inhibits glucose stimulated insulin secretion (GSIS) [85, 86]. Therefore, the accelerated GSIS and hyperinsulinemia in the GPx1 overexpressing mice can be well explained by the downregulated islet UCP2 protein and elevated mitochondrial member potential [61]. Treating islets of wild-type mice with the GPx1 mimic ebselen duplicated suppression of UCP2 protein by GPx1 overproduction. Meanwhile, knockout of GPx1 alone or together with SOD1 upregulated UCP2 protein in pancreas and decreased islet ATP content [42]. Both changes could contribute to the attenuated GSIS in these mice.

20.3.3 Regulation of Insulin Signaling in Insulin Target Tissues

The fact that H_2O_2 serves as a major substrate of GPx1 allows the enzyme to affect insulin sensitivity at multiple sites because H_2O_2 may activate or prolong phosphorylation of key proteins in the insulin signaling [87–89]. By an oxidative inhibition of protein tyrosine phosphatase 1b, H_2O_2 may exert a pro-insulin or insulin-mimic action on phosphorylation of the β subunit of the insulin receptor in rat adipocytes [90]. In general, body insulin sensitivity is largely controlled by the balance between activities of protein kinases (phosphorylation) and protein phosphatases (de-phosphorylation). In the GPx1 overexpressing mice, insulin resistance was associated

with an attenuated phosphorylation of insulin receptor (β subunit) and AKT (Ser^{473} and Thr^{308}) after insulin stimulation in liver and muscle [40]. These decreased phosphorylations were presumably caused by the diminished intracellular ROS that lifted the oxidative inhibition of protein tyrosine phosphatases. In contrast, knockout of GPx1 resulted in enhanced phosphorylation of AKT in muscle [42]. Most interesting, knockout of GPx1 rendered mice resistant to a high-fat diet induced insulin resistance via an increased oxidation of the protein tyrosine phosphatase family member phosphatidylinositol 3-kinase phosphatase with tensin homology in muscle that terminates signals generated by phosphatidylinositol-3-kinase [91]. Reciprocally, the improvement was reversed by supplementing the antioxidant, *N*-acetylcysteine.

While importance of basal levels of ROS in insulin signaling is well illustrated in the above-discussed GPx1 overexpressing and knockout mice, outcomes may be totally different after prolonged exposure to high levels of ROS in diabetic subjects [92]. Another pathway that might also contribute to insulin resistance in the GPx1 overexpressing mice is their elevated body fat deposit. Limited experimental evidence has led to a postulation that high Se supply or high GPx1 activity may affect body lipogenesis via regulation of protein tyrosine phosphatase 1b [93].

20.4 GPx1 and Diabetic Complications

It is well accepted that oxidative stress is implicated in various diabetic complications: neuropathy [94–97], nephropathy [98–100], retinopathy [101], and vasculature and heart disease [102]. In screening 184 Japanese type 2 diabetic patients, variants in GPx1 gene Pro198Leu were found to be associated with increased intima-media thickness of carotid arteries and risk of cardiovascular and peripheral vascular diseases [15]. Supplemental antioxidants were beneficial to prevent or reverse diabetic complications [101]. A novel synthetic antioxidant with GPx-like activity reduced diabetes-associated-atherosclerosis in diabetic ApoE knockout mice [103].

20.5 Conclusions and Perspectives

Linking GPx1 overexpression to type 2 diabetes-like phenotypes reveals a novel role of GPx1 and creates a new field of Se biology, although the full role and the underlying mechanism are far from clear. With the “prevailing” perception of low antioxidant capacity in islet β cells and involvement of oxidative stress in pathogeneses of diabetes, both research and clinical scientists have unquestionably viewed upregulating islet or global antioxidant defense as an effective strategy to prevent and treat diabetes. In fact, many past studies have overly amplified transient benefits of antioxidant treatments against a bolus of ROS, but neglected long-term metabolic consequences of shifting cellular redox status. Demonstrating the type 2 diabetes-like phenotype in the GPx1 overexpressing mice provides a more realistic and

balanced concept of antioxidant enzymes in diabetes. Elucidating the effects of GPx1 overproduction on expression and(or) functions of PDX1, UCP2, p53 and protein tyrosine phosphatases unveils new in vivo regulation of pancreatic β cell mass and insulin physiology. These findings will help study etiology and potential risk associated with the pro-diabetic effects of Se supplements shown in recent human studies.

Overall, this chapter outlines the physiological importance and molecular mechanism for a dual role of the most abundant selenoprotein, GPx1, in diabetes. Clearly, maintaining the physiological level of ROS and a proper balance with GPx1 is essential to avoid dysregulation of islet integrity, insulin function, and glucose homeostasis. However, the desirable balance between ROS and antioxidant defense including GPx1 could differ greatly with diabetic status or at late stage of complications when target tissues or functions are exposed to high levels of ROS for an extended period.

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Chapter 21
Glutathione Peroxidase 2 and Its Role in Cancer

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Abstract Glutathione peroxidase 2 (GPx2) is preferentially expressed in the intestine, but also upregulated in malignant epithelial cells of other organs. Whether this upregulation is beneficial or detrimental for cancer cell growth and thus for the outcome of cancer, is unclear. The localization of GPx2 in the crypt bases of the intestine, where stem cells proliferate under the control of the Wnt pathway, points to a role in the self-renewal of the intestinal mucosa. This assumption is supported by the fact that GPx2 is a target of the Wnt pathway. In GPx2 knockout mice, apoptosis is highly increased in crypt bases corroborating an involvement of GPx2 in mucosal homeostasis. So far, the role of GPx2 appears to be pro-carcinogenic either by supporting cancer cells to escape apoptosis or by directly maintaining proliferation. On the other hand, GPx2 is induced by Nrf2 transcription factor which is generally accepted to induce endogenous defense systems. In addition, GPx2 counteracts COX-2 expression, thereby decreasing inflammation and migration of tumor cells. Collectively, the role of GPx2 may depend on the stage of cancer. GPx2 likely inhibits the initiation of cancer triggered either by oxidative damage or chronic inflammation and might prevent invasiveness and metastasis, but supports progression of established tumors. Evidences for this dual role of GPx2 are presented and discussed.

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21.1 Introduction

GPx2 was first identified as glutathione peroxidase (GPx) specifically expressed in the gastrointestinal tract of humans and rodents and, therefore, named gastrointestinal GPx, or GPx-GI [1]. Due to this specific localization, the physiological function of GPx2 was proposed to act as a barrier against the absorption of food-borne or bacteria-induced hydroperoxides [1, 2], a function which was confirmed in a CaCo-2 cell culture model [3]. The proposed barrier function most likely is not restricted to the intestine, but also was postulated for lung epithelial cells, where GPx2 is localized in basal cells responsible for epithelial regeneration [4].

Later, GPx2 was found to be upregulated in a number of epithelium-derived tumors. These include colon adenocarcinoma [5–8], Barrett's esophagus [9], squamous cell carcinoma [10], or lung adenocarcinomas of smokers [11]. In the intestine, the increase is transient and especially prominent at early stages of carcinogenesis [6, 12]. An upregulation of GPx2 has also been observed in animals treated with chemical carcinogens: N-diethylnitrosamine-induced hepatocarcinogenesis in rats with an increased GPx2 expression in hepatocellular adenomas [13]. In DMH-induced colon tumors, GPx2 expression was increased [14] as well as in rat mammary carcinomas induced by three different carcinogens [15]. GPx2 knockout mice were predisposed to UV-induced squamous cell carcinoma formation [16]. GPx1 and GPx2 double knockout mice developed spontaneous colitis and intestinal cancer [17].

GPx2 is also upregulated in various types of inflammation like ulcerative colitis [12], and inflamed lung in response to cigarette smoke [18, 19] or to acute allergen exposure [4]. Since chronic inflammation is known to facilitate cancer formation [20, 21], the consistent upregulation of GPx2 in inflamed tissue and epithelial cancer cells raises the question in respect to mechanisms and consequences. We will discuss these aspects and try to answer the questions: (1) is the upregulation of GPx2 in cancer cells beneficial or detrimental for the host; and (2) does GPx2 have a dual role in cancer, as described for other selenoproteins, such as thioredoxin reductase or selenoprotein 15 (see Chap. 25). For similarities and differences of GPx2 to other glutathione peroxidases see Chap. 13.

21.2 Lessons from Localization

A more detailed look onto the intestinal GPx2 expression revealed a specific localization in epithelial cells [2] at crypt bases and in Paneth cells [6] (Fig. 21.1). The crypt base is the area where stem cells proliferate to maintain the renewal of the intestinal epithelium [22]. From there cells migrate to the villi in the small intestine or to the top of the crypt in the colon, respectively. During migration they differentiate into absorptive, enteroendocrine or goblet cells. On the top of the villi, cells undergo apoptosis and are disposed into the lumen. Together with many other pathways this process is regulated by the Wnt pathway, which is mainly active in crypt bases. Since GPx2 is also a target for the Wnt pathway (see Sect. 21.5.1), a function of

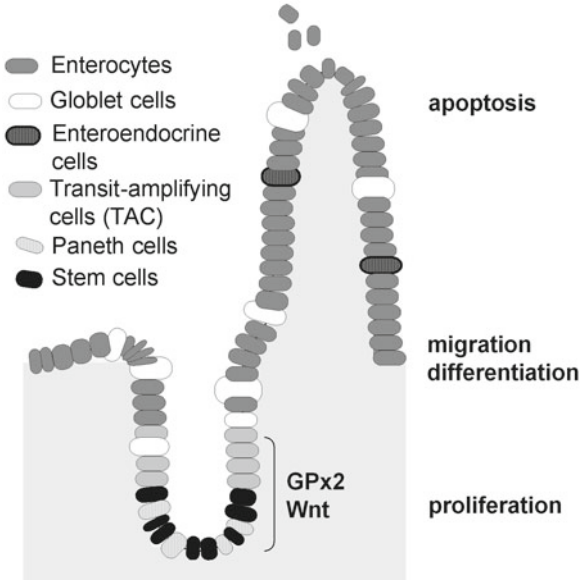

Fig. 21.1 Intestinal morphology and functional areas. The surface of the small intestine is organized into crypts and villi, whereas the colon has only crypts which are organized into crypt bases and tops. The surface is covered with a single layer of epithelial cells consisting of enterocytes (absorptive cells), goblet cells, and enteroendocrine cells. In the small intestine, Paneth cells localize at crypt bases. Since the epithelial layer is renewed every 4–5 days, there is a continuous proliferation of stem cells at crypt bases, migration and differentiation of transit amplifying into the specialized intestinal cells in the middle, and a disposal into the lumen by apoptosis at the top. Paneth cells migrate to the crypt bases. Proliferation and differentiation is controlled by a gradient of Wnt signals, which are high at the base and decrease to the top. A similar gradient was found for GPx2 expression, which fits with the observed effects of GPx2: support of proliferation and inhibition of migration and apoptosis

GPx2 in the regulation of stem cell proliferation might be envisaged. The intriguingly high concentration of GPx2 in Paneth cells, which are involved in mucosal immunity and not in absorption, also points to highly specialized functions that still remain to be elucidated.

21.3 Lessons from the Ranking in the Hierarchy of Selenoproteins

Under conditions of a limited selenium supply, selenoproteins are synthesized according to a hierarchy which mainly depends on the stability of the respective selenoprotein mRNA (see Chap. 11). In selenium-deficiency, the mRNAs of those selenoproteins ranking low in the hierarchy (e.g., GPx1) are rapidly degraded, while

those of others are much more stable. GPx2 has a highly stable mRNA and, hence, can still be detected under selenium-limiting conditions [23] and is preferentially re-expressed upon repletion of selenium after a deprivation period [24–26]. In some cells, including such as HepG2 [23] and those in the duodenum of mice [27], GPx2 mRNA is even increased under selenium-limiting conditions. The latter finding could be explained by its regulation via Nrf2 (see Sect. 21.5.2). An upregulation of Nrf2-dependent phase II enzymes in selenium-deficiency has been observed since the late 1970s [28]. A firm link between selenium-deficiency and Nrf2 activation was finally established by demonstrating a strong increase in Nrf2-driven reporter gene activity in livers of selenium-deficient wild type but not in $Nrf2^{-/-}$ mice [29]. The high stability of and increase in GPx2 RNA under selenium-restriction would enable the immediate translation of GPx2 upon selenium refeeding [24]. All these observations show that GPx2 ranks high in the hierarchy of selenoproteins. The function of high ranking selenoproteins has generally been considered to be more essential than those of selenoproteins ranking low. Having this in mind a knockout of GPx2 should have severe consequences, which obviously is not the case (see Sect. 21.4), at least under unchallenged conditions.

21.4 Lessons from Downregulation and Deletion

21.4.1 Downregulation in Cultured Cells

Searching for functions of GPx2 independent of the other selenoproteins/glutathione peroxidases requires an experimental setup where GPx2 expression can be targeted specifically. This way, the function of other selenoproteins can be eliminated by performing experiments in the absence and presence of selenium. In a selenium-supplemented status, cells in which GPx2 is downregulated are supposed to express all other selenoproteins as do wild type cells with the only difference being the loss of GPx2. To date, a reasonable number of GPx2 knockdown experiments by means of siRNA have been performed [12, 15, 30, 31]. In a stable knockdown of GPx2 in HT-29 colon cancer cells any off-target effects due to the siRNAs or to the transfection process as such were excluded by using two different siRNA oligonucleotides and several independent cell clones chosen for analysis [12]. All GPx2 knockdown clones exhibited a clearly enhanced expression of the inducible cyclooxygenase-2 (COX-2) and of the microsomal prostaglandin E synthase (mPGES-1). This was accompanied by an increased production of the pro-inflammatory prostaglandin E_2 (PGE_2). The opposite, siRNA-mediated knockdown of COX-2 did not result in any changes of GPx2 expression levels [12]. To understand the putative mechanism of the increased COX-2 expression upon GPx2 knockdown, the regulation of COX-2 needs to be considered. Cyclooxygenases as well as lipoxygenases require a certain cellular hydroperoxide tone to be active [32], and glutathione peroxidases, mainly GPx4, were shown to inhibit the activity of both cyclooxygenases [33] and lipoxygenases [34] (see Chaps. 14 and 43). The expression of COX-2 is induced by

pro-inflammatory cytokines, such as IL-1β [35], but also by its own product PGE_2 via an autocrine positive feedback loop [33]. Collectively, the above findings demonstrate that GPx2 may act anti-inflammatory by counteracting COX-2 expression.

The same cell clones were tested for their behavior in cancer-relevant processes such as migration, invasion, and anchorage-independent growth [31]. GPx2 knockdown cells exhibited an increased invasive potential and also migrated faster than cells with normal GPx2 expression in a wound healing assay. Both effects could be adjusted to the level of control cells by inhibition of COX-2 with celecoxib, indicating that the effects were mainly due to increased COX-2 expression and activity in the GPx2 knockdown cells. None of the effects were modulated by cellular selenium status, thus, solely were caused by the absence of GPx2.

Surprisingly, however, cells lacking GPx2 failed to grow in an anchorage-independent manner in a soft agar assay and formed smaller tumors when injected into nude mice [31].

GPx2 knockdown in rat and human breast cancer cells led to a drastic reduction of cell proliferation in cells with an intact p53 but not in cells with a mutated one [15]. In GPx2-overexpressing MCF7 cells, apoptosis was inhibited, whereas a GPx2 knockdown in the same cells enhanced oxidant-induced apoptosis [36]. Inhibition of apoptosis was only observed when it was induced in dependence of p53. It was concluded that GPx2 inhibited the activation of p53 by prevention of an oxidative event required for the activation of p53 [36]. Both observations show that GPx2 may inhibit p53 activation. Whether this is a major function remains to be investigated. However, it is interesting in this context that GPx1 is induced by p53 [37, 38] and upregulated in GPx2 knockout mice (see Sect. 21.4.2).

Taken together, GPx2 by inhibition of COX-2 expression might inhibit inflammation and this way triggered carcinogenesis. It further may inhibit metastasis in accordance with its putative physiological function: inhibiting of migration and differentiation of stem cells in the intestine. GPx2, however, can also support cancer cell growth in accordance with its assumed role in the self-renewal of intestinal mucosa by inhibiting apoptosis.

21.4.2 Deletion in Mice

GPx2 knockout mice have no obvious characteristic phenotype [39]. As GPx2 is mainly localized in the gastrointestinal epithelium, intestine specific effects can be anticipated. Indeed, GPx2 knockout mice display an increased rate of spontaneous apoptosis at crypt bases [40]. This effect was highest in mice with restricted selenium supply. At the same time the proliferative zone was expanded as observed by counting mitotic figures, which obviously is an attempt to compensate for the cell loss due to apoptosis. Selenium supplementation partially prevented apoptosis indicating that another selenoprotein could compensate, at least in part, for the loss of GPx2. This protein might be GPx1, since it was upregulated in crypt bases of GPx2 knockout mice, where otherwise GPx2 is expressed. GPx1 upregulation persisted

even under selenium-limiting conditions, while GPx1 was completely absent in crypt bases of wild-type mice [40]. Thus, GPx1 cannot completely compensate the loss of GPx2, which points to a unique role of GPx2 in the intestinal epithelium.

Due to the lack of an obvious phenotype of a single knockout of either GPx1 or GPx2 double knockout mice were generated [17]. As mentioned, these mice spontaneously develop colitis and intestinal cancer. Colitis development depended on the gastrointestinal microbiota or other stress factors, while unstressed germ-free animals had no symptoms [17]. The presence of one GPx2 allele was sufficient to prevent pathological symptoms, while one GPx1 allele was not [41]. This again shows that GPx2 plays a major role in the prevention of inflammation. The findings also explain why both enzymes have to be deleted before symptoms become manifested. Accordingly, GPx1/2 double knockout mice are now used as a model of spontaneous inflammatory bowel disease predisposing to intestinal cancer [42].

21.5 Lessons from Transcriptional Regulation

Apart from their dependence on selenium supply for translation, selenoproteins were recognized to also undergo transcriptional regulation. Two transcription factors involved in the regulation of GPx2 expression have been studied more extensively and, therefore, will be discussed in more detail below.

21.5.1 Regulation by the Wnt Pathway

The Wnt pathway is essential for embryonic development and tissue homeostasis, especially in the intestine. Constitutive activation results in tumor formation (for review see [43]). In the absence of a Wnt signal, β-catenin is constantly degraded via the ubiquitin/proteasome system. A prerequisite for ubiquitination is the phosphorylation of β-catenin by CK1α (casein kinase 1α) and GSK3β (glycogen synthase kinase 3β), which are all tethered together by the scaffold proteins axin and APC, thus forming the destruction complex. Binding of Wnt proteins to extracellular receptors results in the recruitment of Dvl (disheveled) and axin to the plasma membrane resulting in a destabilization of the destruction complex. Free β-catenin translocates to the nucleus and induces target gene expression together with transcription factors of the TCF/LEF family [43]. Evidence for a link between GPx2 expression and the Wnt pathway came from a microarray analysis which was conducted in a colorectal cancer cell line with an inducibly blocked Wnt pathway [44]. GPx2 together with known Wnt target genes were strongly downregulated upon inhibition of the pathway. The GPx2 promoter indeed contains a functional TCF responsive element [45]. The promoter was highly active in cells with a constitutively active Wnt pathway and could be further stimulated by transfection with β-catenin/TCF.

Mutation of the TCF site reduced the response to β-catenin/TCF by more than 50% [45]. Thus, GPx2 is the first selenoprotein identified as a Wnt target.

The Wnt pathway mainly activates genes required for the stimulation and maintenance of proliferation. If constitutively active in cancer cells, the Wnt pathway supports tumor progression. Wnt signals and GPx2 levels decrease in areas where intestinal cells migrate and differentiate. Thus, GPx2 might rather support proliferation and prevent differentiation, functions which cannot be considered particularly anticarcinogenic.

21.5.2 Regulation by Nrf2

Nrf2 is the basic leucine zipper transcription factor NF-E2-related factor 2 that regulates the basal and inducible expression of many detoxifying and antioxidant enzymes (for review see [46]). Nrf2 binds to a consensus sequence referred to as "antioxidant response element, ARE" [47–49] or "electrophile response element, EpRE" [50], the latter being the more appropriate designation. Under normal conditions, Nrf2 is retained in the cytosol by its inhibitor Keap1 (for review see [51]). Nrf2 is activated by various different substances (e.g., isothiocyanates, Michael reaction acceptors, etc.) whose common denominator is their ability to modify sulfhydryl groups of Keap1, leading to a conformational change and release of Nrf2 [52]. In a list of genes upregulated by sulforaphane in Nrf2 wild type, but not in $Nrf2^{-/-}$ mice, GPx2 showed up [53]. Therefore, a comprehensive analysis of the human GPx2 promoter was performed and two Nrf2 binding sites were found from which one was functional [54]. The functional site was also conserved in mice and rats.

The promoter of GPx2 responded to Keap1 and Nrf2 in a dose-dependent manner. Natural Nrf2 activators such as the isothiocyanate sulforaphane strongly activated the GPx2 promoter, enhanced the binding of Nrf2 to the GPx2-ARE, and increased the expression of GPx2 at the level of mRNA and protein [54]. Also polyphenols (e.g., quercetin) and polyphenol-rich plant extracts (e.g., thyme extract) activated Nrf2 and increased the activity of the GPx2 promoter [55]. Breakdown products of the glucosinolate neoglucobrassicin potently inhibited the sulforaphane induced activation of the GPx2 promoter. Inhibition was dependent on an intact xenobiotic responsive element (XRE) in the GPx2 promoter and, hence, appears to involve the aryl hydrocarbon receptor AhR [56]. Mutation of the ARE within the GPx2 promoter not only abolished the responsiveness of the promoter towards Nrf2, but also lowered the basal promoter activity [54].

An upregulation of GPx2 mRNA in response to Nrf2-activating compounds has been described in various animal experiments, often as a result of global gene expression analysis by means of microarrays. For example GPx2 was induced in the colon of rats upon feeding a polyphenol-rich apple juice [57] or quercetin [58]. The cholesterol-lowering drug simvastatin activated Nrf2 and expression of GPx2 mRNA in liver [59]. Also the inhalation of nanoparticles that cause airway-inflammation

increased GPx2 expression in an Nrf2-dependent manner [60]. GPx2 expression is also increased in the lung of wild type but not in Nrf2-/- mice in response to cigarette smoke [18, 19], to hyperoxia [61, 62], or bleomycin-induced pulmonary fibrosis [63].

So far, upregulation of GPx2 can be considered beneficial due to the antioxidant function attributed to GPx2 and the mainly positive effects observed upon feeding the natural Nrf2 activators mentioned above. However, consequences of an upregulation of GPx2 may also be detrimental. The benzimidazole oxfendazole increased expression of Nrf2 target genes including GPx2 in diethylnitrosamine-induced preneoplastic foci in a model of hepatocarcinogenesis [64, 65]. However, oxfendazole also increased the number of preneoplastic foci. The upregulation of GPx2 and other Nrf2 targets was, therefore, interpreted as response to the oxfendazole-induced oxidative stress resulting in an advantage for cancer cells to survive. This way GPx2 would protect cancer cells from oxidative damage and support tumor cell growth [65].

21.5.3 Regulation by Other Transcription Factors

The GPx2 promoter contains three putative retinoic acid responsive elements (RARE), accordingly GPx2 mRNA was induced by all-trans retinoic acid in some cell lines (MCF7) but not in others (HT-29) [66]. The retinoic acid-mediated induction of GPx2 was also observed in hepatoma cells [30]. However, whether the putative RAREs are responsible for the retinoic acid-induced expression has not been investigated.

GPx2 expression was downregulated in the prostate epithelium in mice, in which Nkx3.1 had been knocked out [67]. Nkx3.1 is a homeobox gene required for differentiation of prostatic epithelial cells and suppression of prostatic cancer, and Nkx3.1 knockout mice develop prostatic intraepithelial neoplasia and later metastatic adenocarcinoma. Both, the human as well as the murine GPx2 promoter, contain three putative Nkx3.1 binding sites (own observation), however, whether GPx2 is a direct target of Nkx3.1, has not been investigated. These observations point to a rather anti-carcinogenic function of GPx2 at the initial stage (Fig. 21.2).

ΔNp63 belongs to the p53 family and is highly expressed in undifferentiated basal epithelial cells [68] and also in tumor cells (for review see [69]& Nμ63j induced GPx2 expression and activated its promoter in MCF7 cells, while p53 had no effect [36]. The human GPx2 promoter contains a functional binding site for ΔNp63γ which turned out to be responsible for this effect [36]. In addition to its direct activation of the GPx2 promoter, ΔNp63γ might also have an indirect effect because it is an activator of β-catenin and stimulates β-catenin nuclear accumulation and signaling [70] (see Sect. 21.5.1). As a target of ΔNp63γ, GPx2 may serve as mediator of ΔNp63γ in maintaining stem cell proliferation supporting the idea that GPx2 is induced during self-renewal of intestinal mucosa. These observations rather point to a pro-carcinogenic function of GPx2.

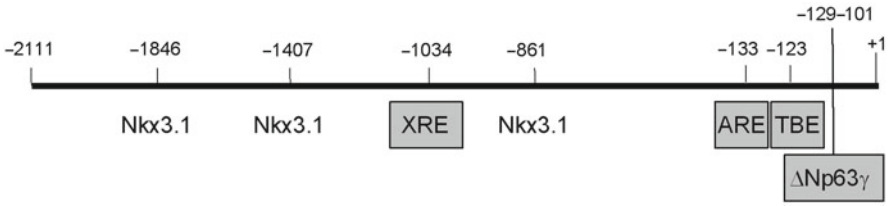

Fig. 21.2 Transcription factor binding sites in the human GPx2 promoter. The position of the ARE and TBE (β-catenin responsive element, TCF/LEF binding element) was taken from [54] and [45], respectively. The area of a novel ΔNp63γ binding site was identified in [36]. Position of Nkx3.1 was found in the MatInspector program also used for ARE and TBE. *Numbers* indicate the 3′-position of the consensus sequence upstream of the ATG start codon. Confirmed elements are *highlighted*

Furthermore, the expression of GPx2 was found to be inversely related with viral replication, i.e., hepatoma cells infected with hepatitis C virus subgenomic RNA showed a drastically reduced GPx2 expression, while a forced overexpression of GPx2 lowered the amount of virus RNA and protein [30].

21.6 The Role of GPx2 in Cancer

The fact of being an Nrf2 target gene and, hence, part of the adaptive response machinery would point towards a protective role of GPx2, mainly at the initiation stage of cancer development or during a preceding inflammation. Chronic inflammation is known to facilitate cancer formation [21], and due to its ability to counteract the pro-inflammatory COX-2 and, hence, to inhibit the COX-2-mediated PGE_2 production in a cell culture model [12], GPx2 might act with priority at the level of inflammation. A characteristic feature of cancer cells is their increased oxidative status [71, 72]. Hence, it is tempting to speculate that GPx2 also in vivo tries to counteract the expression and activity of COX-2. In ulcerative colitis, GPx2 was overlapping with COX-2 [12] which is increased in 85% of human colon cancer specimens [73] and is consistently associated with inflammation [74]. In inflamed tissue, GPx2 may aim at eliminating the excess of locally produced hydroperoxides. A shift in the cellular redox state towards oxidation also triggers the activation of the Nrf2 signaling cascade. There is growing evidence on the positive role of Nrf2 in cancer prevention. Most Nrf2 target genes are either classical phase II enzymes (e.g., glutathione S-transferases) or have antioxidant (e.g., γ-glutamyl cysteine synthetase) or detoxifying functions (e.g., heme oxygenase-1, NQO1). The naturally occurring Nrf2 activating compounds, such as sulforaphane or curcumin are considered to have chemopreventive properties [52], and Nrf2 knockout mice show a higher incidence of chemically induced tumors [52, 75–77] and of DSS-induced colitis [78]. Upon exposure to cigarette smoke Nrf2-/- mice exhibit signs of increased

oxidative stress [19] and pulmonary emphysema [18]. Moreover, the consumption of brassica vegetables rich in SFN and other isothiocyantes is associated with a reduced cancer risk [79]. Additional facts supporting an anti-inflammatory and anti-carcinogenic function of GPx2 are provided by knockout mouse models (see Sect. 21.4).

However, the one-sided conclusion of GPx2 being chemoprotective and anticarcinogenic might be too premature as becomes clear from reduced cell proliferation [15] and reduced tumor growth properties in soft agar and nude mice [31] of GPx2 knockdown cells. Also, being a target gene of the Wnt pathway points into a tumor promoting direction, as most of the Wnt target genes are involved in the promotion of cellular proliferation.

Most likely, the role of GPx2 depends on the stage of tumor development and may be a protective one in healthy cells and in the early phase of cancer initiation, but a promoting one at later time points when cells have already reached a transformed stage and a tumor has developed. This view of GPx2 being a survival factor for cancer cells is supported by the anti-apoptotic activity of GPx2 [36, 40] that provides cancer cells with a growth advantage. Even Nrf2, which has always been considered to solely be cytoprotective and tumor preventive, recently was described to also have a "dark side" at later stages of carcinogenesis, because some of its downstream target genes may provide cancer cells with a growth advantage, protection from apoptosis, and resistance to chemotherapy (for review see [80]). A benefit of GPx2 expression, thus, might depend on the cancer stage [81].

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Chapter 22
Selenoprotein N: Its Role in Disease

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Abstract Selenoprotein N is among the newly identified selenoproteins, initially discovered in silico with no known molecular function. It has become the focus of attention because mutations in the selenoprotein N gene are linked to a group of muscle disorders, now referred as *SEPN1*-related myopathies. An emerging view arising from recent findings is that the loss of selenoprotein N leads to cellular sensitivity to oxidative stress and loss of calcium homeostasis. Studies of animal models for *SEPN1*-Related Myopathies revealed the fate of sensitized muscle may depend on stresses to which it is subjected, and defects in the function of selenoprotein N-deficient muscle progenitor cells during development in zebrafish embryos or during muscle regeneration in fully developed mouse muscle. Dysfunction of these different processes raises significant questions regarding which of the phenotypic manifestations of *SEPN1*-Related Myopathies are initiated by events during development and which are progressive in nature arising from dysfunction of mature muscle.

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22.1 Introduction

Of critical importance to the field of selenium (Se) biology is our understanding of the role that this trace element plays in normal physiological processes and the dysfunctions associated with its deficiency. Se is incorporated co-translationally into selenoproteins in the form of the amino acid selenocysteine. Selenoproteins are thought to be responsible for the beneficial properties of this trace element, although other less characterized low-molecular-weight compounds may have additional roles [1]. The presence of a selenocysteine residue confers increased reactivity due to the low pK_a and unique nucleophilic properties of the selenol group [2, 3]. In the case of selenoenzymes, selenocysteine is found in the catalytic center of the protein and facilitates the catalysis of reductive/oxidative (redox) reactions.

Genomic analysis has succeeded in identifying the full selenoprotein gene repertoire in humans and other organisms [4, 5]. Several novel proteins of unknown function were described including selenoprotein N (SelN) [6], encoded by the *SEPN1* gene. Subsequently, SelN was found to be the first selenium-containing protein directly involved in inherited human disease [7]: loss-of-function mutations in the *SEPN1* gene cause a group of muscle disorders now referred as *SEPN1*-Related Myopathies (*SEPN1*-RM) (reviewed in [8]). Discovery of a myopathic phenotype arising due to mutations in the human *SEPN1* gene established SelN as a potential protein mediator of selenium deficiency-induced muscle syndromes, which have been described in both domesticated livestock and humans (reviewed in [9]). Although several observations suggested a link between selenoprotein W and one of these "nutritional muscular dystrophies," termed white muscle disease [10], the underlying molecular mechanisms remain elusive and may involve altered expression or function of more than one selenoprotein. An understanding of the role selenium plays in normal muscle development, maintenance, and function requires a detailed analysis of the molecular pathways affected by the selenoproteins involved in these processes.

Since its discovery over a decade ago [6], significant advances have been made in our comprehension of the key physiological roles of SelN in muscle. In this chapter, we review recent findings that demonstrate SelN is required for normal muscle development and regeneration; discuss our current understanding of its function and the molecular pathways affected by SelN deficiency; and highlight critical questions remaining to be answered.

22.2 *SEPN1*-Related Myopathies: Clinical Presentation

Mutations in the *SEPN1* gene have been identified as the genetic cause underlying four early-onset, autosomal recessive neuromuscular disorders: congenital Rigid Spine Muscular Dystrophy (RSMD1) [7], Multiminicore Disease (MmD) [11], Desmin-Related Myopathy with Mallory Body-like inclusions (MB-DRM) [12], and congenital fiber-type disproportion myopathy (CFTD) [13]. All of these clinical

phenotypes are now collectively termed *SEPN1*-RM. Affected individuals are characterized by generalized muscle atrophy and predominant weakness of axial muscles, as well as severe contractures of the neck and spine extensor muscles, due to their progressive replacement by connective tissue. One striking feature of these diseases is the predominant effect on muscles with constant tonic activity, such as the postural and respiratory muscles. Disease progression leads to severe scoliosis, spinal rigidity, and life-threatening respiratory insufficiency.

As our understanding of the range of tissue abnormalities and cellular phenotypes associated with *SEPN1*-RM broadened, the similarities between the pathophysiology of disease states associated with mutations in *SEPN1* or in the gene encoding the ryanodine receptor calcium release channel (*RYR1*) became clearer [14, 15]. Many of the histological muscle defects associated with loss of *SEPN1* are also exhibited by a sub-group of patients with *RYR1* mutations, notably sarcomere disorganization (minicores), mitochondrial depletions, and type I fiber abnormalities [16, 17]. In addition some of these histological features are observed in muscle disorders associated with mutations in two others genes, those encoding desmin *DES* (MB-DRM) [12], and alpha-actin *ACTA1* (CFTD) [13]. Thus it is possible that each of these genes contributes to a common process in the muscle cell. Of relevance, observations in patients [18, 19] and recent characterizations of mice heterozygous for a dominant disease-associated allele of *Ryr1* [20] indicate that the histological presentation of mutant muscle evolves with time/age. Thus snapshot characterizations of human biopsies acquired from different muscle types or stages of the disease may have initially provided a limited view of the continuum of disease phenotypes associated with mutations in the *SEPN1* and other phenotypically related genes.

Although the clinical symptoms of *SEPN1*-RM in patients are highly recognizable, patients exhibit variable degrees of severity and a large spectrum of histopathological presentations ([21–23] and reviewed in [8]). This observation, together with the overlap between different genetic conditions for similar muscular disorders suggest a complex pathophysiological mechanism and the possible contribution of other genetic variants and/or non-genetic factors to the progression of the disorder.

22.3 Unraveling SelN Function: Expression

Given the heterogeneity in the histological presentation of *SEPN1*-RM and the inability to study the earliest stages of disease onset in humans, it has been difficult to derive the primary biochemical and cellular functions of SelN from the clinical manifestations of the disease. Initial in vitro studies using cultured human cells revealed SelN to be a transmembrane protein specifically associated with the endoplasmic reticulum, including its perinuclear compartment [24]. SelN was found to be expressed widely, if not ubiquitously, in human fetal and adult tissues, although it appeared less abundant in the adult tissues. As expression was down-regulated upon differentiation of proliferating human myoblasts into myotubes, Petit et al.

[24] hypothesized that SelN had a primary function in cell proliferation and/or tissue development.

Analysis of *Sepn1* gene expression in zebrafish and mice during embryogenesis revealed a pattern of utilization that was consistent with, but not uniquely connected to, muscle cell development or function [15, 25, 26]. *Sepn1* mRNA is highly expressed in the notochord and nascent somites of the zebrafish embryo; it is restricted specifically to the myotomal compartment within the developing somites of the mouse. Analogous to the in vitro studies described above, *SEPN1* expression appears significantly down-regulated upon differentiation of muscle tissue in vivo. However, in both vertebrate embryos *Sepn1* is expressed in many tissue precursor populations: throughout the undifferentiated mesoderm in the zebrafish and in many tissues of the mouse, including the neural tube and neural crest derivatives. Therefore, it may be that SelN function is not restricted to muscle formation during early development.

22.4 Unraveling SelN Function: A Requirement in Muscle Development and Regeneration

Studies using antisense methods to inhibit synthesis of SelN protein in zebrafish embryos demonstrated SelN was required for the normal structural organization of the somite [15, 27]. Under conditions where embryos were significantly depleted for SelN, Jurynec et al. [15] noted a deficit in the production of a specific set of slow muscle fibers, called Muscle Pioneer Cells, whose absence could readily account for the aberrantly shaped somites reported in both studies. In addition, both studies reported indicators of aberrantly formed muscle cells. Newly differentiated embryonic somite muscle cells exhibited sarcomere disorganization reminiscent of that observed in human diseased muscle, and muscle attachments to somite borders were disrupted. Thus two types of dysfunction were uncovered: disruption in the generation of muscle cells, reflecting a defect in the specification and/or development of muscle precursor cells, and disruption of the normal cytoarchitectural organization of muscle cells. Although it is unresolved whether the two kinds of defects reflect one or multiple molecular pathways that SelN function is required to support, these studies clearly pointed to a role for SelN in muscle development.

More recently, *Sepn1* knock-out mice were generated by targeted disruption of the *Sepn1* gene, which produced a null mutant allele [28, 29]. In contrast to SelN-depleted zebrafish, no defect could be observed in somite organization or myogenic gene expression in *Sepn1*$^{-/-}$ embryos [26]. Homozygous *Sepn1*$^{-/-}$ mice were healthy, fertile, and indistinguishable from wild-type littermates into adulthood. Histological and ultrastructural analyses of several muscle groups, including paravertebral muscles and diaphragm, revealed no major defect in muscle architecture. In addition, no modifications in the fiber type or size were observed in *Sepn1*$^{-/-}$ mice, except for a slight fiber hypotrophy observed in the paravertebral muscles of mutants. Furthermore, functional tests, such as rotarod or treadmill exercise, and in situ measurements of

muscle contractility failed to reveal any specific defect. In contrast, when submitted to repeated forced swimming tests (FST), the mutant mice progressively developed a striking phenotype characterized by whole-body rigidity during the test and a reduced mobility following the swimming period. After several weeks of FST, mutant mice displayed a severe kyphosis (curvature of the spine), and after 3 months paravertebral muscles displayed major alterations, including hypotrophy, switch toward slower fibers and tubular aggregates. Interestingly, the phenotype of the stressed mice was reminiscent of the clinical spectrum described in *SEPN1*-RM patients, with a predominant alteration of trunk muscles. Overall, this reveals sensitivity of exercised SelN-deficient mouse muscle to recurrent stress [29]. Although SelN may affect the physiology of additional cell types (see [8]), no obvious defects were observed in any of the other organs analyzed. These studies clearly show that maintenance of normal muscle function is the most critical role of SelN in vivo, but does not exclude the possibility of important functions for SelN in other organs.

Further study of these *Sepn1* knockout mice demonstrated that SelN is required for maintenance of the satellite cell population, muscle progenitors involved in muscle repair in adults [28]. During cardiotoxin-induced muscle regeneration in wild-type mice, SelN expression was strongly up-regulated and appeared most prominent in mononucleated cells, notably identified as muscle precursors. Upon initial injury, mutant and wild-type mice were similarly capable of restoring muscle fibers, although clear defects such as fat deposition or calcification were observed in *Sepn1*$^{-/-}$ muscles. However, following a second cardiotoxin injection, SelN-deficient muscles failed to regenerate, due to the total depletion of the satellite cell pool during the first round of necrosis/regeneration. This loss was not related to increased cell death, and in vitro studies suggested that in the absence of SelN, satellite cells may make an inappropriate cell fate choice altering the balance between proliferation and self-renewal. Moreover, in adult mutant mice, the number of satellite cells is reduced, indicating basal defect in their maintenance. Similarly, analyses of the *Pax7*$^{+}$ cells in muscle biopsies from patients with *SEPN1*-RM revealed a major reduction in the numbers of satellite cells compared to biopsies from control individuals or individuals with other muscle diseases. Overall these results demonstrated that SelN plays an essential role in the maintenance of the satellite cell pool in skeletal muscles, under normal physiological conditions and during injury induced regeneration [28].

In sum, studies of animal models indicate SelN plays a major role in the dynamics/function of muscle progenitors both in embryo and adult, as well as in homeostasis of mature muscle fibers. However, clear differences exist in the unique roles of SelN in humans, mice, and zebrafish. In contrast to its critical function in the zebrafish, SelN is not absolutely necessary for embryogenesis in mammals. Similarly, although SelN contributes to fiber type specification and sarcomere organization of muscle in humans and zebrafish, its importance in mice is observed only in an increased stress context or in the muscle regeneration process. In humans, it remains unclear whether muscle defects arise from altered maintenance/dynamics of muscle progenitors during development or growth, and/or impaired homeostasis of mature fibers. The divergent phenotypes observed may reflect differences in

muscle development and physiology, differential use of specific muscles or different abilities to switch fiber type identity between the different species. For example, differences in disease phenotypes between humans and mouse models for Duchenne Muscular Dystrophy [30], Werner syndrome [31] and ataxia telangiectasia syndrome [32] have been linked to species-specific variation in telomere length. This chromosomal difference appears to have a direct impact on the ability of the relevant stem cell populations to sustain regenerative processes initiated by other primary defects. Alternatively, these differences might reflect new functions acquired by SelN in some species.

22.5 Unraveling SelN Function: Ryanodine Receptor Intracellular Calcium Release Channel and Cellular Redox State

Recent studies support a model that links SelN to the establishment and/or maintenance of the RyR calcium release channel and view the pathological consequences of loss of SelN as arising from a primary defect in the regulation of intracellular calcium mobilization. Given the many similarities in the muscle disorders caused by mutations in *SEPN1* or *RYR1*, the understanding that many selenoproteins can catalyze redox reactions [33] and the fact that intact RyR channels, which contain more than 400 cysteine residues, are sensitive to redox modifications that modulate channel activity [34, 35], Jurynec et al. [15] tested the hypothesis that SelN is required for normal RyR channel function. They showed the tissue defects resulting from loss of *sepn1* in zebrafish embryos could be mimicked precisely by loss of *ryr1* and *ryr3*. Moreover, SelN and RyR3 were both required for a long-lasting elevation of intracellular calcium around the Kupffer's Vesicle, demonstrating SelN is required for calcium fluxes in vivo. Together these studies indicated that SelN and RyR calcium channels contribute to common molecular and cellular processes in the embryo. Moreover, co-immunoprecipitation of SelN and RyR proteins from rabbit muscle or zebrafish embryos supported a potential direct molecular link between SelN and the calcium channels in vivo. Analysis of RyR activity from zebrafish embryos or human disease tissue lacking SelN revealed RyR channels were no longer responsive to changes in the redox potential of the environment, a characteristic that could be reversed by adding in vitro synthesized SelN. One interpretation of these experiments is that SelN is physically associated with RyR channels in some contexts and serves as a redox sensor to mediate signals that result in redox modifications of the RyR channels.

Analyses of muscle from patients carrying null mutations in *SEPN1* also demonstrated that calcium homeostasis was compromised in the absence of SelN [36]. Myotubes from patients appeared to have elevated constitutive levels of free calcium in the cytoplasm and depleted sarcoplasmic reticulum (SR) stores of calcium in comparison with control cells. This condition could result from either inefficient activity of the SERCA pump responsible for loading calcium into the ER/SR or

abnormal leakiness of the RyR channels. However, in contrast to the model invoking a direct association between SelN and RyR, Arbogast et al. [36] suggested that absence of SelN in muscle cells might have a direct effect on maintaining their oxidative state and only an indirect effect on calcium homeostasis. Indeed myotubes from patients exhibited increased basal intracellular oxidative level and protein oxidation, as well as an increased susceptibility to exogenous oxidative stress. These authors concluded that lack of SelN leads to a state of increased sensitivity to oxidative stress and an increase in the abundance of oxidative modifications to proteins, which could account for the defects in the function of the RyR calcium channels.

It is likely that SelN-deficient cells are impaired in both redox and calcium homeostasis. The two appear intimately linked, making it difficult to resolve which defect arises directly from loss of SelN. Domains of mitochondria and ER/SR are tethered in close apposition [37, 38] and there is structural and functional crosstalk between the two organelles [39–41]. Evidence supports a feed-forward interaction between elevated cytosolic calcium and elevated reactive oxygen species (ROS) levels [40, 42]. For example, mice with a primary defect in the regulation of calcium, heterozygous for a mutant *Ryr1* allele (R163C) associated with Malignant Hyperthermia and Central Core Disease, have mitochondria with elevated levels of calcium and elevated ROS production [43]. In sum, further investigation is warranted into the relative roles of redox and calcium homeostasis in muscle diseases, and the contribution of SelN to these processes.

22.6 Expression of *SEPN1*: Informative Mutations

Among the mutations resulting in *SEPN1*-RM, several interfere with the selenocysteine insertion pathway required for SelN expression. As discussed in Chap. 3, co-translational incorporation of selenocysteine requires a 3′ UTR selenocysteine incorporation sequence (SECIS) element that forms a complex with a SECIS binding protein (SBP2) and the specialized selenocysteine elongation factor (EFSec). This complex is required to recruit Sec-tRNA$^{[Ser,Sec]}$ to the ribosome during decoding of the UGA codon.

Compound heterozygous mutations in the *SBP2* gene were recently identified in patients that exhibit a remarkably complex phenotype thought to be due to a global reduction in selenoprotein expression [44]. This multisystem disorder includes defects in spermatogenesis, increased cellular ROS, photosensitivity, impaired T lymphocyte proliferation, abnormal cytokine secretion, telomere shortening, and enhanced insulin sensitivity. Further supporting a role for SelN in normal muscle development and maintenance, these subjects also revealed an apparent lack of full length SelN, an axial muscular dystrophy with minicores, as well as connective tissue and fatty infiltration of select muscle groups similar to those seen in patients with *SEPN1* mutations. Direct evidence that functional interactions between SBP2 and the *SEPN1* SECIS element are required for normal SelN expression are derived from the previous identification of a patient with *SEPN1*-RM containing a single

homozygous point mutation in the *SEPN1* 3′ UTR SECIS element [21]. This disease-causing mutation was shown to prevent SBP2 binding and selenocysteine incorporation, leading to a significant reduction in both *SEPN1* mRNA and protein levels.

In addition to the SECIS element, sequence context information affecting selenocysteine incorporation efficiency resides near the UGA codon in the *SEPN1* mRNA [45–47]. This *cis*-acting element, designated the Selenocysteine codon Redefinition Element (SRE), stimulates selenocysteine insertion and consists of upstream sequences and a highly conserved stem-loop structure downstream of the UGA codon. Phylogenetic analysis of this region reveals it to be highly conserved in chordates [45] and some invertebrates (unpublished, AL and MH).

A study analyzing four disease-causing missense mutations downstream of the UGA codon in *SEPN1* [47] identified one mutation c.1397G>A, that affected selenocysteine incorporation by creating a C:A mismatch near the base of the SRE stem-loop. This mutation was shown to significantly reduce selenocysteine insertion efficiency and resulted in negligible levels of both *SEPN1* mRNA and protein in the patient's muscle. It is notable in this case, and the SECIS mutation described above, that not only was selenocysteine insertion impaired, but mRNA levels were substantially reduced. These studies highlight the importance of both the SECIS and SRE in maintaining the stability of the message and the selenocysteine insertion pathway in vivo. The remaining three mutations (c.1388G>T, c.1405C>T, c.1406G>A) were shown to have negligible effects on selenocysteine incorporation, suggesting these missense mutations might impact SelN catalytic activity directly. Selective pressure near the UGA codon is likely acting not only at the nucleotide level to preserve the *cis*-acting information affecting selenocysteine incorporation, but also at the amino acid level to maintain the integrity of the SelN catalytic site and the specificity of the reaction.

22.7 SelN Through Evolution

Examination of the SelN protein sequence identified a calcium binding EF-hand motif next to a transmembrane domain located near the N-terminus [24], and the presence of a potential redox motif, SCUG, but additional functions or catalytic activity could not be predicted based on sequence comparisons or homology to known proteins.

Classification of a protein into the selenoprotein group is based on the presence of a selenocysteine residue in its sequence and the presence of a SECIS motif in the mRNA, but in many cases the encoded proteins share no other common features. Moreover, many selenoproteins have orthologs or paralogs in different species with cysteine in place of selenocysteine [48]. Interestingly, SelN is one of the rare selenoproteins with no cysteine homolog identified to date.

It was previously reported that SelN is present in all vertebrates; however, analysis of recently available invertebrate genomes reveals that SelN is also found in other phyla. SelN homologs were identified in two chordates, *Branchiostoma floridae*,

and *Ciona intestinalis*; in the hemichordate acorn worm, *Saccoglossus kowalevskii*; within the echinoderm group, the sea-urchin, *Strongylocentrotus purpuratus*; in two cnidarians, *Nematostella vectensis* and *Hydra magnipapillata*; in the annelids, *Capitella teleta* and *Alvinella pompejana*; in three mollusks, *Aplysia californica*, *Mytilus californianus* and *Lottia gigantea*; as well as in five poriferae, *Amphimedon queenslandica*, *Leucetta chagosensis*, *Oopsacas minuta*, *Oscarella carmella*, and *Suberites domuncula*. In addition, SelN orthologs were found in three arthropods, one arachnid, *Ixodes scapularis* and two insects, *Locusta migratoria*, and *Culex quinquefasciatus*. No SelN ortholog was identified in other insect genomes, including *Drosophila* or mosquitoes, two insects with extensive high quality sequence coverage of the genome, further supporting previous observations showing that selenoproteins have been independently lost in several insect species [49]. Altogether, this analysis demonstrates that SelN is more ancient and distributed more broadly in the animal kingdom than was previously appreciated. The *SEPN1* gene is already part of the ancestral parazoa and eumetazoa gene repertoire, but it is not systematically conserved in all animals. These differences, and the lack of compensatory cysteine-containing SelN homologs, suggest that the absence of SelN might reflect species-specific adaptations to different physiological requirements or environmental contexts. Importantly, the presence of SelN in primitive organisms lacking organized muscle structures indicates that its original function may have been unrelated to muscle differentiation and maintenance. One implication of this latter observation is that SelN may have additional functions that remain to be identified.

Sequence alignment of vertebrate and invertebrate SelN orthologs identified two conserved domains, flanked by critical residues at conserved positions (see Fig. 22.1). Interestingly, 9 out of the 10 missense mutations causing *SEPN1*-RM are located in these two domains and affect highly conserved residues (Fig. 22.1). Domain 1 appeared to be a SelN-specific hallmark, since no similar motif could be found in any other protein. The second domain includes the selenocysteine residue within a highly conserved context (LWGALDDQSCUGSGRTLR). This block of conservation is expected to correspond to the active catalytic center, although as discussed above, conservation might also reflect evolutionary pressure acting to maintain both the amino acid sequence and the SRE structure.

It was previously observed that SelN harbors a SCUG catalytic site, reminiscent of the thioredoxin reductase GCUG motif [50]. This similarity is consistent with a reductase activity for SelN. However, the thioredoxin reductases contain two additional functional domains: the FAD and NADPH binding domains. These two domains, essential for reduction of the N-terminal thiol active site and electron transfer to the C-terminal selenylsulfide bond [51], are absent in SelN. Lack of a second redox active site might be compensated by interactions with other redox active partners, which have yet to be identified. In addition, the highly accessible selenolate active site at the C-terminus of the thioredoxin reductases has been proposed to confer a broad range of substrates to these enzymes, from small molecules such as selenite, lipid hydroperoxides, ebselen, and dehydroascorbate, to proteins such as thioredoxin, protein disulfide isomerases or glutathione peroxidases [52, 53].

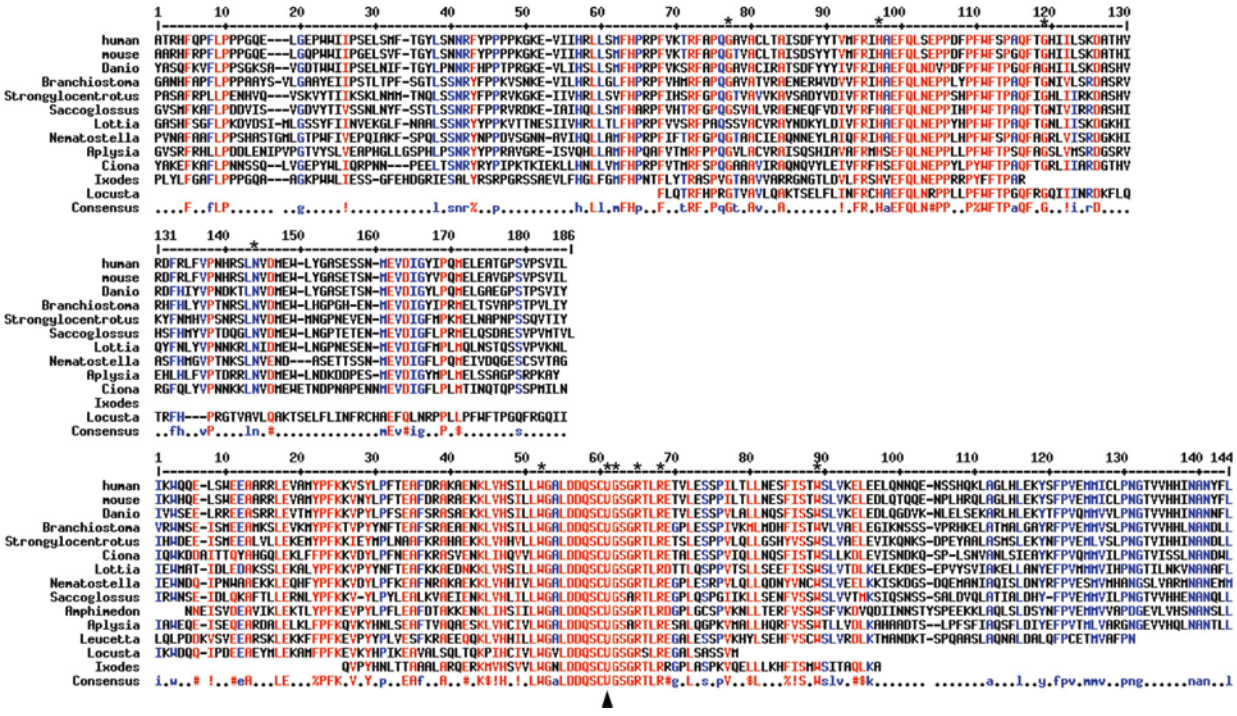

Fig. 22.1 Alignment of vertebrate (human, mouse and zebrafish), chordate (*Ciona intestinalis*) and several predicted invertebrate SelN sequences shows two blocks of conserved residues. Invertebrate SelN homologous sequences were deduced from BLAST search using genomic or EST sequences from NCBI databases. Retrieved sequences were aligned with the multialign program (http://multalin.toulouse.inra.fr/multalin/multalin.html). Conserved residues are highlighted in *red* or *blue* according to their occurrence, presence in at least 75 or 50% of the sequences, respectively. Several sequences are indicate due to the lack of information in the database. Positions of missense mutations causing *SEPN1*-Related Myopathy are indicated by *upper asterisk*. U stands for selenocysteine, its position is indicated by an *arrow head*. These two domains match positions 202–379 and 403–544 of the human SelN isoform 1, respectively

In contrast, the localization of the active site in the central part of SelN together with its extended conservation among SelN orthologs might reflect a higher selectivity of SelN for its substrate(s).

22.8 Concluding Remarks

Significant progress has been made toward understanding the biochemical and cellular processes that rely on SelN and whose perturbation leads to the syndrome known as *SEPN1*-RM. Loss of SelN affects both redox and intracellular calcium homeostasis. Whether SelN functions directly to regulate activity of the RyR channel, acts primarily to maintain the oxidative state of the cell, or both is a matter still to be resolved. It will be important to characterize SelN substrates to define whether SelN acts on specific substrates or plays a more general redox "buffering" role. These proximal effects are likely to underlie the increased susceptibility of SelN-deficient muscle cells to further oxidative stress. SelN deficiency affects muscle embryogenesis in zebrafish and muscle regeneration in mice, by impairing the development, maintenance and function of muscle progenitor cells. The potential role of stress as a factor contributing to the progression of the disease warrants detailed study.

SelN has additional cellular roles that need to be better defined. In terms of muscle development, it is not clear why loss of SelN function affects type I fiber development or the organization of myofibrils and myofibril-associated mitochondria. Finally, whereas the association between *SEPN1* mutations and congenital muscle disorders has drawn attention to the function of SelN in muscle, the ancient evolutionary history of *SEPN1* and its broad tissue expression suggest another layer of activity and a more general function than one restricted to muscle differentiation and maintenance.

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Part III
Selenium and Selenoproteins in Human Health

Chapter 23
Prostate Cancer Prevention and the Selenium and Vitamin E Cancer Prevention Trial (SELECT): A Selenium Perspective

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Abstract The Selenium and Vitamin E Cancer Prevention Trial (SELECT) randomized 35,533 healthy men, ≥55 years (≥50 years if African American), with normal digital rectal exams and prostate-specific antigens <4 ng/mL, to (i) 200 μg/day *l*-selenomethionine, (ii) 400 IU/day all-rac-alpha-tocopheryl acetate (vitamin E), (iii) both supplements, or (iv) placebo for a median of 5.5 years (range 4.2–7.3 years). The hypotheses underlying SELECT, that selenium and vitamin E individually and together decrease prostate cancer incidence, derived from epidemiologic and laboratory evidence and significant secondary endpoints in the Nutritional Prevention of Cancer (NPC) (selenium) and Alpha-Tocopherol Beta-Carotene (vitamin E) trials. Results from SELECT showed that prostate cancer incidence did not differ among the four arms: hazard ratios (HRs) (99% CIs) for prostate cancer: 1.13 (99% CI, 0.95–1.35; $p=0.06$; $n=473$) for vitamin E, 1.04 (99% CI, 0.87–1.24; $p=0.62$; $n=432$) for selenium, and 1.05 (99% CI, 0.88–1.25; $p=0.52$; $n=437$) for selenium + vitamin E vs. 1.00 ($n=416$) for placebo. Statistically nonsignificant increased risks of prostate cancer with vitamin E alone (RR 1.13; $p=0.06$) and newly diagnosed type 2 diabetes mellitus with selenium alone (RR 1.07; $p=0.16$) were observed. SELECT data show that neither selenium nor vitamin E, alone or together, in the doses and formulations used, prevented prostate cancer in this heterogeneous population of healthy men. Although there are many potential explanations for the null findings in SELECT, the most likely reasons appear to be a mismatch between the target population and the intervention agents selected, or that effects were limited to as-yet-undetermined subgroups of susceptible men.

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23.1 Background

Documentation of the anticancer properties of selenium and vitamin E as secondary endpoints in two nutrition intervention trials, the NPC Study [1] and the ATBC Cancer Prevention Trial [2, 3], formed the foundation upon which the Selenium and Vitamin E Cancer Prevention Trial (SELECT) was based. SELECT was only the second NCI-sponsored cancer prevention trial, specifically designed and implemented with the primary objective to prevent prostate cancer [4–7].

23.2 Study Objectives

The hypotheses underlying SELECT, that selenium and vitamin E prevent prostate cancer, were the basis for its primary objective: to assess the effects of selenium and vitamin E alone and in combination on incidence of prostate cancer. Prespecified secondary endpoints included: prostate cancer-free survival; all cause mortality; the incidence and mortality of other cancer types such as lung and colorectal; overall cancer incidence and survival; and disease potentially impacted by chronic administration of selenium and vitamin E. Serious cardiovascular events were also monitored because of concerns over the safety of vitamin E with regard to the risk of hemorrhagic stroke [5, 6]. Additional trial objectives included periodic quality of life assessment, serum micronutrient measurement and prostate cancer risk, and the evaluation of biological and genetic markers associated with the risk of prostate cancer [8].

23.3 Selection of Study Agents

Advice from an NCI-sponsored panel of experts led to selection of *l*-selenomethionine over selenized yeast for SELECT. Although selenized yeast was the form used in the hypothesis-generating NPC trial [1], marked batch-to-batch variability in various forms of selenium in the selenized yeast, lack of commercial availability of the selenized yeast used in the NPC study, and laboratory analysis which showed that *l*-selenomethionine was the predominant selenium species in commercially available selenized yeast at the time the trial was being designed led to the panel's recommendation of the essential nutrient form. A daily dose of 200 μg was selected to mimic the NPC trial dose. The optimum dose and formulation of vitamin E was also the subject of debate. Ultimately, α-tocopherol (*all rac* (*dl*)-α-tocopheryl acetate) was selected because of the observed association of long-term supplementation with this form of vitamin E with reduction in prostate cancer incidence in the ATBC trial [3, 9]. The chosen daily dose of 400 mg was based on its potential benefits for other non-cancer diseases (e.g., cardiovascular disease, Alzheimer's disease, age-related macular degeneration), as well as its inclusion in widely used vitamin supplements, suggesting its safety [10–12].

23.4 Study Cohort, Design, and Statistical Methods

SELECT was a prospective, randomized, double-blind, placebo-controlled, 2×2 factorial design clinical trial, which tested selenium and vitamin E alone and in combination in eligible healthy men. Eligibility was based mainly on elevated risk of disease due to age: ≥55 years in Caucasian men and ≥50 years in African-American men since 50 to 55-year-old black American men have a prostate cancer incidence rate comparable to that of 55 to 60-year-old white men. Full eligibility criteria are shown in Fig. 23.1. At completion of accrual, 35,533 eligible men enrolled in SELECT, exceeding the goal of 32,400. A great strength and advantage in the SELECT study design is that the randomization process should lead to equal participant distribution among the four study arms for all factors (beyond the agents being tested) that might otherwise influence study endpoints, thus avoiding unmeasured or hidden sources of bias in participant characteristics. The study design with randomization groups is shown in Fig. 23.1.

SELECT had a planned sample size of 32,400 men to address five prespecified comparisons – (i) vitamin E vs. placebo, (ii) selenium vs. placebo, (iii) combined vitamin E plus selenium vs. placebo, (iv) combined vitamin E plus selenium vs. vitamin E, and (v) combined vitamin E plus selenium vs. selenium. Each comparison was powered to detect a ≥25% decrease in the incidence of prostate cancer for selenium or vitamin E alone, and an additional 25% decrease for selenium and vitamin E combined, compared with either agent alone. Prostate cancer was assessed based on a recommended routine clinical diagnostic evaluation, including yearly digital rectal exam (DRE) and serum prostate specific antigen (PSA) measurement.

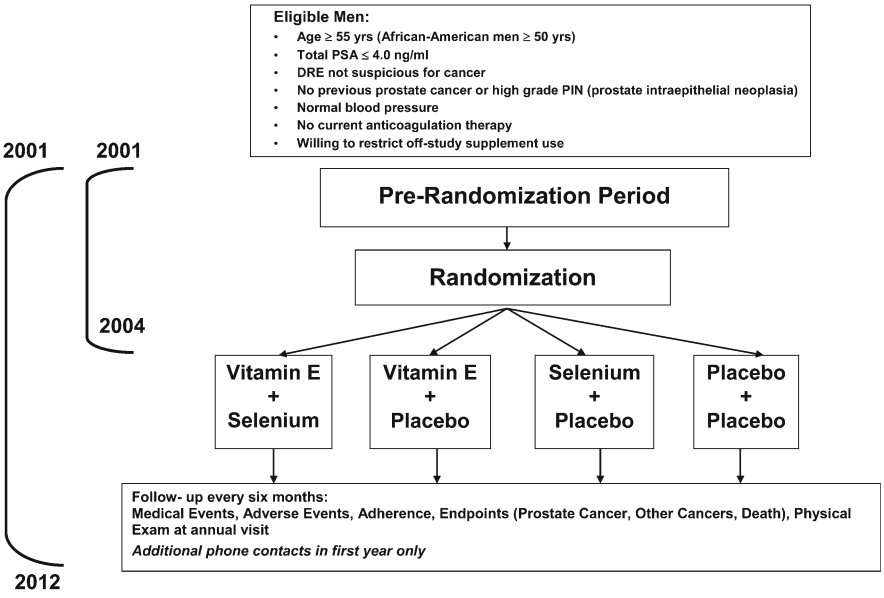

Fig. 23.1 SELECT: study eligibility, schema, and follow-up schedule

23.5 Study Implementation, Recruitment Strategies, and Participant Baseline Characteristics

Eligible men from the US, Canada, and Puerto Rico were enrolled from July 2001 to June 2004, a period 2 years shorter than projected. Although the accrual target was 32,400, a total of 35,533 participants, including 21% minorities (12% African American, 7% Hispanic, and 2% other) were randomized [13] (Table 23.1). Not only was SELECT the largest randomized chemoprevention trial ever conducted, it also had the largest percentage of black participants ever randomized to this type of study [14].

Table 23.1 Select: baseline characteristics – age, race/ethnicity, PSA, serum levels

	Number (%) of participants[a]			
	Placebo	Vitamin E	Selenium	Selenium + vitamin E
Age (year)				
Median (interquartile range)	62.6 (58.1–67.8)	62.3 (58.0–67.8)	62.6 (58.2–68.0)	62.4 (58.1–67.8)
50–54	355 (4)	402 (5)	337 (4)	385 (4)
55–64	5,078 (58)	5,143 (59)	5,076 (58)	5,052 (58)
65–74	2,702 (31)	2,641 (30)	2,733 (31)	2,731 (31)
≥75	561 (6)	551 (6)	606 (7)	535 (6)
Race/ethnicity				
White	6,863 (79)	6,890 (79)	6,942 (79)	6,874 (79)
African American	1,078 (12)	1,107 (13)	1,053 (12)	1,076 (12)
Hispanic (non-AA)	492 (6)	477 (5)	481 (5)	484 (6)
Hispanic (AA)	76 (1)	103 (1)	86 (1)	95 (1)
Other	187 (2)	160 (2)	190 (2)	174 (2)
PSA (ng/mL)				
0.1–1.0	4,122 (47)	4,208 (48)	4,218 (48)	4,213 (48)
1.1–2.0	2,728 (31)	2,653 (30)	2,661 (30)	2,666 (31)
2.1–3.0	1,168 (13)	1,228 (14)	1,211 (140	1,149 (13)
3.1–4.0	666 (8)	634 (7)	652 (7)	659 (8)
>4.0	5 (<1)	3 (<1)	2 (<1)	1 (<1)
Unknown/missing	7 (<1)	11 (<1)	8 (<1)	15 (<1)
Serum levels (μg/mL)[b]				
Median, interquartile range				
Selenium (μg/L)	138 (125–152)	136 (122–148)	135 (123–146)	136 (123–150)
α-tocopherol (μg/mL)	12.5 (10.7–15.0)	12.8 (10.7–15.4)	12.6 (10.4–14.8)	12.2 (10.1–15.4)

SELECT Selenium and Vitamin E Cancer Prevention Trial; *PSA*, prostate-specific antigen; *AA*, African American

[a]Number (%) of participants refers to all entries in this section except for age and serum values where median and interquartile ranges are shown

[b]Serum α-tocopherol levels are cholesterol-adjusted

Table 23.2 Select: study adherence – pill counts by supplement type and study year

	% of Men adherent[a,b] (range)			
	Placebo	Vitamin E	Selenium	Selenium + vitamin E
Selenium/matching placebo				
Year 1 ($n = 34{,}708$)	85 (76–85)	85 (77–85)	84 (76–84)	85 (77–84)
Year 2 ($n = 34{,}163$)	81 (72–81)	80 (72–81)	79 (71–80)	80 (72–80)
Year 3 ($n = 33{,}616$)	76 (68–77)	77 (69–77)	75 (68–76)	76 (69–77)
Year 4 ($n = 32{,}976$)	69 (65–73)	73 (66–74)	71 (64–72)	72 (65–74)
Year 5 ($n = 23{,}419$)	69 (63–71)	71 (64–73)	69 (62–70)	70 (64–71)
Vitamin E/matching placebo				
Year 1 ($n = 34{,}708$)	85 (76–85)	85 (77–85)	85 (76–85)	85 (77–85)
Year 2 ($n = 34{,}163$)	80 (71–80)	80 (71–80)	79 (70–79)	79 (71–80)
Year 3 ($n = 33{,}616$)	75 (67–75)	75 (67–76)	74 (67–75)	76 (69–77)
Year 4 ($n = 32{,}976$)	70 (63–72)	70 (63–72)	69 (62–71)	70 (63–72)
Year 5 ($n = 23{,}419$)	67 (61–69)	69 (62–71)	67 (61–69)	68 (61–70)

SELECT, Selenium and Vitamin E Cancer Prevention Trial

[a]Percent of men adherent defined as taking at least 80% of their study supplements

[b]Ranges are estimates which include those with missing data and assumes that those with missing data were either all not adherent (low estimate) or all adherent (high estimate)

Adherence in SELECT was assessed via pill count (Table 23.2), participant diary, and serum levels (in a bioadherence subcohort), and is described in detail elsewhere [13].

Selenium and vitamin E intervention supplements were discontinued on October 23, 2008 based on an assessment of the SELECT data as of August 1, 2008 by the Data and Safety Monitoring Committee, with a median overall follow-up of 5.5 years (range, 4.2–7.3 years) [13]. This independent committee concluded that the null hypothesis – that no convincing evidence of benefit existed with either selenium or vitamin E or the two in combination – prevailed, according to the SELECT results.

23.6 Results

23.6.1 Adherence to Study Supplements

Adherence, assessed both by pill count and in a subset of men by "bioadherence" metrics (i.e., serum levels of selenium and vitamin E), was high and comparable in all four study arms. Importantly, serum selenium and α-tocopherol levels rose only in participants assigned to the selenium- and vitamin E-containing arms, respectively.

Table 23.3 Select: clinically diagnosed prostate cancers

	Placebo ($n=8,696$)	Vitamin E ($n=8,737$)	Selenium ($n=8,752$)	Selenium + vitamin E ($n=8,703$)
Prostate cancers				
Number[a]	416	473	432	437
5-year incidence[b] (%)	4.43	4.93	4.56	4.56
HR (99% CI)	1.00	1.13 (0.95–1.35)	1.04 (0.87–1.24)	1.05 (0.88–1.25)
p-value	–	$p=0.06$	$p=0.62$	$p=0.52$
Diagnosis by prostate biopsy				
Number[b]	404 (97%)	458 (97%)	419 (97%)	420 (97%)
Reason for biopsy (positive biopsies)[b]				
Elevated PSA[b]	259 (64%)	324 (71%)	296 (71%)	263 (63%)
Abnormal DRE[b]	66 (16%)	58 (13%)	46 (11%)	56 (13%)

SELECT, Selenium and Vitamin E Cancer Prevention Trial; *HR*, hazard ratio; *CI*, confidence interval; *PSA*, prostate-specific antigen; *DRE*, digital rectal exam

[a]Total number of prostate cancers diagnosed

[b]Number or % of participants per treatment arm

These measurements indicated both good compliance with assigned study agents and conversely, minimal "drop-ins" to unassigned supplements from taking over-the-counter selenium and/or vitamin E off-study.

23.6.2 Primary Endpoint: Prostate Cancer

Rates of prostate cancer did not differ statistically among the four intervention arms, with HRs for prostate cancer relative to placebo of 1.13 (99% CI, 0.95–1.35; $p=0.06$) for the vitamin E-alone group, 1.05 (99% CI, 0.88–1.25; $p=0.52$) for the selenium + vitamin E group, and 1.04 (99% CI, 0.87–1.24; $p=0.62$) for the selenium-alone group (Table 23.3). The graph depicting the cumulative incidence of prostate cancer detected during each study year indicated that the vitamin E-alone curve showed some divergence from the placebo and other two intervention curves at about 4 years of follow-up which, although statistically nonsignificant, was of potential concern (Fig. 23.2). Most prostate cancers were diagnosed by prostate biopsy, constituting histological diagnoses (Table 23.2). The majority were early stage and low Gleason grade, which were similar in all four groups [13]. The clinical presentation that prompted biopsy was primarily increased PSA (approximately two-thirds of cases in each of the four groups) or abnormal DRE (11–16% of cases in the four groups). Importantly, the proportion of participants undergoing PSA testing and DREs was similar in all groups, obviating any concern that observed outcomes reflected detection bias associated with differential screening.

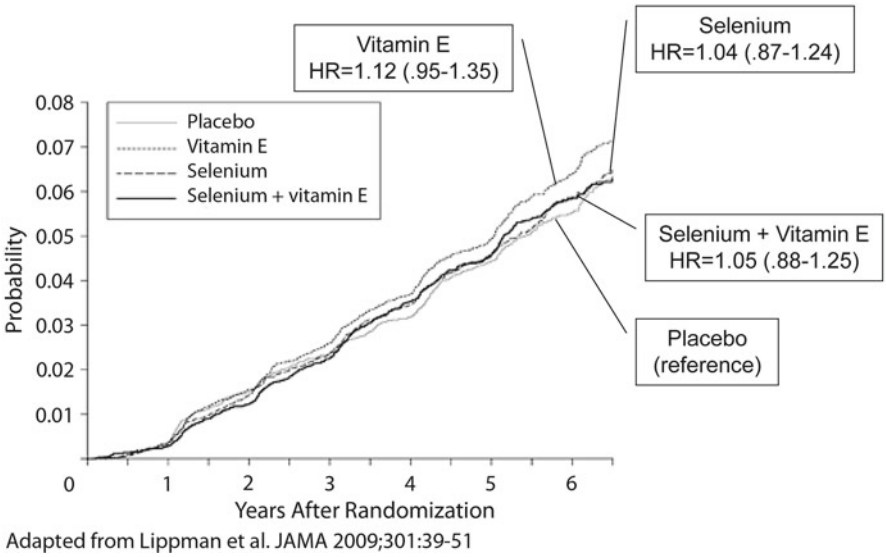

Fig. 23.2 SELECT: cumulative incidence of prostate cancer over time

23.6.3 Secondary Endpoints

Prespecified secondary endpoints included other cancers, especially those influenced by a study supplement in prior nutritional trials [1]. None of these cancers differed significantly in any of the intervention arms compared to the placebo group; all p-values were >0.15 (Table 23.4). Non-cancer secondary outcomes included cardiovascular outcomes, none of which showed a significant difference from the reference placebo arm [13]. In particular, hemorrhagic stroke, which was a potential concern due to the known association of vitamin E with bleeding propensity [15] and the previous association observed at a lower dose (50 mg daily) in the ATBC trial [2], did not differ among the four groups (Table 23.4). Type 2 diabetes mellitus was of interest because of earlier reports linking increased prevalence with higher serum selenium levels, and higher incidence following long-term selenium supplementation [16]. Although there was a hint of increased risk of type 2 diabetes in the selenium-alone arm based on patient-reported outcomes, the observed effect was small and statistically nonsignificant (relative risk (RR), 1.07; 99% CI, 0.94–1.22; $p = 0.16$). Deaths, total and those due to predesignated causes, also did not differ among the four arms (Table 23.4). The only adverse effects that were statistically significantly increased were alopecia and low-grade dermatitis in the selenium-alone group, and halitosis in the selenium + vitamin E group; these are previously known side effects of the interventional supplements (Table 23.4).

Table 23.4 Select: secondary endpoints

	Treatment arm (number of participants)			
	Placebo ($n=8,696$)	Vitamin E ($n=8,737$)	Selenium ($n=8,752$)	Selenium + vitamin E ($n=8,703$)
	No. events HR (99% CI)			
Cancers				
Any cancer	824	856	837	846
(including prostate)	1 (reference)	1.03 (0.91–1.17)	1.01 (0.89–1.15)	1.02 (0.90–1.16)
Lung	67	67	75	78
	1 (reference)	1.00 (0.64–1.55)	1.12 (0.73–1.72)	1.16 (0.76–1.78)
Colorectal	60	66	63	77
	1 (reference)	1.09 (0.69–1.73)	1.05 (0.66–1.67)	1.28 (0.82–2.00)
Cardiovascular events				
Any (including death)	1,050	1,034	1,080	1,041
	1 (reference)	0.98 (0.88–1.09)	1.02 (0.92–1.13)	0.99 (0.89–1.10)
Hemorrhagic stroke	11	7	11	12
	1 (reference)	0.63 (0.18–2.20)	0.99 (0.33–2.98)	1.09 (0.37–3.19)
Diabetes[a]	669	700	724	660
	1 (reference)	1.04 (0.91–1.18)	1.07 (0.94–1.22)	0.97 (0.85–1.11)
Deaths				
Total	382	358	378	359
	1 (reference)	0.93 (0.77–1.13)	0.99 (0.82–1.19)	0.94 (0.77–1.13)
All cancers	125	106	128	117
	1 (reference)	0.84 (0.60–1.18)	1.02 (0.74–1.41)	0.93 (0.67–1.30)

Prostate cancer	0	0	1	0
	1 (reference)	N/A	N/A	N/A
Cardiovascular	142	119	129	117
	1 (reference)	0.84 (0.61–1.15)	0.91 (0.66–1.24)	0.82 (0.60–1.13)
Supplement-specific adverse events[b]				
Alopecia	206	220	265	238
	1 (reference)	1.06 (0.83–1.36)	1.28 (1.01–1.62)	1.15 (0.91–1.47)
Dermatitis, grades 1–2	516	591	605	554
	1 (reference)	1.14 (0.98–1.32)	1.17 (1.00–1.35)	1.07 (0.92–1.25)
Halitosis	427	493	503	531
	1 (reference)	1.15 (0.97–1.36)	1.17 (0.99–1.38)	1.24 (1.06–1.46)

SELECT, Selenium and Vitamin E Cancer Prevention Trial; *HR*, hazard ratio; *CI*, confidence interval; *N/A*, not applicable

[a]Diagnoses based on self-report or reported use of diabetes medication excluding prevalent cases at randomization

[b]Point estimates expressed as relative risk (RR) and 99% CIs

23.7 Discussion

The results of SELECT – that neither selenium nor vitamin E supplementation alone or in combination reduced prostate cancer incidence – are at odds with results from the NPC and ATBC trials, upon which the SELECT hypotheses were based. Furthermore, the nonsignificant increased prostate cancer incidence in the vitamin E-alone arm raises a largely unexpected concern that vitamin E might, in fact, has undesirable effects in prostate carcinogenesis. These outcomes of SELECT have been debated extensively, generating a series of potential explanations for the negative results.

23.7.1 Why Didn't Selenium Reduce the Clinical Incidence of Prostate Cancer?

Kristal enumerated a general list of categorical reasons why cancer prevention trials can fail [17]: the intervention dose was too high or low, the intervention period was too short, unexpected side effects resulted in early termination, adherence was poor, too many controls "dropped in," susceptibility was limited to subgroups, and the intervention itself affected detection of the endpoint. It is also possible that a lag-to-effect may occur such that benefit (or harm) appears only much later, after the conclusion of the intervention, as was evident in one of the tamoxifen vs. placebo breast cancer prevention trials [18]. Yet, another alternative is that intervening in middle-aged to elderly adults is simply too late in life and misses the true prevention window of opportunity to alter early carcinogenic events.

For SELECT in general and selenium in particular, a number of potential explanations for the null findings stand out as most likely, including, the dose and form of selenium chosen, the study population targeted for the intervention, effects were restricted to subgroups, and among others, the play of chance, as discussed below.

23.7.1.1 Selenium Dose

The dose and, more importantly, the formulation (see below) of selenium used in SELECT have been cited as major contributors to the failure of the selenium-containing arms to show a reduction in prostate cancer incidence. Yet, these features of the selenium intervention were chosen with great care. Although an optimum dose of selenium supplementation for cancer prevention has not been established, the selenium dose chosen for SELECT was the same 200 μg/day dose used in the hypothesis-generating NPC trial. Based on this, plus the efficacy and safety data derived from a series of preclinical studies, an expert panel convened in December 1998 concurred that 200 μg would be an appropriate daily dose. One idea is that a narrow window exists for the most beneficial dose of dietary selenium. Selenium intake, and more importantly the actual selenium concentration in tissues,

does not exhibit a linear relationship to DNA damage, the regulation of which is a major mechanism by which selenium is presumed to serve as a chemopreventive agent in the prostate. Waters et al. [19] demonstrated that a nonlinear U-shaped dose-response curve characterized the relation between selenium (as toenail selenium concentration) and genotoxic stress in the prostate of dogs. Tissue concentrations either above or below the optimal selenium range might be either ineffectual or even toxic. Importantly, this U-shaped relationship between intake/concentration and biological function appears to have more general applicability to trace elements beyond just selenium [20].

23.7.1.2 SELECT Study Population: Baseline Selenium Status

The net tissue concentration of selenium reflects not only selenium intake, or dose, but also baseline selenium status. Thus, differences in the study populations between the SELECT and NPC trials with respect to mean baseline selenium status could explain the difference in their prostate cancer outcomes. Unlike SELECT, the NPC trial was conducted in a study population located in east coast areas of the United States where environmental selenium levels are low [1, 21, 22]. The baseline mean plasma Se levels in both the selenium and placebo arms of this trial were 114 μg/mL. The Se levels rose about 67% in the Se-treated arm, reaching a mean plasma level of 190 μg/mL. Patients with baseline plasma Se levels in the lowest (<106.4 μg/mL) and middle (106.4–121.2 μg/mL) tertiles showed significant reductions in prostate cancer, with RRs of 0.08 ($p=0.002$) and 0.30 ($p=0.03$), respectively. In contrast, among those in the highest tertile (>121.2 μg/mL), only a nonsignificant reduction was observed, with an RR of 0.85 ($p=0.75$) [23]. The low baseline selenium levels in the NPC participants appear to have accentuated the beneficial effects of selenium supplementation in reducing prostate as well as total cancer incidence [23, 24]. Unlike the NPC trial, the men participating in SELECT came from multiple regions all over the United States and Canada and were replete in selenium levels at baseline, with median serum selenium levels of 135 μg/mL (Table 23.1) compared to the median of 114 μg/mL observed in the NPC trial. In fact, 78% of SELECT participants entered the trial with serum levels that were higher than the lower two tertiles of NPC participants, namely those with lower serum selenium levels who benefited from the selenium intervention in the NPC trial [13].

23.7.1.3 SELECT Study Population: Genetics

In addition to environmental factors feeding into the response of a trial population to the selenium intervention, polymorphisms in the 25 identified selenoprotein genes [25] or in genes encoding proteins involved in selenium metabolism and activity may influence health outcomes. For example, manganese superoxide dismutase (MnSOD), a mitochondrial antioxidant enzyme encoded by the *SOD2* gene, participates in processes that depend on selenium [26]. In a case-control study nested within the Physicians' Health Study, homozygosity for a functional variant

of MnSOD containing an alanine (A) in place of a valine (V) in codon 16 in men who also had the highest pre-diagnostic levels of serum selenium was associated with a reduced risk of prostate cancer (relative risk or RR = 0.47, 95% confidence interval (CI) 0.26–0.85, compared to VV/VA genotypes and low serum selenium for all prostate cancers; and RR = 0.35, 95% CI 0.15–0.82 for aggressive prostate cancer) [26]. An analysis of prostate cancer mortality, also from the Physicians' Health Study, showed that three polymorphisms in the selenoprotein gene *SEP15* significantly affected survival time in men with prostate cancer, and that the survival effect for one of these variants was further influenced by plasma selenium levels [27]. These results suggest that stratification of SELECT participants according to allelic status for relevant genes such as *SOD2* or *SEP15* may well elicit relations between selenium supplementation and prostate cancer risk that did not emerge in the trial population as a whole.

23.7.1.4 Selenium Formulation

The choice of the formulation of selenium, which exhibits a complex metabolism [28–31], posed an even greater challenge. Inorganic forms of selenium, such as selenite, were considered because they are more active than organoselenium compounds in suppressing prostate cancer cell growth and inducing apoptosis of prostate cancer cells [32]. However, in contrast to the organoselenium compounds, the anticancer properties of inorganic forms are linked to genotoxicity, specifically the rapid induction of DNA single-strand breaks [33]. Potential genotoxicity, particularly in view of the prolonged use anticipated in the prevention setting, argued against using an inorganic selenium compound despite the potential of greater efficacy. A similar view confronted the promising compound methylseleninic acid, which exhibited greater potency in vitro and in vivo relative to its organic precursor, Se-methylselenocysteine [29]. Methylseleninic acid was new at the time SELECT was being designed and concern that its toxicity and safety were not well understood, together with its commercial nonavailability, discouraged the panel from further consideration of this form of selenium [7]. The remaining options were selenomethionine and selenized yeast. Although selenized yeast was used in the NPC trial, incomplete characterization and concern over large batch-to-batch variation in concentration of specific organoselenium compounds led the panel to reject yeast as the form of intervention. *L*-selenomethione was the primary active ingredient in the selenized yeast used in the NPC trial, pointing to this form of selenium as the optimal intervention in SELECT.

23.7.1.5 SELECT vs. NPC Trial Designs: Statistical Issues

Perhaps the most important difference between the SELECT and NPC cancer prevention trials and their prostate cancer outcomes is that prostate cancer was the primary

endpoint in SELECT, but merely a secondary endpoint in NPC. Although statistical design in clinical trials typically focuses on assuring adequate power to address the primary endpoint, this is not necessarily true of secondary endpoints [34]. In a trial containing multiple outcomes, prospectively defining a given outcome as the primary endpoint protects that endpoint from concerns that the observed result is due to chance from multiple testing [35]. This leaves secondary endpoints at risk of precisely that, representing findings that are due to chance alone. In this manner, the NPC trial was designed to evaluate the effect of selenized yeast on the incidence of non-melanoma skin cancers as the primary endpoint. Observations regarding secondary endpoints, including other cancers such as prostate cancer, were at risk of being due to chance. In essence, it is as if "all available statistical power had been 'spent' on the primary outcome and the play of chance could have considerable influence even though the secondary outcomes seemed to be statistically significant" [35]. The NPC trial was especially vulnerable to the possibility of a chance finding in a secondary endpoint since it was a small trial, with only 1,312 participants, and it had multiple secondary endpoints.

Statistical concerns regarding interpretation of trial outcomes apply to secondary endpoints irrespective of the significance of the accompanying primary endpoint. These concerns are especially pertinent to outcome data relating to interventions being tested for cancer prevention, because prevention trials lay the foundation for broad health policy decisions affecting healthy populations. Since health policy should be based on the high level of evidence provided by rigorously conducted clinical trials, adoption of a cancer preventive intervention based on a statistically significant secondary endpoint alone is insufficient. However, a significant secondary endpoint may generate a hypothesis that, in turn, serves as the basis for the primary endpoint in a derivative clinical trial. This is exactly the role played by prostate cancer incidence in the NPC trial, which laid the groundwork for the selenium intervention incorporated into the factorial design of SELECT [36]. SELECT was justified because equipoise existed regarding the expectation that selenium would reduce prostate cancer incidence as a primary endpoint.

23.7.2 Ancillary Studies

Several ancillary studies were incorporated into SELECT and results from these studies will ultimately enrich the overall output from SELECT. These studies include: the Prevention of Alzheimer's Disease with Vitamin E and Selenium (PREADVISE), which enrolled ~6,500 men to evaluate Alzheimer's, other neurodegenerative diseases, and normal aging; the SELECT Eye Endpoints (SEE) study to evaluate cataract and macular degeneration events in SELECT participants; the Respiratory Ancillary Study (RAS), which enrolled ~2,900 men to evaluate change in pulmonary function during the intervention; and the Adenomatous Colorectal Polyp (ACP) study, which enrolled over 2,000 men to evaluate adenomatous polyps.

23.8 Conclusion

The absence of positive findings in SELECT for either selenium or vitamin E was surprising in view of the abundant laboratory and epidemiologic data that supported associations between these nutrients and decreased prostate cancer risk. Among the candidate explanations for the negative results, the most likely reasons involve a mismatch between the target population and intervention agents selected, or that effects were limited to as-yet-undetermined subgroups of susceptible men. The choice of dose and formulation for each agent tested, together with selection of a cohort most likely to benefit from supplementation, should be the focus of future trial design. In the case of selenium, a trial cohort that has low selenium intake or status would be most likely to benefit from supplementation. In general, nutritional agents appear to exhibit an optimal "window" of activity (a "U-shaped" dose-response curve), below and above which their benefits disappear and toxicity may even ensue. Unlike purely synthetic drugs, nutrients derive from natural products, and the state of endogenous nutritional repletion of an individual participant must be prospectively factored into the trial designs aimed at achieving this optimal level. Similarly, trial design in the future will be aided enormously by improved understanding of the underlying biologic effects of the intervention agents themselves, particularly as they relate to potentially susceptible subgroups (e.g., genetic susceptibility defined by genotypes, or concurrent environmental exposures such as tobacco or alcohol use).

In summary, SELECT was an enormously important effort. It was the largest nutritional intervention trial ever conducted in the US to prevent cancer, its implementation was a model of methodologic rigor and care, and it produced highly informative (albeit null) answers regarding the potential role of selenium and vitamin E in the prevention of prostate cancer under the conditions the trial was conducted. But SELECT is not yet done, and we await further analyses of these most valuable data, especially those regarding baseline serum levels, subgroups of environmental exposures such as smoking and genetic factors, as well as findings from postintervention follow-up and the several studies of ancillary endpoints incorporated into the SELECT.

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Chapter 24
Selenium as a Cancer Preventive Agent

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Abstract The known metabolic functions of selenium, which appear to be discharged by a fairly small number of selenoproteins, do not fully explain the anticarcinogenic effects of selenium, particularly those observed in response to selenium-supplementation of non-deficient subjects. While anticarcinogenic roles are possible for at least some selenoproteins, i.e., those involved in antioxidant protection, redox regulation and hormonal regulation of metabolism, anticarcinogenic effects of selenium have been shown in individuals with apparently full selenoenzyme expression, suggesting additional mechanisms. Seleno-compounds have been shown to alter gene expression, affect DNA damage and repair, affect cell-signaling pathways, inhibit cell proliferation, stimulate apoptosis, and inhibit metastasis and neo-angiogenesis. Underlying these effects are metabolic activities of various seleno-metabolites: redox cycling, modification of protein-thiols, and methionine mimicry. It is, therefore, likely that selenium deprivation may increase cancer risk by compromising selenoprotein expression, and that supranutritional exposures to Se reduce cancer risk in non-deficient subjects.

24.1 Evidence for a Selenium-Cancer Link

24.1.1 Emergence of Evidence

The nutritional essentiality of selenium (Se) was recognized in the late 1950s when the element was found to spare vitamin E in the diets of rats and chicks for the prevention of vascular, muscular, and/or hepatic lesions [1]. That Se may be anticarcinogenic

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was suggested a decade later based on empirical observations of inverse relationships of cancer mortality rates and blood and forage crop Se contents in the United States [2, 3]. Subsequent evidence has shown Se status to be inversely associated with cancer risk, cancer cases tending to have lower prediagnostic serum Se levels than controls, and Se-treatment can reduce tumor yields in Se-adequate animal models [4]. Almost all have shown that supranutritional Se doses reduced the tumor yields.

24.1.2 Clinical Trial Evidence

Several clinical trials have been conducted to determine the efficacy of Se in reducing cancer risk in humans (Table 24.1). Those results [4] include reports of protection by selenite-enriched table salt against primary liver cancer [5, 6], and by Se-containing, multiagent supplements against esophageal cancer [7–12], precancerous oral lesions [13, 14], and prostate cancer [15].

The strongest evidence of anticancer efficacy of Se in humans comes from the NPC[1] Trial [16–20], a randomized, placebo-controlled clinical trial that tested the hypothesis that a daily oral dose of Se (200 μg/day as Se-enriched yeast) could reduce the rate of recurrent non-melanoma skin cancer in a high-risk group of 1,312 older Americans. The initial results [16] showed no effects on the incidences of basal or squamous cell carcinoma (BCCs or SCCs) of the skin; however, they showed significant reductions in risks to total cancer, cancer deaths and carcinomas of the prostate, lung, colon-rectum, and total non-skin. Follow-up analyses [17, 18] supported those findings and showed that, while Se-treatment did not affect BCC risk, it appeared to delay diagnosis of the first BCC [18]. The Trial showed that, for men with plasma prostate specific antigen (PSA) levels <4 ng/mL, Se-treatment caused a 65% reduction in prostate cancer risk, while for men with PSA >4 ng/mL, there was no protection [20], suggesting protection only in early stage(s) of carcinogenesis. Protection was noted mostly (86% risk reduction) among subjects with baseline plasma Se levels <106 ng/mL, i.e., in the lowest tertile of the cohort,[2] to a lesser extent (61% reduction) among those in the middle tertile (107–123 ng/mL), but not for those in the highest tertile (>123 ng/mL) [17].

The largest clinical trial of Se conducted to date, SELECT[3] [21], found no protection by Se against prostate cancer over a 5-year intervention period. That trial, while large (>32,000 subjects) used a cohort of relatively high baseline Se status (plasma Se 136 ng/mL). For this reason, those negative findings are consistent with those of NPC [20], which found Se to have no cancer-protective effect for subjects with relatively high plasma Se levels.

[1] Nutritional Prevention of Cancer.

[2] The cohort level was 114 ± 23 ng/mL; very few subjects had levels <80 ng/mL, the level Nève [20] found to be the upper limit for GPx responses to supplemental Se in healthy adults. These levels suggest an average Se intake of ≥85 μg/day, or at least 155% of the RDA [21].

[3] Selenium and Vitamin E Cancer Trial.

Table 24.1 Results of randomized clinical trials of Se for cancer prevention

Trial (country)	Treatment			Period (year)	Subjects	Results – RR-cancer site	References
	Dose μg Se/day	Se form	Other agents				
NPC (USA)	200	Se-yeast	None	13	1,313	0.63 – All sites 0.51 – Prostate 0.46 – Colorectal	[16, 20]
SELECT (USA)	200	SeMet	None	5	32,400	0.99 – Prostate N.S.	[21]
Qidong study (China)	200	Se-yeast	None	2	2,065 (HSA^+)	0.51 – Liver	[5, 6]
Qidong table salt study (China)	15 ppm, salt	Selenite	None	3	20,847	0.63 – Liver	[6]
Linxian general population trial (China)	50	Se-yeast	Vitamin E β-carotene	5.25	29,584	0.91 – All sites 0.87 – Cancer mort. *10 year follow-up* 0.89 – Gastric cancer mortality 0.83 – Esophageal cancer mortality	[7–9] [10]
Linxian esophogeal dysplasia trial (China)	50	Se-yeast	Vitamin E β-carotene	5.25	3,318	0.93 – All (N.S.) 0.96 – Cancer mortality (N.S.)	[7, 8]
SU.VI.MAX (France)	50	Selenite	Vitamin E Vitamin C β-carotene	8	12,749	0.51 – Prostate	[15]

RR relative risk

24.2 Mechanisms of Selenium Anticarcinogenicity

24.2.1 General Theory of Selenium-Anticarcinogenesis

That Se deficiency may increase cancer risk might be expected on the basis of the known functions of selenoenzymes in antioxidant protection, the glutathione peroxidases (GPxs) and thioredoxin reductases, (Txnrds), as mutagenic oxidative stress is thought to be a major factor in the initiation of human carcinogenesis. However, it is clear that Se intake in *excess* of the nutritional requirement can inhibit tumorigenesis: antitumorigenically effective Se-exposures in animal models (≥1.5 mg/kg diet) have often been much greater than those required to prevent Se deficiency or to support maximal expression of selenoproteins (<0.2 mg/kg diet). We proposed a theory of Se-anticarcinogenesis accommodating these various findings [22]. Our multitiered model (Fig. 24.1) links known features of Se metabolism to anticarcinogenesis through underlying actions of Se-metabolites affecting cellular mechanisms.

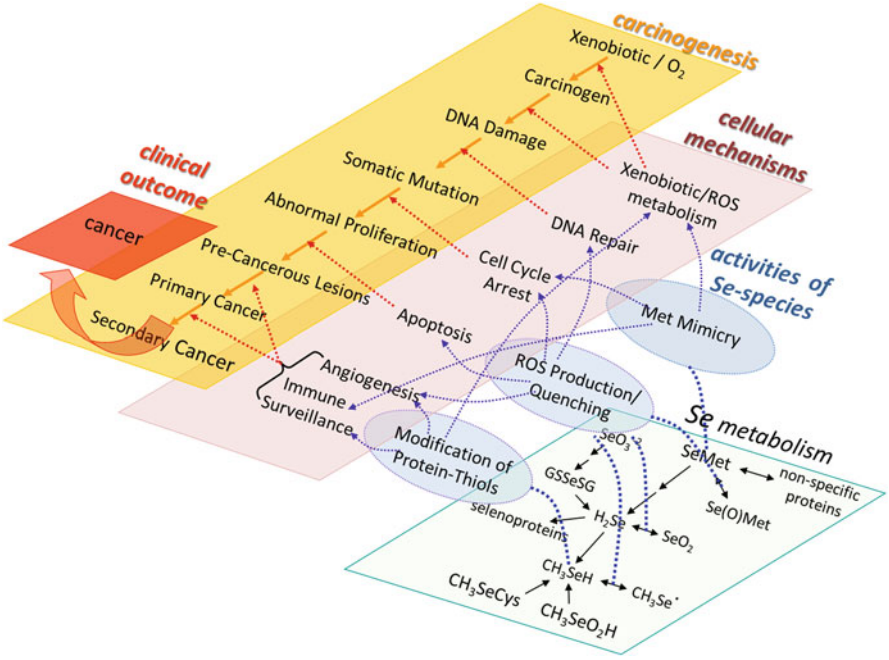

Fig. 24.1 Theory of Se-anticarcinogenesis. Figure is taken from Jackson and Combs [22] with permission. SeO_3^{-2} selenite; *SeMet* selenomethionine; *Se(O)Met* selenomethionine selenoxide; *GSSeSG* selenodiglutathione; H_2Se hydrogen selenide; SeO_2 selenium dioxide; CH_3SeH methylselenol; $(CH_3)_3Se^+$ trimethylselenonium; CH_3SeCys methylseleno-cyeteine; CH_3SeO_2H methylseleninic acid

24.2.1.1 Roles of Selenoenzymes

Etiologies of some cancers are believed to involve mutagenic oxidative stress, thus antioxidant selenoproteins are expected to have anticarcinogenic impact by removing DNA-damaging H_2O_2 and lipid hydroperoxides, blocking production of reactive oxygen species (ROS) and malonyldialdehyde, and regulating the redox signaling system critical to growth of many cancers. Partially through these actions, Se has been shown to modulate p53 activity by redox modification of cys275,277 mediated by Ref-1, enhancing repair of DNA damage [23, 24]. As p53 suppresses expression of angiogenic factor VEGF [25] and induces angiogenesis-suppressing thrombospondin-1 [26], a Se-mediated increase of p53 could play a pivotal role in switching off angiogenesis in early lesions.

The association of selenoprotein allelic variation with cancer risk responses to Se suggests the involvement of one or more selenoprotein in cancer protection. A single nucleotide polymorphism (SNP) at codon 198 of human GPx1, resulting in a leucine-for-proline substitution, has been associated with increased risks of cancers of the lung [27], breast [28], head and neck [29], bladder [30] and prostate [31]. The 198-leucine genotype may be less responsive to Se exposure than the 198-proline genotype [27], suggesting that increased cancer susceptibility of individuals with that allele may involve their reduced ability to utilize Se for selenoprotein expression.

The frequency of SNPs in the promoter of selenoprotein P (Sepp1) [32] is similar in colorectal adenoma patients and controls [33], but malignant colon tissues showed lower levels of Sepp1 than adjacent normal tissue [34, 35]. Prostate cancer cells also have low Sepp1 expression, although they express the Sepp1 transporter (ApoER2) [36]. The SNP 25191 of Sepp1 predicts increase in plasma Se level with Se-supplementation [37], most of which is associated with Sepp1. Apart from the effects of SNPs, the risk of prostate cancer decreased by 11% for every 10 μg/mL of plasma Se increase [38].

Jablonska et al. [31] found lung cancer risk related to SNPs of the 15 kDa selenoprotein (Sep15); individuals with the 1125AA genotype appeared to benefit most from higher Se status. Reduced expression of Sep15 has been observed in malignant liver and prostate [39], and malignant mesothelioma cells, which also showed resistance to Se-induced growth inhibition [40]. Reduced expression of Sep15 by mouse colon cancer cells (short hairpin RNA) decreased expression of gene pathways involved in cell growth and proliferation, [41] and reduced the cell's ability to produce metastatic tumors upon injection into surrogate mice. Lewis lung carcinoma cells were not affected by Sep15 knockdown, indicating the tissue specificity of the Sep15 effects.

The selenoprotein, methionine sulfoxide reductase A (MsrA), which reduces oxidized protein methionyl residues, is downregulated in a number of human breast cancers [42], resulting in increased tumor aggressiveness and derepression of the phosphoinositide proliferation pathway due to decreased levels of PTEN tumor suppressor protein.

Thus, one or more selenoproteins may have anticarcinogenic roles that would be limited under conditions of insufficient Se supply and by mutations affecting incorporation of selenocysteine into selenoproteins. Therefore, correction of nutritional Se deficiency can be expected to have anticarcinogenic effects; however, that hypothesis has not been extensively tested.

24.2.1.2 Roles of Se-Metabolites

Anticarcinogenic activities have been demonstrated for several intermediary metabolites of Se: selenodiglutathione (GSSeSG), the reductive metabolite of the oxidized inorganic salts (selenite, selenate); hydrogen selenide (H_2Se), the common intermediate of that reductive pathway and of the catabolism of selenoamino acids; methylated metabolites of selenide ($[CH3]_xSeH$), excretory forms; and selenomethionine (SeMet), a methionine analog and dominant food form of Se. These metabolites execute several functions that effect Se-anticarcinogenesis at underlying and intermediate levels (see Fig. 24.1).

24.2.2 Underlying Mechanisms

24.2.2.1 Redox Cycling

Redox cycling and covalent protein-thiol modification appear to constitute competing pathways available to Se. The disposition of Se-metabolites through these pathways would appear to determine their biological effects. Selenite, diselenides, and the oxidation product of H_2Se, selenium dioxide (SeO_2), are reduced by GSH producing selenolate ion (RSe^-) and oxidized glutathione (GSSG) [43]; in the presence of molecular oxygen $(O_2)^-$, they can redox cycle to deplete GSH and produce the ROS, superoxide (O_2^-) and hydrogen peroxide (H_2O_2) [44]. Selenite elicits biological effects through cell damage responses initiated by such ROS, leading to DNA damage and thiol modification [45, 46]. This appears to be the basis of: (i) caspase-independent apoptosis in selenite-treated cervical cancer cells, suppressible by antioxidants and exacerbated by prior GSH depletion [47]; (ii) DNA damage by chronic selenite feeding [48]; and (iii) increased Txnrd associated with hepatotoxic selenite doses [49]. MeSeH can also redox cycle; but the anticarcinogenic effects of MeSeH-precursors are qualitatively different from H_2Se-precursors, indicating different mechanisms.

Free and peptide-bound forms of SeMet scavenge ROS and are regenerated nonenzymically by GSH; the SeMet/Se(O)Met couple may, thus, serve as a cellular defense mechanism. Met(O) formation can alter protein activity; calmodulin kinase is activated by ROS from angiotensin signaling [50]. SeMet is more readily oxidized than Met [51], thus Met → SeMet substitution may sensitize regulatory proteins to ROS.

24.2.2.2 Modification of Protein-Thiols

Application of Se compounds alters protein-thiol redox status, driving cell-signaling mechanisms [52]; products derived from both H_2Se and MeSeH react with protein-thiols, resulting in covalent adduction, altering protein activity. Similarly, thiols in cell surface proteins may react with oxidized Se to become crosslinked [53]. The dominant species of both intracellular H_2Se and MeSeH is likely to be a mixed selenosulfide of GSH, i.e., GSSeSG for H_2Se and MeSeSG for MeSeH. Se-species can act through protein-thiol modification; for example SeMet-treatment affected the expression of redox-sensitive proteins of prostate cancer cells (and see reference [54]).

Se-induced inhibition, presumably by such reactions, has been demonstrated for several relevant enzymes: ribonuclease [55], Na^+, K^+-ATPase [56], and PKC [57]. Inhibition of PKC would be expected to trigger a number of downstream effects including cell cycle arrest, apoptosis, and angiogenic switch regulation. Some of these effects have been reported after treatment with MeSeH-precursors, including decreased cdk2 kinase activity [58] and inhibition of vascular endothelial MMPs and VEGF expression [59]. These effects target certain factors, rather than affecting the far-reaching perturbations in cellular redox control exerted by Se-proteins, as Se-metabolites are present at much lower levels and are not always catalytic in action.

24.2.2.3 Methionine Mimicry

SeMet competes with Met in general metabolism including protein synthesis. It can charge $tRNA^{Met}$, resulting in substitution of SeMet for Met in proteins [60], trapping Se and limiting its conversion to anticarcinogenic H_2Se and MeSeH. Li et al. [61] showed that SeMet raised tumor Se levels eight-fold more than MeSeH-precursors did, but failed to affect tumor burden. This may be relevant to cancer management under circumstances of restricted Met intake [62]. SeMet is converted to analogues of Met-metabolites, and as such is more effective than Met as a substrate for Met-adenosyl transferase [63], forming Se-adenosylselenomethionine (SeSAM). Further, SeSAM is a better substrate for methyltransferases than *S*-adenosylmethionine [64]; these apects of Se metabolism may be relevant to anticarcinogenesis, as methyltransferases play roles in gene silencing, repair of damaged proteins, and activation of oncogenes.

24.2.3 Intermediate Mechanisms

24.2.3.1 DNA Damage and Repair

Selenite can cause DNA damage in both malignant and normal tissues [48, 65]. Letavayová et al. [66] found selenite to induce DNA double-strand breaks and frame-shift deletions in yeast, effects not seen for SeMet or a MeSeH-precursor.

Se has been shown to induce the ATM mismatch repair pathway by facilitating an interaction with hMLH1 in colorectal cancer cells, allowing cells to respond to and correct nascent DNA mutations [67]. The findings of Hu et al. [68] suggest that DNA repair secondary to damage can impair carcinogenesis: a high-Se milk protein enhanced the removal of carcinogen-induced DNA lesions in mice. That Se-yeast failed to produce comparable effects suggests an active principle other than SeMet.

24.2.3.2 Cell Cycle and Apoptosis

SeMet or MSA increases expression of genes associated with apoptosis in transformed cell lines, and androgen-regulated genes in prostate cells[69]. High Se intakes can arrest the cell cycle in different ways: selenite in S-phase leading to caspase-independent apoptosis; methylated Se in G1-phase leading to caspase-mediated apoptosis [65]. In contrast, SeMet transiently activates Akt before inactivating it in a PTEN-dependent fashion resulting in its degradation through caspase and proteosome pathways [70]. Rudolf et al. [47] showed that selenite can activate a p53-dependent pathway, increasing p21 and phosphorylated p53, as well as a p38 pathway leading to accumulation of Bax. The product of the thiol-dependent reduction of selenite, GSSeSG, has been shown to inhibit the DNA-binding of AP-1 [71], inhibit cell proliferation [72], and enhance apoptosis [73]. Wang et al. [74] showed that methylated Se produced transient upregulation of p21/CIP1 and p27/KIP1 in G1-arrested endothelial cells, with a modest increase in p16/INK4a, indicating a link between cell cycle and Se-antiangiogenesis. Differential sensitivity has been found for cell types to apoptosis induced by methylated Se, on the order of: breast carcinoma cells > hepatoma and neuroblastoma cells > colon cancer cells and nonmalignant mammary epithelial cells [75]. Hu et al. [76] showed the response to methylated Se involves downregulated expression of two anti-apoptosis proteins, Bcl-XL, and survivin. The MeSeH-precursor, CH_3SeCys, can inhibit mammary cell growth, arresting cells in the G_1 or early S-phase and inducing apoptosis in a caspase-dependent manner involving mitochondrial cytochrome C release, poly (ADP-ribose) cleavage, and nucleosomal DNA fragmentation [77, 78]. In cell lines that lack functional p53, the pro-apoptotic action of methyl-Se is caspase-dependent [79, 80]. In addition to apoptotic mechanisms, subapoptotic levels of methyl-Se have been shown to reduce androgen receptor protein expression [81], reduce PSA expression, and cause rapid PSA degradation [82] and inhibit androgen-stimulated PSA promoter transcription [83–85], suggesting a unique basis for the apparent sensitivity of the prostate to Se-anticarcinogenesis.

24.2.3.3 Metastasis and Angiogenesis

Both selenite and SeMet can inhibit the growth of secondary tumors in animal models [83, 84]. Hurst et al. [85] showed that this involves altered collagen gene expression preferentially affected by methylated Se. Kim et al. [86] showed SeMet decreased

tumor cell invasion by decreasing ROS and blunting Akt-dependent matrix metalloproteinase secretion. In a murine model of melanoma invasiveness, Se application did not reduce primary tumor size, but did reduce tumor metastasis and in vitro cell culture growth, suggesting a role in periods of adaptation during metastasis [87]. The MeSeH-precursor MSA reduced NFKb protein expression, resulting in decreased IL-6, MCP-1, COX-2, and iNOS expression in osteoblasts challenged with conditioned media from breast cancer cells. This implies that osteoblast/osteoclast-induced bone demineralization, which occurs with cancer metastasis to bone, may be ameliorated by Se-treatment [88].

MeSeH-precursors inhibit expression of matrix matalloproteinase-2 in vascular endothelial cells and of vascular endothelial growth factor in cancer cells [77, 78, 89, 90]. This suggests that methyl-Se inhibits cellular proliferation and survival of activated endothelial cells by inhibiting neo-angiogenesis. Jiang et al. [65] found Se-treatment to impair microvascular development of tumors. They also found methyl-Se to reduce microvessel density in tumors developing from prostate cancer cell xenografts by inducing cell cycle arrest in microvascular endothelial cells [76]. Li et al. [61] found methylated Se more effective than selenite in this regard, an effect that Bhattcharya et al. [91] showed can provide therapeutic synergy with anticancer drugs, finding CH_3SeCys to reduce vascular permeability of carcinoma xenografts and consequent tumor uptake of doxorubicin.

24.3 Conclusions

Se compounds, including those in foods, can inhibit and/or delay carcinogenesis. These effects may involve the protective, nutritional functions of Se as an essential constituent of metabolically important selenoenzymes; such functions may be compromised in Se-deficient individuals and those with allelic variants of certain selenoproteins. In addition, certain Se-metabolites appear to inhibit carcinogenesis through mechanisms unrelated to the nutritional functions of Se. These appear to involve ROS production, protein-thiol modification and replacing Met in critical proteins, resulting in alterations of DNA damage/repair, cell cycle/apoptosis and metastasis/angiogenesis. Because most ingested forms of Se can be metabolized to one or more of these species, competing metabolic pathways would appear to underlie differences in their relative anticarcinogenic activities. Understanding the interplay of these processes with individual metabolic differences will be necessary to determine who will likely benefit from increased Se intake.

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Chapter 25
Selenoproteins Harboring a Split Personality in Both Preventing and Promoting Cancer

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Abstract Selenium has long been known to have a role in preventing cancer, but only in recent years has a deficiency in this element also been shown to function in preventing cancer. Selenoproteins have also been shown to serve as selenium-containing components in cancer prevention, but we are learning that specific members of this protein class can also function in cancer promotion. The involvement of two of these selenoproteins, thioredoxin reductase 1 and the 15 kDa selenoprotein, in promoting cancer is examined in this chapter.

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25.1 Introduction

Selenium has been associated with many health benefits in humans and other mammals that include roles as a cancer chemopreventive agent, a preventive of heart disease, other cardiovascular diseases and muscle disorders, and in mammalian development, slowing the aging process, delaying the onset of AIDS in HIV-positive patients, and inhibiting viral expression [1, 2]. The benefit of selenium in health that has received the most attention by far is its role in preventing cancer [1, 2]. Whether small molecular weight selenium-containing compounds (selenocompounds) or selenium-containing proteins (selenoproteins), or both of these classes of agents, are responsible for the health benefits of this element in cancer has been the subject of considerable debate in the selenium field [3–9]. Evidence supporting both proposals has been reported [3–12], but the emphasis of the research in this area appears to be shifting in favor of the role of selenoproteins (see other chapters in this book) of which there are 25 selenoprotein genes in humans and 24 in rodents [13]. There are, of course, more selenoprotein forms in humans and rodents than genes due to transcription variants, different start signals for initiating selenoprotein synthesis and splicing variants in a single mRNA (e.g., human thioredoxin reductase 1 (TR1) has at least six known transcription variants [14] and glutathione reductase 4 (GPx4) has alternative transcripts with two distinct promoter regions [15]). In addition, multiple Sec UGA codons may occur in a single mRNA that results in both partial termination and partial read-through at UGA codons downstream of the initial selenocysteine (Sec) codon (i.e., selenoprotein P (SelP)). Partial termination of SelP at the second and third Sec codons yields two truncated, likely functional, proteins [16].

Of the many selenoproteins known in mammals [8], there are at least three that have a split personality in that they apparently have roles in both preventing and promoting cancer. These selenoproteins are TR1 and the 15 kDa selenoprotein (Sep15), which are described below, and glutathione peroxidase 2 (GPx2), which is described in Chap. 21.

25.2 TR1

TR1 has long been recognized as one of the major antioxidant and redox regulators in mammalian cells [8, 13], and more recently, as an essential protein in mammalian development [14]. Both the selenium-containing form, wherein the selenium atom occurs at the active site as the amino acid, Sec, and the corresponding cysteine-containing form in which cysteine is inserted in place of Sec, are known. Since a major function of TR1 is to control the redox state of thioredoxin and therefore protect normal and malignant cells from oxidative stress, it would seem that TR1's role in both promoting and preventing cancer may be, at least in part, the same. That is, TR1 prevents oxidative damage in normal cells by helping maintain them in a

healthy state, and regulates redox homeostasis in cancer cells by assisting them in overcoming the burdens of oxidative damage. These plausible roles of TR1 in normal and cancer cells are further discussed below.

There are other activities of TR1 that provide further evidence of its role in normal cells as a cancer preventive agent. For example, this selenoenzyme is known to activate the p53 tumor suppressor and to have other tumor-suppressor activities [17], and is specifically targeted by carcinogenic electrophilic compounds [18, 19]. Thus, TR1 clearly is an anticancer protein.

TR1 also has roles in promoting cancer. This selenoenzyme has been shown by several investigators to be overexpressed in many cancer cell lines and cancers [20–23]. In addition, cancer-related properties of malignant cells have been shown to be altered by specifically targeting and inhibiting TR1 activity by using a variety of anticancer drugs and potent inhibitors [20–25]. Furthermore, rendering cancer cells TR1 deficient has been shown to reverse several of the cancer characteristics [26, 27]. Thus, TR1 clearly is a procancer protein.

A major focus of our laboratories over the past several years has been to elucidate the underlying cellular mechanisms of how TR1 can have a role in preventing cancer and then switch its function to one of promoting cancer [26–28]. We initially targeted the removal of TR1 in a mouse lung cancer cell line, designated LLC1 [26]. More than 90% of TR1 expression was lost in LLC1 cells carrying the knockdown vector compared to control cells carrying the same vector except that it lacked the targeting sequence (Fig. 25.1 a1). As a result, the morphology, expression of two cancer-related mRNAs, *Hfg* and *Opn1*, anchorage-independent growth and other of the cells' malignant properties were altered to become more similar to those of normal cells following the downregulation of TR1 expression [26]. Injection of TR1-deficient LLC1 cells into wild type, genetically compatible mice resulted in a dramatic reduction in tumor development in mice injected subcutaneously (Fig. 25.1 a2) or in lung metastasis in mice injected in the tail vein (Fig. 25.1 b1) compared to wild-type mice injected with the control vector. Tumors arising in the flank were much smaller in size than the corresponding tumors that developed in mice injected with the control vector. Most importantly, the smaller tumors in mice injected with the TR1 knockdown vector had lost the vector (Fig. 25.1 a3) demonstrating that tumor development caused by LLC1 cells is dependent on TR1 expression. Pathological analysis of lungs of mice injected with TR1-deficient cells had no detectable pathological changes, while those injected with the control TR1-sufficient cells showed extensive malignant alterations (Fig. 25.1 b2).

The above studies demonstrate a direct dependency of the malignancy process on an enriched TR1 expression in LLC1 cells. They do not, however, show the underlying mechanism(s) of how TR1 is responsible for promoting and/or sustaining this process. One major drawback in pursuing an elucidation of TR1's role in this process is that LLC1 cells do not have a parental, normal cell line for comparison. We therefore undertook a study of the knockdown of TR1 in DT cells, which represent an oncogenic *k-ras* cell line derived from parental, normal NIH 3T3 cells [27]. Interestingly, the morphological changes that resulted in DT cells following the targeted removal of TR1 were more characteristic of the parental NIH 3T3 cells than

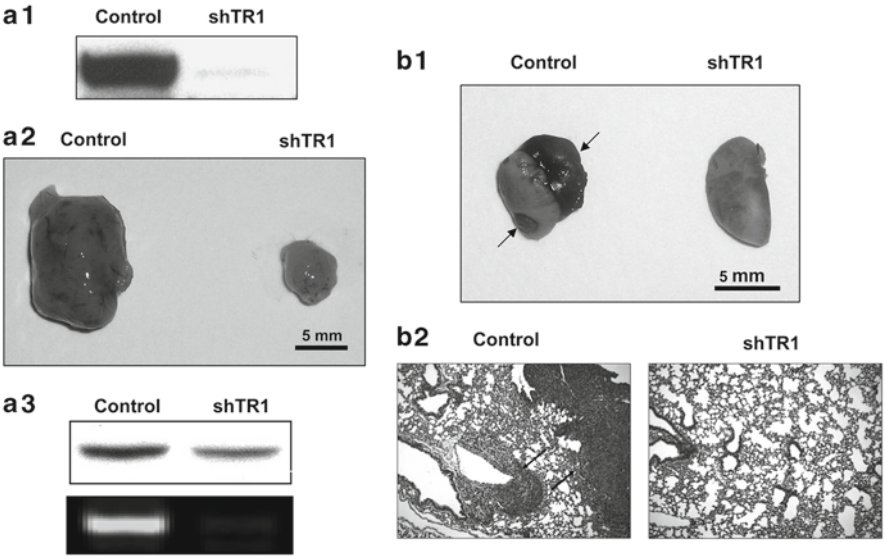

Fig. 25.1 Tumorigenicity and metastasis of TR1-deficient LLC1 cells. In (**a1**) western blot analysis of TR1 in LLC1 cells encoding the control or TR1 knockdown construct is shown. TR1 was more than 90% removed in knockdown cells. In (**a2**) tumors removed from mice that had been injected subcutaneously with LLC1 cells expressing either the control or TR1 knockdown construct are shown. Tumor size was much larger in mice injected with the control vector. In (**a3**) western blot analysis of TR1 levels that were present in the two tumors from **a2** (*upper panel*) and PCR analysis of genomic DNA isolated from these tumors (*lower panel*) are shown. TR1 is present in the smaller tumor (*upper panel*) as the TR1 knockdown vector is lost in the tumor from mice injected with the knockdown vector (*lower panel*). The data clearly show that both tumors express TR1 and that the knockdown vector is lost in the smaller tumor that resumes TR1 expression; and most importantly, that TR1 expression is essential for tumor growth. In (**b1**) tumor formation as a result of metastasis in the lung of the mouse injected with cells encoding the control vector and the normal appearing lung from the mouse injected with cells encoding the TR1 knockdown vector are shown. In (**b2**) tissue slices are shown from the lungs in (**b1**) that were analyzed for pathological changes; and the lung from the mouse injected with control vector manifested severe pathological abnormalities while the lung from the mouse injected with the knockdown vector appeared normal. See [26] for details. The figure was taken from [26] and modified slightly as shown here with permission from the *Journal of Biological Chemistry*. In the figure, shTR1 designates small hairpin RNA directed towards knocking down TR1 expression

DT cells transfected with the control vector. Compared to the TR1 overexpressing DT control cells, the corresponding TR1-deficient cells were found to have (i) lost their self-sufficiency growth properties, (ii) a defective progression in the S growth phase, and (iii) a decreased expression of DNA polymerase α, which is important in DNA replication [27]. Studies using DT TR1-deficient and sufficient cells and the corresponding parental (normal) NIH 3T3 cells, have provided considerable insight into the direct role of this selenoenzyme in many of the requirements governing the malignancy process and suggest possible novel avenues for inhibiting cancer.

25.3 Sep15

Sep15 was originally reported in 1998 as a 15 kDa selenium-containing protein in human T cells [29] and was subsequently shown to occur in a complex with UDP-glucose:glycoprotein glucosyltransferase (UGTR) [30]. UGTR is localized in the endoplasmic reticulum in mammalian cells and is involved in the quality control of protein folding [31]. The gene for Sep15 resides on chromosome 1p31 and contains five exons and four introns [32]. Recent studies on Sep15 have provided various insights into its structure and possible function. NMR structural analysis of Sep15 suggests a role in the reduction or isomerization of disulfide bonds of glycoprotein substrates of UGTR [32].

There are numerous lines of evidence suggesting that Sep15 has a role in cancer prevention. For example, earlier studies had shown that Sep15 exhibits redox properties [32], is structurally similar to the thioredoxin superfamily [33], belongs to the class of thioldisulfide oxidoreductase-like selenoproteins [33], and its gene exists on a chromosomal locus that is often deleted or mutated in human cancers [32, 34]. The highest levels of Sep15 expression were reported to occur in human and mouse liver, brain, testes, and prostate, but these levels were found to be reduced in hepatocarcinoma, lung cancer, and a prostate cancer cell line [32]. Furthermore, two polymorphic sites were found in *sep15* at nucleotide positions 811 (C/T) and 1125 (A/G) in the 3′UTR, and the latter polymorphism resides in the Sec Insertion Sequence (SECIS) element [29]. Interestingly, these two polymorphisms show different responses to selenium supplementation with regard to the efficiency of Sec incorporation into the growing polypeptide chain of Sep15 during translation [35]. Differences in frequencies of these two alleles were found to be associated with breast or head and neck tumors within African Americans and among different ethnic groups [36]. Recently, the A/T polymorphism in *sep15* was correlated with an increased risk of rectal cancer in Korean men [36].

There are additional studies that also suggest a role of Sep15 in cancer prevention. Malignant mesothelioma cells that were found to be sensitive to selenite toxicity have decreased Sep15 expression and further downregulating this protein by targeting its knockdown demonstrated that the cells were less sensitive to selenite [37]. In addition, this malignant cell line containing the A^{1125} polymorphic allele was also less sensitive to the effects of selenite than the corresponding cell line carrying the G^{1125} allele [38]. An increase in lung cancer risk was observed among smokers encoding a GG^{1125} or GA^{1125} genotype compared to those carrying an AA^{1125} genotype [38].

Many of the above studies suggest a role of Sep15 in cancer protection. However, other studies suggest a role of Sep15 in cancer promotion. The National Cancer Institute maintains a Developmental Therapeutics Program Database (http://dtp.nci.nih.gov/mtweb/targetdata) that screened 60 human tumor cell lines for molecular targets. An analysis of the data on these cells lines revealed an increase in Sep15 expression in colon cancer cell lines relative to other selenoproteins and to other cancers. These observations suggest that, by analogy to many other malignant cells, wherein TR1 is overexpressed and is used as a target for cancer therapy, Sep15

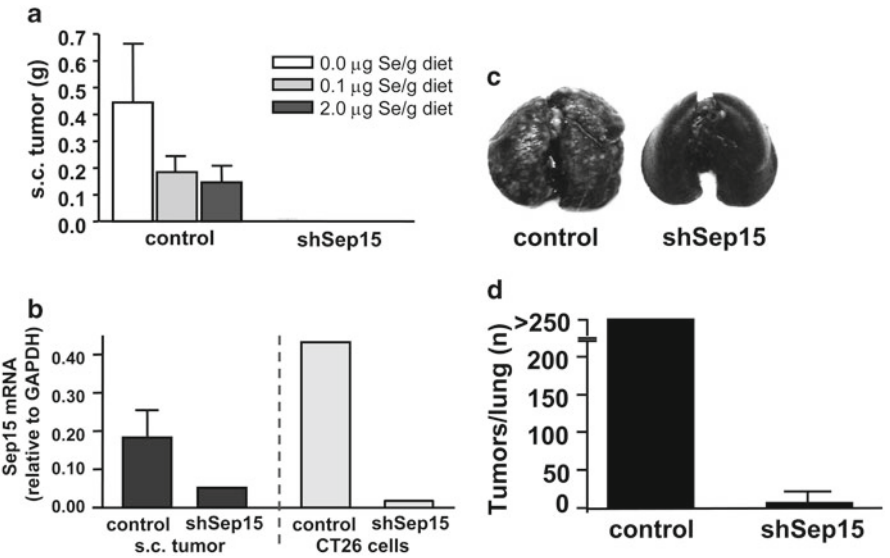

Fig. 25.2 Tumorigenicity and metastasis of Sep15-deficient CT26 cells. In (**a**) the weights of tumors removed from mice fed selenium-deficient (0 ppm selenium in the feed), selenium-adequate (0.1 ppm selenium) and selenium-enriched diets (2.0 ppm selenium) that were injected in the flank with the control or Sep15 knockdown vector are shown. Mice maintained on the selenium-deficient diet clearly had larger tumors. In (**b**) quantitative RT-PCR analysis of Sep15 mRNA from tumors of mice injected with control or Sep15 knockdown vector (*left two bars*) and mRNA from control and Sep15 deficient cells (*right two bars*) are shown. The amount of Sep15 knockdown vector relative to the amount of control vector is proportionally much higher in tumors formed in the flanks of mice than in Sep15 control and deficient cells. In (**c**) tumor formation as a result of metastasis in lungs of mice injected with control or Sep15-deficient cells. The tumors are shown in *white* on the surface of the lungs as a result of the black ink stain used for injection [39]. In (**d**) tumors on the surfaces of the lungs were counted and lungs with >250 lesions were designated as 250. The data are from three independent experiments. See [39] for details. The figure was reproduced from [39] with the permission of *Cancer Prevention Research*. In the figure, shSep15 designates small hairpin Sep15 RNA directed towards knocking down Sep15 and s.c. designates subcutaneous

might be a target for cancer therapy in colon cancer cells. We therefore undertook a study to evaluate the role of Sep15 in colon cancer. Sep15 mRNA was targeted for removal in a mouse colon cancer cell line, CT26, that had elevated Sep15 levels [39]. The resulting Sep15-deficient cells had a reduced growth rate and their ability to grow in soft agar was also reduced. Injection of the CT26 cells encoding the Sep15 knockdown vector into wild type, genetically compatible mice maintained on selenium-deficient, selenium-sufficient, or selenium-supplemented diets resulted in a large reduction in tumor number and size in mice injected subcutaneously compared to mice injected identically, but with control cells (i.e., those encoding the control vector). Fourteen of 15 mice injected with control cells developed tumors and the tumor size varied depending on the selenium level in the diet (Fig. 25.2a). Only one tumor was observed in mice injected with the Sep15 knockdown vector,

and an examination of this tumor for the presence of the knockdown vector showed that it had been lost (Fig. 25.2b). Similar to the experiments carried out with the knockdown of TR1 in LLC1 cells described above, wherein tumor development was found to be dependent on TR1 expression, it also appeared that tumor development in the CT26 cells was dependent on Sep15 expression [39]. The Sep15 deficient, CT26 cell line also manifested a dramatically reduced metastatic ability in forming tumors in the lungs of mice compared to control cells when the two cell lines were injected into mice intravenously (Fig. 25.2c, d) [39].

To elucidate the possible means of how Sep15-deficient CT26 cells can have such pronounced effects on colon cancer cell growth, anchorage-independent cell growth in soft agar, tumorigenicity and metastasis, these Sep15 knockdown cells were compared to the corresponding Sep15-expressing CT26 cells using microarray analysis [39]. Those genes that were the most significantly up- or downregulated indicated primarily biological functions related to cancer, and cellular growth and proliferation as the main molecular and cellular functions affected. The gene with the highest upregulation (13.5-fold increase in Sep15-deficient cells) encoded for the cyclin B1-interacting protein 1, which was subsequently validated using real-time RT-PCR. Fluorescence-activated cell sorting analysis confirmed the G_2-M cell arrest in Sep15-deficient cells suggested by microarray analysis. It appeared that Sep15 loss resulted in reversal of the original cancer phenotype of the murine colon cancer cells that was mediated, at least in part, by upregulation of cell cycle-related genes, and likely resulting in subsequent G_2-M cell cycle arrest.

Currently, we are investigating the effects of Sep15 loss in colon cancer in vivo, quantitating chemically induced aberrant crypt foci development in colonic epithelia of Sep15 knockout mice. Preliminary results indicate that lack of Sep15 is protective against formation of aberrant crypt foci (PA Tsuji, BA Carlson, S Naranjo-Suarez, M-H Yoo, X-M Xu, DE Fomenko, VN Gladyshev, DL Hatfield, CD Davis, submitted for publication). Furthermore, a cytokine-regulated GTPase was highly upregulated in Sep15 knockout animals and its possible link to Sep15 is being elucidated further.

25.4 Conclusions and Other Roles of Selenium in the Cancer Process

It is most interesting that the malignancy of a lung cancer mouse cell line, LLC1, appeared to be dependent on TR1 expression, and similarly, a mouse colon cancer cell line, CT26, appeared to be dependent on Sep15 expression. If, indeed, these cell lines are being driven by two different selenoproteins, which also have been shown to have roles in cancer prevention, a question arises as to how TR1 and Sep15 can have such overall opposing roles in their behavior. One seemingly plausible explanation is that their roles in cancer and normal cells are much the same in that they help maintain a balanced redox system. However, the burdens of oxidative stress are most certainly far greater, and the metabolic demands far broader, in maintaining an

efficient redox system in cancer cells than in normal cells. Hence, the higher levels of expression of these two selenoproteins in their respective malignant cell lines are likely a reflection of greater demands for the services of these two selenoproteins that may include expanded roles in cancer. In support of such a proposal, we have recently shown that TR1 assumes new roles that appear to be in large part independent of its known role in reducing thioredoxin in DT cells suffering from selenite toxicity as compared to the parental, NIH 3T3 cell line or to DT cells not exposed to selenite (R Tobe, M-H Yoo, N Fradejas Villar, BA Carlson, S Calvo, VN Gladyshev, DL Hatfield, submitted for publication).

To our knowledge, there are only two studies showing that selenium deficiency can also play a role in cancer prevention. The progression of peritoneal plasmacytoma (PCT) in mice was found to be dependent on chronic peritoneal inflammation following injection with pristane [40]. Interestingly, virtually no PCT occurred in selenium-deficient mice injected with pristine, whereas about 40% of the mice fed selenium-adequate diets developed PCT. In another study, liver tumor formation was inhibited in TGFα/c-Myc transgenic mice, which are well known to develop hepatomas [41], maintained on a selenium-deficient diet compared to mice maintained on the same diet but supplemented with 0.1 or 0.4 ppm selenium [42]. These studies are surprising, as are those showing that specific selenoproteins have roles in promoting cancer, in that selenium has long been heralded as a cancer preventive. It is of further interest that the latter study involving TGFα/c-Myc transgenic mice also demonstrated that mice fed a diet supplemented with 2.25 ppm selenium appeared to be protected from liver cancer compared to mice maintained on the other two selenium diets. This study showed that a selenium-deficient and a highly enriched selenium diet reduced liver tumor formation. Very importantly, these studies reveal the complexities and the consequences involved in an imbalance of the selenium population (and selenium, small molecular weight selenocompounds and/or selenoproteins may be involved) on the resulting affected cells. They also provide clear illustrations of how insufficient our knowledge is regarding the biology of selenium. Furthermore, the studies discussed in this chapter suggest how extremely important it is not to pursue human clinical trials involving selenium until we learn far more about the underlying selenium metabolic pathways and how they act in promoting as well as preventing cancer.

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Chapter 26
An Emerging Picture of the Biological Roles of Selenoprotein K

Peter R. Hoffmann

Abstract Recent insight has been made regarding the biological role(s) for selenoprotein K (SelK) in humans and other organisms. Suggested functions for mammalian SelK include protection against oxidative stress in cardiomyocytes, regulation of endoplasmic reticulum (ER) stress in HepG2 cells, and facilitation of calcium flux in immune cells during receptor-mediated activation. The data supporting these functions as well as other aspects of SelK are summarized in this chapter.

26.1 Introduction

Selenoprotein K (SelK) was one of several novel human selenoproteins identified by the Gladyshev laboratory in the landmark 2003 study in which members of the human selenoproteome were revealed [1]. Since then, progress has been slow in elucidating the biological role or roles of SelK in humans or other species. However, some recent in vitro and in vivo data have provided key insights into SelK function. This chapter will cover various aspects of SelK in terms of expression patterns, structural features, and biological functions.

26.2 *Drosophila* SelK

Prior to the "discovery" of the mammalian SelK gene and protein in 2003, some experimental data had been obtained pertaining to the SelK ortholog in *Drosophila*, dSelK (also called dSelG or G-rich). The first published report involving dSelK

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identified the gene encoding this 110 amino acid protein [2]. Similar to its mammalian counterpart, dSelK contains one Sec residue near the C-terminus. In addition, this report described a cysteine-paralog encoded by a gene only 320 base pairs (bp) from the gene encoding the Sec-containing dSelK. Using in situ hybridization, the mRNA for dSelK was found to be expressed ubiquitously in embryos throughout development. It was unclear how much in situ signal was attributable to dSelK vs. its cysteine paralog. Still, these data are consistent with those presented in a subsequent study using RNAi to inhibit expression of dSelK in developing embryos, which found that dSelK expression was necessary for normal embryonic development in *Drosophila* [3]. Interestingly, dSelK was not as important as dSelH for maintaining antioxidant status in embryos. Topological studies in *Drosophila* demonstrated that the Sec residue in dSelK was located in the cytoplasm, making it a type III transmembrane protein (N-terminus in lumen with no N-terminal signal peptide) [4]. In addition to this important revelation, this study also identified dSelK as a Golgi-localized protein, although whether it also localized to adjacent endoplasmic reticulum (ER) is not clear from the data. Of course, there are fundamental differences between insect and mammalian biology and SelK may carry out quite different functions in these different species. More comparisons to features of mammalian SelK are described below.

26.3 Tissue Distribution and Subcellular Localization of SelK in Mice

Based on Northern blot analysis, expression of SelK mRNA was suggested to be relatively high in the heart [5]. However, real-time RT-PCR data published by our laboratory demonstrated that SelK mRNA expression levels are more widely distributed throughout tissues, with particularly high levels detected in spleen and testes [6]. Analyses of SelK protein expression in our laboratory subsequently demonstrated the highest expression in immune cells and lymphoid tissues [7], which is consistent with immunohistochemical survey of human tissues [8]. To our knowledge, this is the first selenoprotein exhibiting enriched expression in immune cells or tissues. Interestingly, increasing the dietary Se intake to above-adequate levels in mice (from 0.25 to 1.0 ppm Se) increased western blot detection of SelK in nearly all tissues examined, with the exception of heart [7]. The relative abundance of SelK in different tissues is shown in Fig. 26.1.

Overexpression of GFP-tagged SelK resulted in its localization to the ER [5], while other data have suggested that SelK may also localize to the plasma membrane [1]. Immunofluorescence data from our laboratory involving primary immune cells support the notion that SelK is predominantly localized to the ER [7], and we have not observed any pattern of SelK staining that reflects plasma membrane localization. However, it may be that only small amounts of SelK are transported to the plasma membrane or subcellular localization may differ between tissues or cell types. Also, regions of the ER come very close to the plasma membrane (within 10–25 nm) that

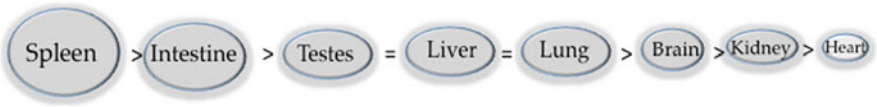

Fig. 26.1 Relative abundance of SelK in mouse tissues. Eight different mouse tissues were examined for assessing the relative amounts of SelK

are commonly referred to as puncta [9], and SelK may be localized to these regions. Puncta provide microenvironments in which key signaling events occur between the ER and the plasma membrane, particularly during Ca^{2+} influx and activation of T cells and other immune cells [10]. Whether SelK resides in puncta or is distributed throughout ER membranes proximal or distal to plasma membranes has yet to be determined. However, it is tempting to speculate that SelK may be integral in the ER membrane and enriched in puncta, providing it the opportunity to interact with plasma membrane-proximal proteins. This certainly is consistent with the functional data described in more detail below linking SelK with ER Ca^{2+} flux during immune cell activation.

SelK is localized to the ER, but how does it get there? The amino acid sequence of SelK contains a predicted transmembrane domain, a feature found only in four other selenoproteins: Dio2, SelI, SelN, and SelS [11]. Cellular fractionation of human Jurkat T cells confirmed the notion that SelK resides in the membrane fraction [7]. But the transmembrane domain is not sufficient for directing SelK into the ER membrane. Curiously, the SelK amino acid sequence contains no motifs corresponding to a signal peptide or localization signals, leaving the means by which SelK is inserted into the ER membrane a mystery. SelK could be bound by chaperones or other ER-localized proteins immediately upon translation, and guided to the ER membrane in this manner. Experimental confirmation of the means by which SelK is localized to the ER membrane is needed.

26.4 Structure of SelK

SelK is a small (94 amino acid) protein predicted to be an integral, single-spanning transmembrane protein (Fig. 26.2). SelK amino acid sequences from human and mouse share 91% identity, with the Sec residue located near the C-terminus for both species. As mentioned above, topological studies in *Drosophila* demonstrated that the Sec residue resides in the cytoplasm, making dSelK a type III transmembrane protein (N-terminus in lumen with no N-terminal signal peptide). Presumably, mammalian SelK is also situated with its Sec residue in the cytosol, although this has not been experimentally confirmed. The predicted molecular mass of SelK is 10.6 kDa, but we have found that it migrates on western blots closer to 15–16 kDa. This is most likely due to the abundance of positively charged residues (estimated pI = 10.8), because mutation of three or four positively charged amino acids to the

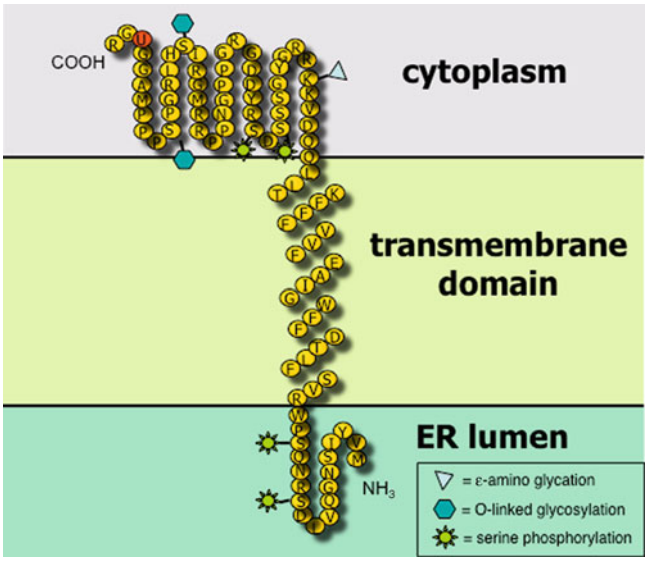

Fig. 26.2 Predicted structural features of SelK. NCBI amino acid sequence (NP_064363) was used to predict secondary structure and domains using SOSUI system software, Mitaku group, Nagoya University. Predicted transmembrane domain was confirmed using TMHMM2.0, glycosylation sites were identified using NetGlycate 1.0 and NetOGlyc 3.1, and phosphorylation sites identified using NetPhos 2.0. The Sec residue is represented by U

neutral amino acid, alanine, causes a shift toward the predicted 10.8 kDa (our unpublished data). Posttranslational modifications that contribute to a higher molecular mass cannot be ruled out, but no modifications have been experimentally confirmed. The cytosolic region of SelK is rich in both proline and glycines. The region of SelK protruding into the lumen has no identifiable features, with a distinct absence of any Ca^{2+}-binding motifs such as EF-hands, epidermal growth factor (EGF)-like repeats, cadherin repeats, and thrombospondin repeats. Structurally, the amino acid sequence of SelK provides very little clues regarding potential roles for either the cytosolic or luminal regions of SelK.

26.5 Phenotype of the SelK Knockout Mice

26.5.1 Development and Growth

As reported by our laboratory, SelK knockout (KO) mice are healthy and fertile with no apparent phenotype [7]. Preliminary tests involving behavior, anxiety, and motor skills suggested no differences between KO and wild-type (WT) mice (our unpublished data). Similarly, cardiac function was measured using ultrasound-based

echocardiography and no physiological differences were found between KO and WT mice. This is in contrast to the early in vitro studies suggesting an important protective, antioxidant function in cardiomyocytes [5]. Given the relatively high expression levels of SelK in immune cells and tissues, we examined the SelK KO mice for signs of immune system development. However, immune system development was not affected in SelK KO mice, as the numbers of total cells and cell subtypes were similar to WT in primary lymphoid tissues (bone marrow and thymus), secondary lymphoid tissues (spleen and lymph nodes), and nonlymphoid tissues (lung and liver) [7]. Overall, SelK does not appear to be required for growth or development of mice. However, studies are in progress for fully characterizing these mice regarding various aspects of health.

26.5.2 Immune System Challenges and Ca^{2+} Flux

Despite SelK-deletion having no effect on immune system development in mice, the impact on the immune system became apparent when the KO mice were challenged with inflammatory agents [7]. For example, treatment of the mice with a viral mimetic, poly(i:c), produced significantly lower levels of inflammatory chemokines, such as MCP-1 and KC, and lower infiltration of neutrophils compared to WT controls. KO macrophages secreted lower levels of inflammatory cytokines, IL-6 and TNFα, in response to poly(i:c)- or LPS-treatment. KO macrophages also exhibited decreased oxidative burst upon phagocytosis through the Fcγ receptors. T cells, neutrophils, and macrophages showed impaired migration in response to chemotactic agents. When infected with West Nile virus (WNV), SelK KO mice produced ineffective immune responses that failed to clear the virus, resulting in higher neuropathology and significantly higher mortality rates. These immune cell defects can be explained by the decreased Ca^{2+} flux induced in the SelK KO immune cells as described below.

26.6 Questions Regarding the Function of SelK

26.6.1 Is SelK an Antioxidant Enzyme?

One of the first studies to describe a potential function for mammalian SelK was that of Lu et al. in which an antioxidant, cardioprotective role was proposed [5]. In this study, overexpression of SelK in neonatal rat cardiomyocytes was shown to reduce endogenous ROS produced in the cells. Furthermore, overexpression of SelK protected these cells from a challenge with hydrogen peroxide. Based on these results, the authors suggested an antioxidant role for SelK in cardiomyocytes. However, all of these experiments involved comparisons of overexpressed SelK to

overexpressed GFP, and it is possible that overexpression of any Sec-containing protein may shift the redox balance toward a proreducing environment. In fact, overexpression of other ER-localized selenoproteins, such as SelM and SelS, also has shown to protect against hydrogen peroxide challenge [12, 13]. Does this indicate that all of these ER selenoproteins serve to mitigate oxidative stress? One must consider the possibility that an overabundance of nearly any selenoprotein may reduce ROS and this does not necessarily reflect on the biological role of that selenoprotein at physiological levels. Specific mechanisms by which SelK may act as an antioxidant have not yet been described and the *Drosophila* homolog of SelK (dSelK) was not found to contribute to overall antioxidant potential [5]. Furthermore, SelK lacks defined redox motifs such as Cys–X–X–Sec or Cys–X–X–Ser (X is any amino acid) found in antioxidant selenoproteins like the GPx enzymes [14]. Thus, the current data do not clearly support the notion that SelK is an antioxidant enzyme in vivo. However, it is possible that SelK forms a complex with other proteins that utilize its Sec residue for its reducing capacity.

26.6.2 Is SelK Involved in the ER-Stress Response?

A recent report demonstrated a potential role for SelK in both being regulated by and regulating ER-stress in the HepG2 cell line [15]. SelK expression was increased in a dose- and time-dependent manner in response to ER-stress reagents. Decreasing SelK levels with siRNA induced the ER-stress marker, GRP78, with or without the addition of ER-stress reagents. Cell viability was slightly lower in cells with diminished SelK expression when exposed to ER-stress. Overall, in HepG2 cells there appears to be a relationship between SelK and ER-stress. However, cells and tissues from SelK KO mice show no signs of ER stress [7]. This discrepancy may be due to a difference of cell type or species. Alternatively, in vivo effects of deleting SelK on ER-stress may be alleviated by redundant or compensating systems (perhaps SelS?), and this redundant system may not exist in the HepG2 cells. At this point in time, it appears that SelK may play a role in regulating ER-stress, but its specific role in this process remains unknown and the lack of apparent ER-stress in the SelK KO must be explained.

26.6.3 Is SelK Involved in Calcium Flux from the ER?

Based on data from cells purified from the SelK knockout mouse, there appears to be a specific role for SelK in promoting receptor-mediated Ca^{2+} flux [7]. In three different types of immune cells (T cells, neutrophils, and macrophages), SelK deletion was shown to significantly reduce receptor-mediated Ca^{2+} flux. To illustrate the step at which SelK may influence or regulate Ca^{2+} from the ER, T cell stimulation through the T cell receptor (TCR) receptor is shown in Fig. 26.3. Upon TCR crosslinking

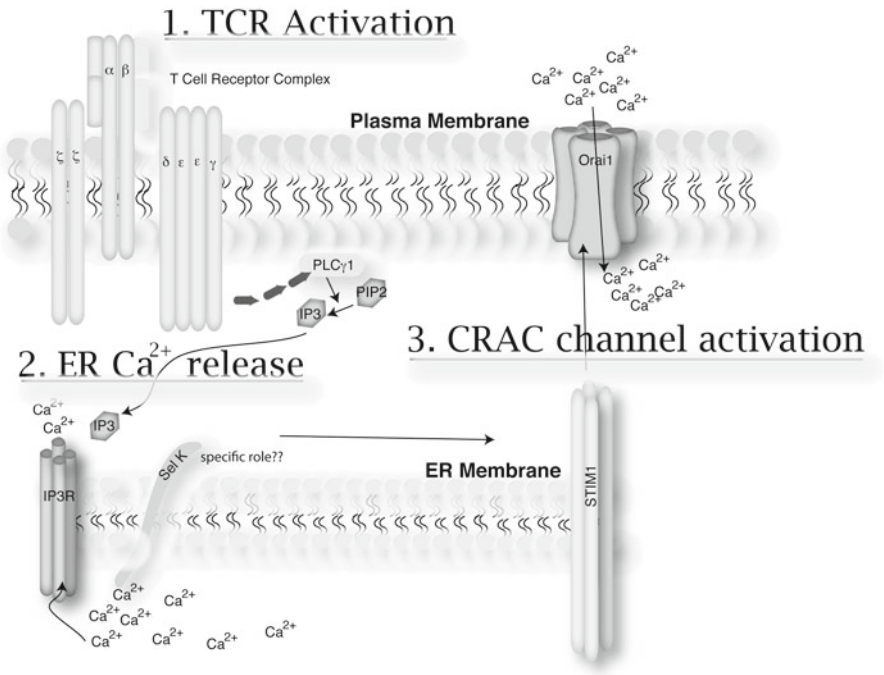

Fig. 26.3 Diagram of signaling events occurring during activation of T cells. Engagement of the T cell receptor (TCR) activates phosphoinositide-specific phospholipase C (PLCγ1), which catalyzes the degradation of phosphatidylinositol-4,5-bisphosphate to generate inositol-1,4,5-triphosphate (IP3) and diacylglycerol. IP3 binds to its receptor, located on the surface of internal Ca^{2+} stores, primarily the ER. Binding of IP3 with the IP3 receptor results in release of Ca^{2+} from ER lumen to the cytosol, which causes oligomerization of STIM1 and subsequent translocation of STIM1 oligomers to the plasma membrane where they interact with the pore-forming unit of Ca^{2+} release-activated Ca^{2+} (CRAC) channels. CRAC channel activation results in an influx of extracellular Ca^{2+}, which leads to activation of multiple signaling pathways indispensable for cellular proliferation and differentiation. SelK modulates the effects of IP3 receptor stimulation

on the surface of T cells, the organization of puncta allows rapid association of ER and plasma membrane proteins, providing interactions during the Ca^{2+}-dependent signaling crucial for their activation. While many of the early steps of T cell activation have been elucidated, other puncta-associated proteins participating in this process have yet to be identified. The predominant pathway of Ca^{2+} entry in T cells involves inositol-1,4,5-triphosphate (IP3)-receptor mediated Ca^{2+} release from the ER Ca^{2+} store, which subsequently induces the opening of plasma membrane-expressed store-operated Ca^{2+} channels, also known as calcium release-activated Ca^{2+} (CRAC) channels. The overall process is known as store operated Ca^{2+} entry (SOCE) [16]. During SOCE, TCR engagement activates phosphoinositide-specific phospholipase C, which catalyzes the degradation of phosphatidylinositol-4,5-bisphosphate to generate IP3 and diacylglycerol (DAG). IP3 binds to the IP3 receptor,

located on the surface of internal Ca^{2+} stores, primarily the ER [17]. Binding of IP3 with the IP3 receptor results in release of Ca^{2+} from ER lumen to the cytosol. The loss of Ca^{2+} from ER causes entry of Ca^{2+} through CRAC channels on the plasma membrane, which leads to activation of multiple signaling pathways indispensable for cellular proliferation and differentiation [18, 19]. SOCE is crucial not only for T cell activation, but also for IgE-dependent mast cell activation [20] and for specific aspects of macrophage activation [21].

Our data suggest that SelK deletion impairs SOCE in immune cells, but is this merely due to ER-stress? If SelK deletion caused ER stress or dysfunctional storage of Ca^{2+} in the ER, one would expect that a Ca^{2+}-mobilizing reagent such as thapsigargin or ionomycin would result in decreased Ca^{2+} flux in KO cells. However, these reagents produced no differences in Ca^{2+} flux in KO compared to WT cells. Similarly, the defects in Ca^{2+} were not mediated by ER stress in SelK KO cells due to the fact that ER stress markers did not differ between KO and WT cells or tissues. In fact, no evidence of ER stress has been detected in the KO mice. Thus, SelK deletion impairs Ca^{2+} from ER stores through a receptor-dependent mechanism, not by disrupting ER function in a general manner. This suggests SelK is an important component of the signaling network operating between cell surface receptors and ER membrane receptors. Effective Ca^{2+} flux is crucial for proper cellular responses to stimulation induced through a number of receptor systems including the TCR, chemokine receptors, Toll-like receptors, and Fcγ receptors. Our data clearly show that Ca^{2+} flux induced by these receptors was impaired in KO cells compared to WT controls. The role of SelK in regulating immune cell activation and in vivo immune responses via Ca^{2+} flux is not completely understood, but a clearer picture is emerging for SelK in this important process and is discussed in more detail below.

26.7 Similarities and Differences Between SelK and SelS

There are some important similarities between SelK and SelS. Both are transmembrane proteins localized to the ER membrane. Expression of both selenoproteins is increased in cells treated with reagents that cause ER stress [13, 15]. This may suggest similar roles for SelK and SelS in mitigating ER stress. The role of SelS in retrotranslocation of misfolded proteins has been experimentally demonstrated [22], whereas any data showing a similar function for SelK have yet to be published. Other than the transmembrane domain and the Sec residue, SelK and SelS share no similar structural features. Also, SelK is a type III transmembrane with the Sec residue in the cytosol, but SelS has been suggested to be a type II transmembrane protein with the Sec residue in the ER lumen [4, 23]. The latter requires experimental confirmation and, if indeed this proves to be true, it would be an important difference that may be related to the functional roles of these proteins. Perhaps the most important difference between SelK and SelS is the effect on inflammation in the absence of either of these two selenoproteins. Diminished SelS expression increases inflammatory cytokines, whereas SelK KO mice show no signs of increased inflammation [7, 24].

Table 26.1 Comparison of Sel K and Sel S

Similarities	Differences
Both localized to ER membrane	Sel K is type III protein with Sec in cytosol, Sel S is suggested to be a type II protein with Sec in lumen
Both exhibit increased expression with ER stress	Sel S is also induced with glucose-deprivation, glucose-dependent expression of Sel K has not been determined
Decreased expression of either increases ER stress	Sel K-knockdown in HepG2 cells produces increased ER stress, but no apparent ER stress in cells or tissues from Sel K KO mice
Lowered expression affects inflammatory cytokines	Sel K KO mice and cells secrete *decreased* inflammatory cytokines but Sel S knockdown cells secrete *increased* inflammatory cytokines compared to WT controls

In fact, SelK KO macrophages secrete lower levels of proinflammatory cytokines upon stimulation with various inflammatory agents. Overall, it remains to be determined whether SelK and SelS are functionally similar and/or if they have biological roles completely independent of each other (Table 26.1).

26.8 Concluding Remarks

Overall, many questions remain pertaining to the biological role(s) of SelK. The low abundance of SelK throughout most tissues together with relatively higher expression in immune cells suggests to this author that SelK may play multiple roles. For example, in most tissues SelK may act to mitigate ER stress that may arise from misfolded proteins, viral infection, Ca^{2+} imbalance, or other conditions. SelK is unlikely the sole protein involved in this function, as the corresponding KO mice and cells appear to function normally with no apparent ER stress. This is in contrast to the siRNA studies in HepG2 cells described above, but this must be repeated in other cell types and in vivo to better define the role of SelK in ER stress. In addition to this ER stress-related function, SelK appears to serve an important role in immune cells to promote activation of these cells during receptor-mediated Ca^{2+} flux. The data from KO mice need to be corroborated in human cells and tissues, but the immune system in mice clearly exhibits a dependence on SelK for optimal function.

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Chapter 27
Polymorphisms in Selenoprotein Genes and Cancer

Margaret E. Wright and Alan M. Diamond

Abstract Human selenoproteins comprise a diverse group of peptides whose role in cancer etiology might be presumed from what is currently known about their functions. For a subset of these, genetic data (1) demonstrating allelic loss during cancer development and/or (2) revealing an association between specific polymorphisms in selenoprotein genes and either cancer risk or survival have provided support for this association. Additional factors such as lifestyle, diet, gender, and interactions with polymorphisms in other genes may modify this level of risk. These data provide useful information that may eventually be used to identify individuals at increased risk of cancer and aid in the design and development of novel strategies to prevent and treat some of the more common cancer types.

27.1 Introduction

Interest in selenium as a means to reduce cancer risk has persisted for decades. Initial studies established that supplementation of the diets of animals with low, nontoxic doses of selenium could reduce tumor incidence, and these observations served as the foundation for hundreds of published papers showing that selenium was effective in most, if not all, organs tested in rodents. Focus on the anticancer benefits of selenium was further stimulated by a series of epidemiological studies showing an inverse association between dietary selenium intake and cancer risk. While the mechanism(s) underlying the likely benefits of dietary selenium intake have yet to be resolved, the identification of a class of proteins that include selenium in the form of the amino acid selenocysteine has led to speculation that one or more of these proteins are responsive to selenium availability and mediate selenium's

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Table 27.1 Cancer sites that have been linked to variants in selenoprotein genes

Gene	Linked cancer sites[a]
GPx-1	Bladder, breast, colon, head and neck, liver, lung, lymphomas, prostate
GPx-4	Breast (mortality), colorectal
SePP	Colorectal (advanced distal adenoma and cancer), prostate
Sep15	Breast, colorectal, head and neck, lung, prostate (mortality)
SepS	Colorectal, gastric

[a]See references in text

anticancer properties. Moreover, the antioxidant enzyme activity attributed to several of these selenoproteins offered a likely mechanistic appreciation as to how elevated levels of these proteins could be protective. Although proof of this speculation has yet to be realized, a role for a subset of selenoproteins in cancer etiology has been supported by human genetics.

The genomes of humans differ by approximately 0.1% or three million nucleotide positions. Many of these differences may not have functional consequences, while a subset can influence the activity and/or expression levels of the encoded gene products. This genetic diversity among members of our species has provided significant evidence that specific selenoproteins can affect both cancer risk and clinical outcome. In general, this has occurred in one of two ways. In the first, the ability to detect heterozygosity at selected genetic loci has facilitated the analysis of the loss of one of two gene copies during tumor development. Loss of heterozygosity (LOH) is typically indicative of the increased risk of cancer associated with the reduced dosage of a beneficial gene, or alternatively, the unmasking of a recessive mutation that promotes clonal cellular expansion. The second means by which human genetics supports a role for a gene in cancer etiology is by the identification of germline polymorphisms whose presence can be linked to a greater risk of developing or dying from cancer. In this chapter, the genetic evidence implicating several selenoproteins in cancer etiology is presented (see Table 27.1 for a summary), as is a comprehensive model for the interaction among several selenoprotein genes and additional modifying factors.

27.2 GPx1

GPx1 was the first selenoprotein characterized in detail and is an antioxidant enzyme located both in the mitochondria and the cytoplasm. This selenoprotein catalyzes the detoxification of reactive oxygen species (ROS) using glutathione as a source of reducing equivalents. GPx1 levels are responsive to selenium availability and it has long been considered a potential mediator of some of the consequences of selenium deficiency and perhaps also the benefits of its supplementation. In 1994, Moscow et al. reported the existence of two variants in the coding region of the human GPx1 gene: a codon 198 polymorphism resulting in either a leucine or proline at that

position and a variable number of nucleotide triplet repeats resulting in either 5, 6 or 7 alanines in the amino terminus of the GPx1 protein [1]. Differences in the *GPx1* allele frequency were observed between DNA obtained from lung tumors as compared to DNA obtained from individuals without evidence of cancer with significantly fewer *GPx1* heterozygotes noted in tumors from lung cancer patients [1]. Similar results were obtained many years later by establishing that LOH at the *GPx1* locus occurred frequently in the DNA obtained from two other tumor types, breast cancer [2] and cancers of the head and neck [3]. These results could be explained by either the loss of one *GPx1* allele during malignant progression (as would occur if, for example, GPx1 had tumor suppressor activity) or if certain genotypes predisposed an individual to cancer development. Evidence in support of the former possibility comes from studies indicating that there was a loss of one of two *GPx-1* alleles in colon tumor DNA [4] and cancers of the head and neck [3] as compared to noncancerous tissues obtained from the same individuals.

While LOH may be indicative of the loss of a genetically linked gene with beneficial properties and not GPx1, epidemiological and functional data support a role for GPx1 genetic variants in cancer risk and outcome. Some studies have indicated that the number of alanine repeats is associated with increased cancer risk, but there does not appear to be a consistent pattern for a particular number of repeats [5–7]. In contrast, there is significant literature indicating that the identity of the amino acid encoded by codon 198 contributes to cancer risk with the majority of these studies identifying the *leu*-encoding allele as the one associated with increased risk (recently reviewed in [8, 9]). The types of malignancies whose risk increases with the presence of the *GPx1 leu* allele include cancers of the lung, breast, bladder, liver, and lymphomas, and these associations have been found in populations from Finland, USA, Korea, Denmark, Japan, the United Kingdom and France [10–18]. Of note were the results of a recent meta-analysis indicating an association between the *leu* allele and breast cancer risk, but only among African American women [19]. In contrast, three studies investigating the association between *GPx1* alleles and cancer risk reported an increased risk of cancers of the lung and prostate among carriers of the *pro* allele [11, 20, 21]. These apparently conflicting results, as well as those showing that *GPx1* genotype has no effect on cancer risk [22–29], may be explained by other variables, some of which are discussed below.

27.2.1 Interaction Between GPx1 Genotype and Selenium Status

The functionality of the leu/pro variant in GPx1 has been investigated using both cells in culture and in humans. Taking advantage of the observation that human MCF-7 breast carcinoma cells produce negligible quantities of GPx1, derivative cell lines were generated that exclusively produce the *leu* or *pro GPx1* allele and it was shown that there was a differential response to the amount of the selenium available in the culture media with cells expressing the *leu* allele requiring more selenium to achieve the same level of GPx1 activity as compared to those expressing the *pro*

allele [2]. These results were expanded to show that the difference in response to selenium availability occurred only when the *leu* polymorphism was associated with 5 alanine repeats, indicating an interaction between the amino terminus and carboxy terminus of the protein [30]. Less clear is whether the GPx1 genotype affects the corresponding enzyme activity *in vivo*. Several studies have reported lower GPx activity associated with the *leu* allele [13, 31–33]; there was one report of this relationship existing only among women [34] and two studies failed to find any genotype–phenotype association at all [21, 35]. Jablonski et al. were only able to detect an association between plasma selenium levels and GPx activity among individuals who were *leu* homozygotes [36]. While most of these studies reported a reduced GPx1 enzyme activity associated with the same *leu* allele most frequently linked to increased cancer risk, all GPx1 assays reported in these manuscripts examined activity in erythrocytes, which may not reflect the consequences of genotype in the particular organs where the cancers investigated arise. It is of interest to note that the urine of individuals that were either heterozygous or homozygous for the *leu* allele contained higher levels of the DNA oxidation product 8-OHdG than those who were homozygous for the *GPx1 pro* allele [12].

In addition to selenium status, there may be other effect modifiers of associations between *GPx1* genotype and cancer risk. For example, it has been shown that the nature of this relationship depends on smoking habits, alcohol intake, age, gender, and vitamin use, although the emerging patterns can be complicated to discern [20, 24, 33]. One example of this comes from a study of 237 lung cancer patients and 234 community-based controls enrolled at the Mayo Clinic. An interaction was observed between the *GPx1* Pro198Leu polymorphism and smoking status among older individuals (>80 years): among smokers, the homozygous *pro*/*pro* genotype was associated with a threefold increased risk of lung cancer (relative risk (RR) = 3.3, 95% confidence interval (CI): 1.3–8.4), whereas among never smokers, this genotype was linked with more than an eightfold lower risk of disease (RR = 0.12, 95% CI: 0.02–0.7) [11].

27.3 GPx4

Another member of the GPx family of antioxidant selenoproteins is GPx4 – the only member that is associated with membranes where it functions in the detoxification of lipid hydroperoxides [37, 38]. There is an abundance of evidence indicating that enhanced expression of GPx4 can protect against oxidative stresses and also against carcinogenesis (reviewed in [8]). A single nucleotide polymorphism (SNP) in the 3′-untranslated region of the *GPx4* gene at position 718, which results in either a C or T, has been identified and implicated in the regulation of lipoxygenase metabolism [39]. The functionality of this SNP was substantiated by both *in vivo* and *in vitro* studies, which indicated that the SNP influenced the ability of the 3′-UTR to function as a SECIS element required for the proper insertion of selenocysteine into

the growing peptide in response to the in-frame UGA codon present in the *GPx4* coding sequence [40]. The identity of the position 718 SNP also influenced the amount of GPx4 present in individuals following selenium withdrawal and this effect was modified by gender [40, 41]. This same polymorphism has been shown to be associated with the risk of colorectal cancer in two separate populations, one Czech and one English, although the results of these studies were inconsistent as to which allele was associated with increased cancer risk. [41, 42]. While these studies showed an association between the *GPx4* SNP and risk of colon cancer, others have examined this variant in relation to clinical outcome. For example, data obtained from the Studies of Epidemiology and Risk Factors in Cancer Heredity (SEARCH) breast cancer study demonstrated that the *GPx4* 3-UTR 718C polymorphism was associated with increased risk of death from breast cancer among women previously diagnosed with this disease [29].

27.4 SePP

If the levels of GPx's impact cancer risk and progression and the levels of these proteins are affected by selenium availability, then it follows that factors that influence the levels of selenium in organs would also have an effect on cancer risk. Selenium levels in the body are regulated in the liver where selenium is designated either for excretion or for further processing for use in selenoproteins, which includes the major transport selenium-containing protein, SePP [43]. SePP is an extracellular protein containing ten selenocysteines in humans comprising the major form of selenium in plasma. SePP accounts for approximately 44% of the selenium in plasma [44]. SePP enters the tissue where the protein is catabolized by Sec-β-lyase and the products are funneled into selenium metabolism [45].

Several polymorphisms in the *SePP* gene have been identified two of which are common in multiple ethnicities and have been shown to influence SePP levels in the blood and/or SePP levels in response to selenium supplementation [46–48]. A SNP that causes an amino acid change at codon 234 was recently found to increase the risk of sporadic colorectal cancer by 39% in females, although this association was only of borderline significance [42]. The other SNP is located in the 3′ UTR of the mRNA, yielding a G-to-A base change. Individuals with the variant *AA* genotype have been shown to have increased risks of prostate [48] and colorectal [42] cancers, with the latter association limited to females. Other, less commonly studied polymorphisms and haplotypes have also been characterized and linked with advanced distal colorectal adenoma [28] and overt colorectal cancer [42]. Of note is the finding by Méplan et al. that carriage of at least one variant *T* allele of rs2972994 (a polymorphism located in the promoter region) conferred an increased risk of colorectal cancer in males but a significantly decreased risk of this disease in females [42]. Furthermore, results from this study indicated that several SNPs in *SePP* interact with polymorphisms in either *Sep15* or *GPx4* to affect disease.

27.5 Sep15

A 15-kDa selenoprotein was identified in human T cells by virtue of its ability to be labeled with ^{75}Se and was shown to be encoded by a gene on chromosome 1; the highest levels of this gene's product are found in the thyroid, parathyroid, and prostate [49]. This selenoprotein was subsequently shown to reside in the lumen of the endoplasmic reticulum (ER) where it associated with UDP-glucose:glycoprotein glucosyltransferase [50] – a protein with an established role in maintaining the quality of folded proteins – and likely functions in the response to unfolded proteins and ER stresses [51]. The *Sep15* gene includes two polymorphisms in the 3′-UTR at positions 811 (C/T) and 1125 (G/A) that result in only two observed haplotypes: either a 811C/1125G or 811T/1125A with the haplotype shown to be functional in determining the amount of the Sep15 protein produced for a given level of available selenium [52, 53]. In addition, the 811T/1125A is relatively uncommon in Caucasians where only 7% are homozygous, as compared to African Americans where 31% are homozygous [53].

A role for Sep15 in cancer etiology is supported by recent data indicating that reducing its levels in colon cancer cells could attenuate the tumorigenic and metastatic potential of CT26 colon cancer cells in BALB/c mice [54]. In humans, the *Sep15* allele frequency was shown to be different in breast cancer cases and cancer-free individuals, and LOH was demonstrated in both breast cancers and cancers of the head and neck [53, 55]. Evidence that *Sep15* genotype can specifically influence cancer risk in humans was provided by a Polish study designed to investigate the relationship between the *Sep15* allelic identity, selenium status, and risk of lung cancer [56]. In this study, there was a reduced risk of lung cancer among individuals with higher plasma selenium levels; however, there was an increased risk of lung cancer for those who carried at least one copy of the Sep15 variant G allele at position 1125 [56]. A more recent study in a Korean population demonstrated an increased risk of colorectal cancer among male (but not female) carriers of the *Sep15* 811T/1125A haplotype [57]. In contrast, a search for an association among SNPs in a region of DNA including 5 kb upstream and downstream of *Sep15* failed to identify any association of these SNPs with the risk of prostate cancer [58]. In the same study, however, a haplotype consisting of five SNPs (including the 1125 variant) was significantly associated with higher prostate cancer mortality and the presence of one of these SNPs abrogated the observed protection against prostate cancer mortality seen with high levels of plasma selenium [58].

27.6 SEPS

Selenoprotein S (SEPS), also referred to as SELS or VIMP, is an ER-associated protein that functions in the removal of misfolded proteins from the ER to the cytoplasm [59]. It is also involved in the regulation of the inflammatory response and

stimulating the production of proinflammatory cytokines [60]. Allelic variation at *SEPS* has been investigated with regard to a variety of conditions, including cardiovascular disease and preeclampsia, the latter of which is a condition in which there is pregnancy-associated excessive inflammation (reviewed in [9]). One particular polymorphism located in the promoter region at position −105 (yielding either a G or A) has received considerable attention. The A-containing allele is associated with reduced levels of mRNA [60, 61]. Promoter polymorphisms in SEPS have been associated with risk of gastric cancer in a Japanese population [62] and increased colorectal cancer [42], although one recent study revealed the latter relationship only among women [57].

27.7 The Interaction Among Selenium and the Genotypes of Selenoproteins and MnSOD

MnSOD is a major protective mitochondrial enzyme that detoxifies superoxide radicals produced during electron transport to the less toxic hydrogen peroxide. A variant *MnSOD* allele containing an alanine (A) rather than a valine (V) at codon 16 has been described, and in several reports, has been associated with an elevated risk of several cancer types, including prostate cancer, in human epidemiological studies [17, 63–65]. As a consequence of alanine being at this position in the mitochondrial import signal peptide, there is increased transport of MnSOD into the mitochondria [66]. While it might be counterintuitive that elevated levels of an antioxidant enzyme would increase risk, several studies have shed light on the likely explanation. Li et al. reported an impressive tenfold swing in the risk of prostate cancer among men who expressed the *AA* genotype (those being homozygous for the allele encoding alanine at codon 16) between the lowest quartile of total antioxidant consumption and the highest with those consuming the lowest levels of dietary antioxidants being at greatest risk [64]. A separate analysis also showed that there was a threefold increase risk of prostate cancer for *AA* men with low carotenoid status [$P = 0.02$, confidence interval 1.37–7.02] [65]. As originally proposed by Li, it is therefore likely that increased mitochondrial transport of MnSOD as a consequence of a codon 16 alanine is beneficial when antioxidant activity is high and the MnSOD dismutation product, H_2O_2, can be reduced to water [64]. A low antioxidant status, defined either by individual genetics and/or dietary intake, would facilitate the cycling of H_2O_2 to more ROS that are potentially mutagenic and therefore carcinogenic.

As described above, the at-risk *GPx1 leu* allele encodes a protein that is less responsive to selenium as compared to the protein with a proline at the same position [2, 30]. Cox et al. initially reported that there was no association between the at-risk *leu* allele of *GPx1* and breast cancer risk among participants in the Nurse's Health Study [22]. However, a follow-up study from the same authors indicated that there was indeed a significant risk for breast cancer among participants of the same cohort when the *MnSOD* genotypes were also considered; carriers of both the *AA* and

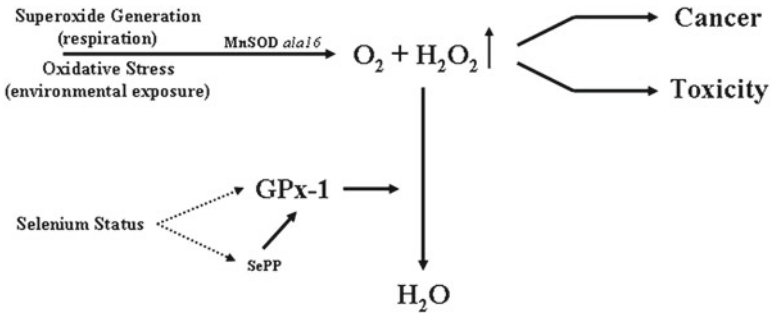

Fig. 27.1 Model for the interaction among endogenous and environmental sources of oxidative stress, individual genotype and risk of cancer and other degenerative disease. Elevated expression and/or activity of MnSOD will generate an additional load of H_2O_2, which if detoxified, will be beneficial. In contrast, reduced levels of GPx-1, as a result of result of polymorphisms in GPx1 or SePP1, or reduced selenium/antioxidant levels, will increase the levels of H_2O_2 and contribute to cancer and degenerative disease risk

leu/leu genotype were at increased risk of breast cancer with an odds ratio of 1.87 [95% CI, 1.09–3.19] [14]. These human data indicate a direct interaction between MnSOD and GPx1 in influencing cancer risk. GPx1 may be a particularly important H_2O_2-detoxifying enzyme because of its cellular location in the mitochondria as well as in the cytoplasm. Further support for this concept comes from human data indicating that polymorphisms in the gene for the selenium transport protein SePP that result in less SePP in the plasma and reduced levels of GPx1 are associated with a significant risk of prostate cancer only in men also expressing the *ala16 MnSOD* allele [67]. Furthermore, the observed gene–gene interaction was strongest in current and former smokers, a group with higher levels of oxidative stress due to exposure to free radicals in tobacco smoke and poor antioxidant nutrient intake. A diagrammatic representation of the dietary and genetic factors that interactively influence the risk of cancer and perhaps other degenerative diseases due to the expression of the *ala16 MnSOD* allele is presented in Fig. 27.1.

27.8 Concluding Remarks

Animal studies, in vitro data and human epidemiology have supported a role for selenium in cancer risk and survivability, and selenoproteins such as those described in this chapter are likely to be important mediators of at least some of these effects. While this seems likely given the functions of selenoproteins in processes such as selenium delivery to tissues, antioxidant defenses, and the maintenance of correct protein folding, this concept is directly supported by human genetic data indicating allelic loss of selenoprotein genes during cancer development or the presence of polymorphisms that predispose to cancer or predict clinical outcome. The impact of allelic variants in selenoprotein genes and/or the loss of one of two gene copies

during carcinogenesis may be influenced by a number of factors, such as genetics, gender, and a host of modifiable behaviors. Future studies that clarify these relations and establish the mechanisms by which they occur offer the potential to develop new strategies to predict, diagnose, prevent, and treat a wide variety of cancer types.

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Chapter 28
Schizophrenia, Oxidative Stress and Selenium

Matthew W. Pitts, Arjun V. Raman, and Marla J. Berry

Abstract Schizophrenia is a complex, crippling mental illness that is influenced by multiple environmental and genetic factors. Oxidative stress is among the most prominent factors implicated in schizophrenia. Many components of the oxidative stress pathways influence cell-signaling cascades that regulate several neurotransmitter systems. One of the characteristic features of schizophrenia is altered dopaminergic, glutamatergic, and GABAergic neurotransmission, which is influenced by oxidative stress and exacerbated by certain drugs of abuse. Selenoproteins play critical roles in defense against oxidative stress and include glutathione peroxidases, thioredoxin reductases, and iodothyronine deiodinases. Based upon their integral function in protection against oxidative stress, impaired selenoprotein synthesis and function may contribute to the pathogenesis of schizophrenia.

28.1 Introduction

Selenoproteins are a unique class of proteins, which play critical roles in defense against oxidative stress. They include glutathione peroxidases (GPxs), thioredoxin reductases (TXNRDs), and iodothyronine deiodinases (DIOs). Selenoproteins are characterized by the incorporation of selenium as selenocysteine, the 21st amino acid, at UGA codons, which typically serve as stop codons [1]. The majority of selenoprotein mRNAs contain single UGA codons, which encode one selenocysteine residue per polypeptide chain. Selenocysteine residues are inserted cotranslationally by means of a selenocysteine insertion sequence (SECIS) located in the 3′-UTR (untranslated region) of selenoprotein mRNAs, which direct incorporation of this

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unique amino acid [2]. In all selenoproteins characterized to date, selenocysteine residues are catalytically active in redox processes.

The involvement of selenium in health-related processes was discovered in 1973, when selenium was found to be an essential component of the detoxifying enzyme, glutathione peroxidase (GPx) [3]. At present, 25 selenoproteins have been identified in humans, including five GPxs, three TXNRDs, and three DIOs. Genetic knockout studies in mice have demonstrated that at least three selenoproteins are essential, as deletion of thioredoxin reductase 1 (*Txnrd1*), thioredoxin reductase 2 (*Txnrd2*), or glutathione peroxidase 4 (*Gpx4*) results in embryonic lethality [4–6]. GPxs protect cells from the deleterious effects of oxidative stress by catalyzing the reduction of hydrogen peroxide (H_2O_2). The antioxidant, glutathione (GSH), is an essential cofactor that normally acts as the electron donor in enzymatic reactions involving GPxs. TXNRDs comprise a family of homodimeric flavoenzymes that catalyze the NADPH-dependent reduction of oxidized thioredoxin in cellular redox pathways. In this class of proteins, selenocysteine is incorporated as the penultimate C-terminal residue [7], where it is essential for enzymatic activity [8]. Another important class of selenoproteins is the DIOs, which catalyze the activation and inactivation of thyroid hormones by reductive deiodination, and hence, play fundamental roles in both development and maintenance of thyroid hormone homeostasis.

In addition to the aforementioned classes of characterized selenoproteins, several additional selenoproteins have been identified and the functions of these proteins have been distinguished to varying degrees. Of these, one of the most intriguing and best characterized is selenoprotein P(Sepp1). Sepp1 is distinct, due to the fact that it contains multiple selenocysteine residues (ten in humans) and has two SECIS elements in the 3′-UTR of its mRNA. It is a secreted glycoprotein that is synthesized in many tissues, with the highest level of expression occurring in the liver. Sepp1 can be divided into two functional domains, an N-terminal domain containing one selenocysteine (U) in a U-x-x-C redox motif, and a shorter C-terminal domain containing multiple selenocysteines (nine in humans) [9]. Based upon its structural characteristics, Sepp1 is widely believed to be multifunctional. The enzymatically active N-terminal domain is thought to be involved in the maintenance of extracellular redox balance, while the primary function of the selenocysteine-rich C-terminal domain is speculated to be selenium transport [10]. Sepp1 binds to the lipoprotein receptors, ApoER2 and megalin, which mediate its uptake into the brain and testis (ApoER2), and kidney (megalin), respectively [9]. In mice, deletion of selenoprotein P (*Sepp1*$^{-/-}$ mice) results in impaired motor coordination, seizures, deficits in spatial learning, and defects in synaptic plasticity [11]. Likewise, deletion of the Sepp1 receptor, ApoER2, produces many similar deficits [12].

28.2 Schizophrenia and Oxidative Stress

Schizophrenia is a neuropsychiatric condition characterized by a heterogeneous mixture of positive (hallucinations, delusions), negative (flat affect, catatonia), and cognitive (attention, memory) symptoms. This devastating disorder affects roughly

1.0% of the population, emerges during late adolescence/early adulthood, and is subject to chronic relapses with intermittent periods of remission [13]. As with most psychiatric conditions, schizophrenia appears to be influenced by a complex array of environmental and genetic variables.

One of the primary factors thought to influence the development and course of schizophrenia is oxidative stress. Reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), can damage neurons by means of lipid peroxidation, protein carboxylation, DNA strand breaks, and altered cell signaling. The link between oxidative stress and schizophrenia is supported by a number of both clinical and genetic studies. Decreased levels of GSH in schizophrenic patients were first noted in 1934 [14], but there was little follow-up on these initial findings. However, several recent studies have documented a correlative relationship between low GSH levels and schizophrenia. One report found a 27% reduction in cerebrospinal GSH levels [15] in untreated patients with schizophrenia, whereas another study documented a 41% decrease in GSH levels in the caudate nucleus postmortem [16]. Several recent studies have also provided genetic evidence for a link between schizophrenia and impaired GSH synthesis. A trinucleotide repeat (TNR) polymorphism in the 5′-UTR of the catalytic subunit of glutamate cysteine ligase (GCLC) was reported to be associated with schizophrenia in humans [17]. Moreover, lower GCLC expression, glutamate cysteine ligase (GCL) activity, and GSH levels were found among the subjects with genotypes that positively associated with schizophrenia. Finally, a global parallel analysis of transcripts, proteins, and metabolic intermediates in the prefrontal cortex of schizophrenia patients identified oxidative stress pathways including the GSH and thioredoxin systems, as being substantially altered [18].

28.3 Schizophrenia, Dopamine and Oxidative Stress

Altered dopamine signaling has long been implicated as a key feature of schizophrenia. Elevated dopaminergic neurotransmission was initially considered to be intrinsically related to psychosis, as dopamine-releasing drugs, such as amphetamines, induce psychosis and the first clinically effective antipsychotics antagonized dopamine receptors [19]. The dopamine hypothesis was further refined during the 1970s, when it was shown that the clinical potency of antipsychotic medication for alleviating the positive symptoms of schizophrenia was highly correlated to its ability to block dopamine D_2 receptors [20]. As additional scientific evidence accumulated and it became apparent that schizophrenia was far more complex than excessive dopaminergic neurotransmission, the role of dopamine in schizophrenia was reconceptualized in the early 1990s. In a seminal review paper, Davis and colleagues proposed a modified dopamine hypothesis of schizophrenia that added regional specificity and attempted to account for both negative and positive symptoms [21]. Based upon multiple lines of evidence from human and animal studies, the authors suggested that schizophrenia is not the result of a hyperdopaminergic brain, but rather dysregulated dopaminergic transmission in multiple brain regions.

More specifically, they hypothesized that positive symptoms are caused by enhanced striatal dopaminergic tone, whereas negative symptoms are influenced by a lack of dopaminergic transmission in frontal brain regions.

In addition to the positive and negative symptoms, patients with schizophrenia typically exhibit impaired cognition, including deficits in semantic and explicit memory, attention, working memory, and executive function [13]. Loss of dopamine signaling in the prefrontal cortex severely disrupts performance of executive tasks in nonhuman primates [22, 23] and schizophrenia patients suffer from defective executive function [24], lending support to the hypothesis that the cognitive impairments in schizophrenia result from hypoactivity in the mesocortical dopaminergic system.

Additionally, several lines of evidence indicate that levels of both dopamine and dopamine receptors are elevated in the striatum of schizophrenic patients. Increased levels of striatal dopamine and its metabolite homovanillic acid [21], of striatal uptake of dopamine [25, 26], and of amphetamine-induced dopamine release [27, 28] have all been reported in patients with schizophrenia. The increase in striatal dopamine availability in schizophrenia is coupled with an increase in striatal dopamine D_2 receptors [29, 30], but whether D_2 receptor increases are apparent before the onset of symptoms, especially during development, is uncertain. This is significant because transgenic mice that transiently overexpress the D_2 receptor within the striatum exhibit impaired performance in cognitive tests as well as increased D_1 receptor activation and decreased dopamine turnover in prefrontal cortex [31, 32]. Moreover, if overexpression is limited to developmental stages, the behavioral deficits are still exhibited in adults, implying that hyperdopaminergic signaling in the striatum during development induces compensatory changes in prefrontal cortex that last well into adulthood.

In the current genomic age, the link between altered dopamine signaling and schizophrenia has been further solidified. Multiple genes involved in dopamine signaling have been associated with schizophrenia, including *DRD2* [33, 34] and *COMT* [35, 36], providing additional evidence for dopamine dysregulation in schizophrenia. Furthermore an association of *DRD2* and *COMT* polymorphisms with impaired performance on working memory and executive function tests has been reported [37–39]. These findings support the idea that genetic susceptibility loci are functionally relevant in schizophrenic patients and for the symptoms they present.

In addition to its role in neurotransmission, dopamine is also a source of ROS in the brain, as dopamine metabolism (Fig. 28.1) produces H_2O_2 and can spontaneously generate highly reactive quinone and superoxide molecules [40]. Furthermore, elevated dopamine metabolism depletes available antioxidant defense systems and renders neurons more susceptible to the negative effects of oxidative stress. This notion is well supported by experiments performed by Do and colleagues looking into effects of dopamine on GSH levels in cultured cortical neurons [41]. In this study, dopamine application resulted in a 40% reduction in intracellular GSH content. This effect appeared to be due to the direct conjugation of dopamine semiquinone/quinone with GSH, as it was not dependent upon D_1 or D_2 receptor activation, monoamine oxidase (MAO) activity, or the generation of ROS. Additional studies

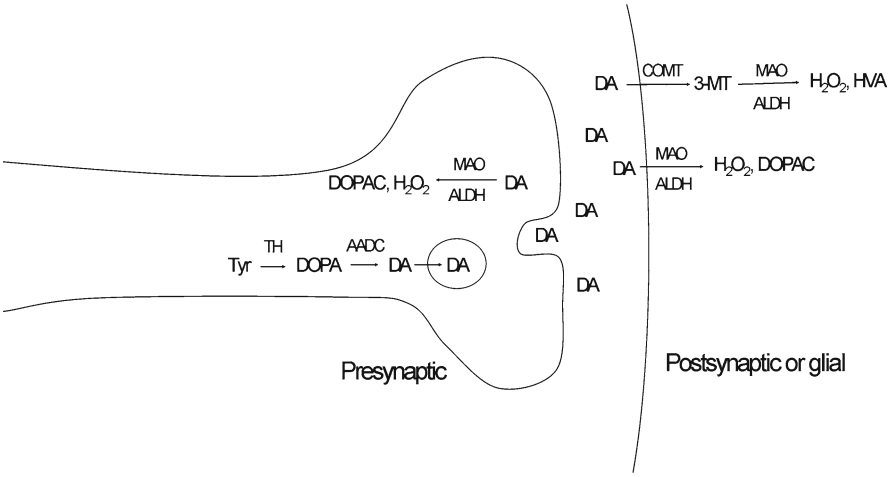

Fig. 28.1 Dopamine metabolism at the synapse. The initial step in the synthesis of dopamine (DA) is hydroxylation of tyrosine (Tyr) by tyrosine hydroxylase (TH), resulting in dihydroxyphenylalanine (DOPA). DOPA is subsequently decarboxylated by aromatic amino acid decarboxylase (AADC) to produce DA, which is sequestered into vesicles by the vesicular monoamine transporter. Presynaptic DA that is not sequestered in vesicles is catabolized primarily by monoamine oxidase (MAO). Oxidative deamination of DA by MAO generates hydrogen peroxide (H_2O_2) and an aldehyde that is subsequently oxidized by aldehyde dehydrogenase (ALDH) to its corresponding acid, dihydroxyphenylacetic acid (DOPAC). Postsynaptic and glial metabolism of DA are similar, with an additional route, whereby DA is first methylated by catechol-O-methyltransferase (COMT) generating 3-methoxytyramine (3-MT). As before, oxidative deamination of 3-MT by MAO generates peroxide and an aldehyde that is oxidized by ALDH to homovanillic acid (HVA). Note that the aldehyde intermediates and the membrane transporters have been omitted for clarity

using ethacrynic acid (EA), an inhibitor of glutathione-S-transferase, decreased GSH levels in a dose-dependent manner. Combined application of dopamine and EA further diminished GSH levels and this additional decrement was inhibited by either D_1/D_2 receptor or SOD antagonists, suggesting a mechanism involving the activation of dopamine receptors and the generation of superoxide. Coadministration of both dopamine and EA for a period of 24 h was also found to result in a 30% reduction in the number of neuronal processes. These results provide evidence for a functional relationship between dopamine and GSH that influences neuronal connectivity.

28.4 Schizophrenia, NMDA Receptors and Oxidative Stress

In recent years, extensive evidence has demonstrated that glutamatergic signaling is also dysregulated in schizophrenia and this appears to be largely due to compromised function of NMDA receptors (NMDARs). Hypofunction of NMDARs is thought to be a key facet of schizophrenia, as administration of NMDAR antagonists,

such as ketamine and phencyclidine (PCP), induce a psychotic state in humans similar to schizophrenia [42]. This notion is further substantiated by findings that transgenic mice containing mutations in NMDAR subunits exhibit some behaviors analogous to schizophrenia [43, 44] and by reports that pharmacological enhancement of NMDAR function can alleviate human schizophrenic symptoms [45]. Additional support is provided by postmortem studies reporting reduced expression of the NR2A subunit in schizophrenic patients [46, 47].

There also appears to be a distinct relationship between oxidative stress and NMDA-dependent synaptic plasticity. NMDA receptors have extracellular redox-sensitive sites, by which reducing agents, such as GSH, can enhance function [48, 49]. Recent experiments on tissue slices of the rat hippocampus provide evidence that GSH deficits alter synaptic transmission [50]. In this study, a 40% decrease in brain GSH levels was induced via administration of an inhibitor of GSH synthesis. This resulted in enhanced excitability and impaired NMDAR-dependent long-term potentiation (LTP) in GSH depleted slices. These findings demonstrate that oxidative stress can impair the function of NMDA receptors.

Interestingly, NMDAR hypofunction has also been associated with increased glutamatergic neurotransmission [51, 52]. Specifically, in vivo microdialysis experiments revealed that systemic administration of low doses of ketamine produce elevated glutamate levels in the prefrontal cortex [53]. In turn, elevated levels of extracellular glutamate can have adverse effects on antioxidant defense mechanisms. Increased levels of glutamate have been shown to inhibit the uptake of cystine [54], a required precursor for GSH synthesis [55]. Glutamate also acts postsynaptically on both NMDARs and non-NMDA ionotropic AMPA and kainate receptors, triggering the accumulation of cytosolic calcium. Excessive intracellular calcium has multiple potential deleterious effects, such as the activation of catabolic enzymes and the generation of free radicals [56]. In sum, elevated extracellular glutamate levels may lead to intracellular calcium overload, enhanced production of ROS, and a hyperexcitable brain that is more susceptible to seizures.

28.5 Schizophrenia, Parvalbumin Interneurons and Oxidative Stress

Another characteristic feature of schizophrenia is dysfunctional cortical inhibition. Individuals with schizophrenia exhibit altered neural oscillations [57] and show reduced expression of the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD67) upon postmortem examination [58]. The link between cortical inhibition and schizophrenia is further supported by the finding that single nucleotide polymorphisms in the regulatory region of *GAD1* (gene coding for GAD67) are correlated with an early onset of schizophrenia [59]. The decrease in GAD67 levels commonly observed in schizophrenia occurs mainly in a subset of interneurons expressing the calcium-binding protein, parvalbumin (PV) [60, 61]. PV-positive interneurons control the firing rates of pyramidal neurons and are critically involved

in generating gamma frequency neural oscillations [62–64]. It has been proposed that the impaired cortical inhibition characteristic of schizophrenia may be a result of flawed maturation of PV-interneurons caused by excessive oxidative stress during neurodevelopment [65]. This idea is corroborated by recent findings that deletion of the gene coding for the modifier subunit of glutamate cysteine ligase (GCLM), a rate-limiting enzyme for GSH synthesis, results in reduced numbers of PV-interneurons and altered gamma oscillations in the ventral hippocampus [66].

Several contemporary reports demonstrate that NMDAR hypofunction and dysfunctional cortical inhibition are intricately intertwined. Experiments in cultured interneurons, wherein the NMDAR antagonist, ketamine, was applied at sublethal concentrations, revealed a time- and dose-dependent decrease in GAD67 and PV immunoreactivity [67]. Repeated in vivo exposure to NMDAR antagonists has also been demonstrated to decrease PV expression in rodents [68] and nonhuman primates [69]. Additionally, electrophysiological studies found that inhibitory GABAergic interneurons were approximately tenfold more sensitive to the effects of NMDAR blockade than excitatory pyramidal neurons [70]. This suggests that the intriguing pairing of NMDAR hypofunction with increased glutamatergic neurotransmission may be the result of insufficient activation of NMDARs on inhibitory GABAergic interneurons. The notion that NMDA hypofunction on GABAergic interneurons is a key component of schizophrenia is further supported by recent studies in which the NR1 subunit was selectively eliminated on PV-interneurons during the early postnatal period [71]. These mutant mice were impaired in several rodent behavioral assays thought to correlate with schizophrenia, including deficits in social recognition, prepulse inhibition (PPI), and spatial working memory.

28.6 Schizophrenia, Oxidative Stress and Selenium

While accumulating evidence provides support for an interconnected relationship between oxidative stress, dopamine signaling, NMDAR function, and cortical inhibition in the symptoms of schizophrenia, reports on the involvement of selenium and selenium-related proteins in schizophrenia are limited. Selenium is a necessary component of glutathione peroxidases and GPx levels have been demonstrated to correlate with whole blood selenium levels up to 0.100 ug/mL, above which GPx levels plateau [72]. In groups of patients with schizophrenia receiving treatment with antipsychotic medication, significantly reduced GPx activity has been reported [73, 74]. Of additional significance, an inverse relationship between blood GPx activity and structural assessments of brain atrophy has also been observed in a population of patients with chronic schizophrenia, suggesting a potential relationship between redox dysregulation and neurodegeneration [75].

There is also circumstantial evidence suggesting that altered function of the mitochondrial selenoprotein, thioredoxin reductase 2 (*TXNRD2*), may contribute to schizophrenia. *TXNRD2* is located on chromosome 22q11.2, a region highly implicated in schizophrenia that also contains the *COMT* gene. A hemizygous deletion of

a 3-Mb region of chromosome 22q11 occurs in approximately 1 in 4,000 humans and produces 22q11 deletion syndrome [76]. Individuals with 22q11 deletion syndrome typically exhibit cardiovascular defects, craniofacial abnormalities, and impaired cognition, as well as an increased likelihood to develop schizophrenia. Moreover, *TXNRD2* has also been reported to be significantly upregulated in the prefrontal cortex of patients with schizophrenia [18].

Of further potential significance within the United States, higher incidences of schizophrenia have been reported in states with low levels of selenium in the food chain [77]. Soil selenium concentration and the long-term rate of schizophrenia have been investigated and although there is a significant relationship, the association is not entirely consistent [78]. In addition, impaired selenium transport was previously hypothesized to be a risk factor for a subtype of schizophrenia characterized by negative symptoms [79], which is supported by findings that platelet and erythrocyte GPx activity is reduced in schizophrenic patients [80, 81].

Several studies also provide convergent evidence for altered dopaminergic signaling in response to dietary selenium intake, suggesting a potential indirect relationship to schizophrenia. Dietary selenium deficiency elevates and prolongs high potassium-induced dopamine release in the striatum, and increases the turnover rate of dopamine in the substantia nigra, prefrontal cortex, and hippocampus [82–85]. Furthermore, selenium deficiency upregulates both tyrosine hydroxylase and dopamine transporter mRNAs in nigrostriatal neurons, with concomitant increases in dopamine synthesis and uptake [86]. Conversely dietary Se supplementation reduces the activity of the dopamine catabolic enzyme, monoamine oxidase (MAO) [87]. Additionally, dopamine deamination by MAO generates H_2O_2, and MAO-catalyzed peroxide generation is coupled to the enzymatic activity of the selenoprotein, GPx1 [88]. Collectively, these findings suggest that dietary selenium modulates the turnover and metabolism of dopamine, which may profoundly affect the pathogenesis of schizophrenia. However, recent genome wide association studies have not identified any polymorphisms in selenoprotein coding genes that significantly associate with schizophrenia.

Nevertheless, studies on mice with targeted disruption of selenoprotein expression provide some intriguing parallels with endophenotypes of human schizophrenia patients. Transgenic mice with neuron-specific deletion of selenoprotein biosynthesis (*Tα1-Cre/Trsp*$^{fl/fl}$) exhibit growth defects and lack of postural control, and rarely survive past P12 [89]. When Cre-mediated recombination is restricted to forebrain neurons (*CamK-Cre/Trsp*$^{fl/fl}$), the mutant mice are able to walk, albeit poorly, and their life span is only moderately extended to P13–P15. Further experiments on *CamK-Cre/Trsp*$^{fl/fl}$ mutants revealed dysfunctional development of the GABAergic system, as PV-interneurons failed to develop and spontaneous epileptiform activity was observed in hippocampal slice preparations of P10 animals. In additional studies, the researchers generated *CamK-Cre/Gpx4*$^{fl/fl}$ mutant mice in order to assess the effects of forebrain-specific GPx4 deletion and compare with a complete blockade of selenoprotein synthesis. In comparison to *CamK-Cre/Trsp*$^{fl/fl}$ mutants, *CamK-Cre/Gpx4*$^{fl/fl}$ mice exhibit similar deficits, albeit milder, as these mice are hyperexcitable, display an awkward gait, and have reduced numbers of

PV-interneurons on P13. In summary, these results indicate a critical role for GPx4 in the development of PV-interneurons [89]. These findings may have particular relevance for schizophrenia, given the established relationship between oxidative stress and impaired cortical inhibition.

Selenium appears to be particularly critical for proper brain function, as selenium is preferentially retained under conditions of selenium deficiency and upon selenium reintroduction into the diet, the brain is the first organ to be re-supplied [90, 91]. In the brain, the primary means of selenium delivery is via ApoER2-mediated uptake of selenoprotein P (Sepp1) [9]. Transgenic mice with targeted deletion of Sepp1 have diminished levels of selenium and reduced GPx and TXNRD activity in the brain [92, 93]. *Sepp1*$^{-/-}$ mice also display a distinct neurological phenotype, which includes occasional seizures, motor dysfunction, and accelerated neurodegeneration [94, 95]. Although administration of a high selenium diet (1 mg/kg) from birth can attenuate many of the adverse symptoms of Sepp1 deletion, *Sepp1*$^{-/-}$ mice maintained on a high selenium diet exhibit motor incoordination, impaired spatial learning, and altered synaptic plasticity in the hippocampus [11]. These findings suggest that Sepp1 may directly influence synaptic plasticity, most likely via the ApoER2 receptor. Transgenic mice without a functional ApoER2 receptor exhibit several symptoms analogous to those of *Sepp1*$^{-/-}$ mice, including reduced brain selenium levels, deficits in spatial learning, and impaired LTP in the hippocampus [11, 12, 92, 93, 96]. Within the membrane, the ApoER2 receptor forms a complex with several signaling proteins, including NMDARs, postsynaptic density protein of 95 kDa (PSD-95), and Disabled-1 (Dab-1) [96, 97]. In addition to Sepp1, several additional ligands interact with ApoER2, including ApoE and Reelin [98]. By means of stimulation of the ApoER2 receptor, Reelin critically influences both neuronal migration and synaptic plasticity and, in recent years, the Reelin signaling pathway has been extensively characterized [96–99]. In particular, an alternatively spliced intracellular domain of ApoER2 has been shown to be required for Reelin-mediated modulation of LTP and synaptic plasticity [96]. This alternatively spliced transcript encodes a 59 amino acid sequence, which contains binding sites for PSD-95 and JNK interacting proteins. Yet, further studies revealed that this intracellular domain was not required for selenium uptake, demonstrating independent roles for this domain in cell signaling and selenium transport [100]. It remains to be determined whether Sepp1 acts as a signaling protein in addition to its role in selenium delivery.

Preliminary findings in this laboratory suggest that Sepp1 may influence PV-interneuron function. Immunohistochemical evidence indicates that the ApoER2 receptor is expressed on PV-interneurons in several brain regions, including the hippocampus, medial septum, and cingulate cortex. As impaired cortical inhibition appears to be one of the cardinal features of both *Apoer2*$^{-/-}$ and *Sepp1*$^{-/-}$ mice, this phenotype may result in part from a disturbance in ApoER2-mediated delivery of Sepp1 to PV-interneurons. Impaired selenium delivery would lead to diminished selenium content, deficient selenoprotein synthesis, and elevated oxidative stress in PV-interneurons (Fig. 28.2).

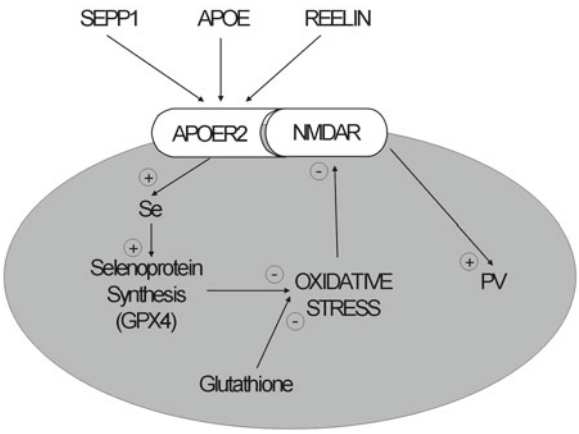

Fig. 28.2 Putative model of PV-interneuron function and oxidative stress. Selenoprotein P (Sepp1), ApoE, and Reelin all interact with the ApoER2 receptor. Preliminary data indicate that ApoER2 is expressed in PV-interneurons. ApoER2-mediated uptake of Sepp1 may act as a mechanism to provide selenium for selenoprotein synthesis in PV-interneurons. Selenoproteins, of which GPx4 is essential, protect against oxidative stress. The antioxidant, glutathione (GSH), is an essential cofactor for the glutathione peroxidases and low GSH levels have been implicated in schizophrenia. Elevated oxidative stress impairs NMDA-mediated neurotransmission, which, in turn, leads to diminished expression of parvalbumin (PV)

Additional evidence suggests that administration of selenium compounds may be preventive against psychosis. Administration of the organic selenium compound $[(F_3CPhSe)_2]$ was found to attenuate apomorphine-induced stereotypy in mice, an animal model of psychosis [101]. Of further potential relevance, upregulation of the selenium-binding protein (*SELENBP1*) has been reported in both the blood and brain of schizophrenic patients [102]. *SELENBP1* does not contain a selenocysteine residue and its functional role in the brain is currently unclear. Yet, given the fact that it binds selenium, *SELENBP1* may reduce levels of free selenium that are available for incorporation into selenoproteins. Thus, increased levels of *SELENBP1* may result in diminished selenoprotein synthesis and elevated oxidative stress. Further experimentation is required to adequately assess this possibility.

28.7 Conclusion

In summary, extensive evidence demonstrates an interconnected relationship between oxidative stress, dopamine dysregulation, NMDA hypofunction, and impaired cortical inhibition in schizophrenia. As selenoproteins comprise one of the key lines of defense against oxidative stress, compromised selenoprotein function may contribute to the dysregulated neurotransmission, impaired cognition, and

behavioral alterations that are characteristic of schizophrenia. The current evidence for the involvement of selenium-related proteins in schizophrenia is suggestive, but limited. Genetic association studies have yet to identify a single selenoprotein as a candidate gene for schizophrenia, but genetic polymorphisms and/or copy number variations in multiple selenoprotein coding genes may, in part, determine the capability of the antioxidant defense system and, thus, may either predispose or protect against the development of schizophrenia. Moreover, selenoprotein functionality may also be influenced by genetic variation in nonselenoprotein coding genes that impact the bioavailability of selenium and the synthesis of selenoproteins. In order to better distinguish the potential relationship between schizophrenia and selenium, additional research is needed to both characterize selenium-related proteins and probe human genetic variation in selenium-related genes.

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Chapter 29
Control of Thyroid Hormone Activation and Inactivation by the Iodothyronine Deiodinase Family of Selenoenzymes

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Abstract The iodothyronine deiodinases both activate and inactivate thyroid hormone, thus controlling thyroid hormone action. These enzymes are also selenoproteins, containing the rare amino acid selenocysteine in their active center. SBP-2 is an important component of the selenoprotein synthesis machinery, and its binding to selenocysteine insertion sequence (SECIS) elements is crucial for selenocysteine incorporation at the UGA codon vs. termination. Recently, several patients have been identified with impaired selenoprotein synthesis due to mutations in SBP-2. One hallmark of this syndrome, found in all patients to date, is abnormal serum thyroid hormone profiles, with low 3,3′,5-triiodothyronine (T3), elevated thyroxine (T4), and inappropriately normal or elevated TSH due to alterations in deiodinase activity. Thus, the constraints that influence selenoprotein synthesis are also relevant to thyroid hormone metabolism. New work in the deiodinase field is also advancing the paradigm that the type 2 deiodinase (D2), the thyroid hormone activating enzyme, and the type 3 deiodinase (D3) the thyroid-inactivating enzyme, play an important role in the modulation of T3 locally within a tissue, without changing circulating levels of T3. One recent example of this can be found in the role D2 plays in providing intracellular T3 necessary during muscle differentiation and regeneration. We have recently shown that in primary muscle precursor cells D2 increases prior to the upregulation of other T3-dependent genes that are necessary for muscle differentiation such as MyoD. Further, when D2 activity is knocked down in this system via an RNAi strategy, myoblast differentiation is dramatically impaired. We have also found that in a mouse model of muscle injury D2 activity increases transiently when muscle is regenerating, and declines when this process is complete. Remarkably, in mice without D2 (D2 knockout) there is a significant delay in muscle repair after injury. While wild type animals have completed the

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regeneration process by 15 days after injury, D2 knockout mice had a much greater number of newly formed centrally nucleated immature myofibers, indicating an impairment in the muscle differentiation process. Thus, the impaired expression of selenoproteins such as the deiodinases can result in alterations in both circulating and intracellular levels of thyroid hormone leading to significant pathophysiologic consequences.

29.1 Introduction

Thyroid hormone (3,3′,5-triiodothyronine or T3) regulates a variety of processes including growth, development, and metabolic rate. In order to produce these effects, T3 enters the nucleus and binds to thyroid hormone receptors, which in turn bind to specific DNA sequences in T3-responsive genes, regulating their transcription in both a positive and negative manner [1]. However, the thyroid gland predominantly produces thyroxine (T4), which has intrinsically low biological activity, having about a tenfold lower affinity for thyroid hormone receptors than T3 [2]. Thus, the enzyme-catalyzed removal of an outer ring iodine from T4 by the type 1 deiodinase (D1) and D2 to produce T3 is an essential step in thyroid hormone physiology. Conversely, D3, and under some conditions D1, can inactivate T3 and T4 by the elimination of an inner-ring iodine, generating the biologically inactive T2 or reverse T3 (rT3) respectively. Accordingly, the iodothyronine deiodinases modulate T3 action by regulating both its production and degradation [3, 4].

29.2 Deiodinases Are Integral Membrane Selenoproteins Containing a Thioredoxin-Fold

All three deiodinases are homodimeric integral membrane proteins containing one transmembrane domain [5–8]. Both D1 and D3 are found in the plasma membrane, while D2 is located in the endoplasmic reticulum [9, 10]. Although the sequence identity between the deiodinases is lower than 50%, all share a conserved active center of approximately 15 amino acids containing the rare amino acid selenocysteine (Fig. 29.1) [4]. D2 also has an additional UGA codon positioned seven amino acids before a UAA stop codon, and although ^{75}Se labeling studies indicate that selenocysteine is incorporated at this site, mutational studies suggest that this second UGA plays no role in D2 enzyme function [11].

Insight into the structure of the deiodinase enzymes has been obtained through in silico protein modeling using hydrophobic cluster analysis [12]. Using this method it was determined that the deiodinases share a common overall structure with a single transmembrane segment in the N-termini, and several clusters of α-helices or β-strands composing the deiodinase globular domains [13]. These are arranged to

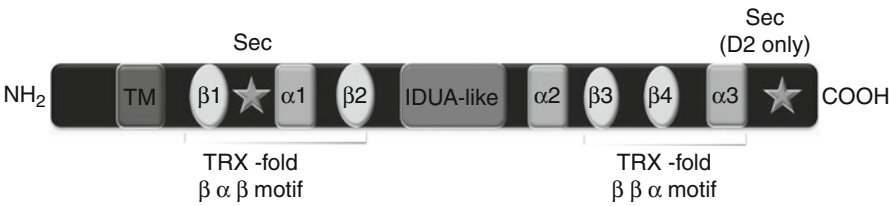

Fig. 29.1 General deiodinase structure. The deiodinases are integral membrane proteins containing one transmembrane domain (TM), and a thioredoxin (TRX)-fold, defined by βαβ and ββα motifs, interrupted by an intervening element 47–60% similar to α-L-iduronidase (IDUA-like). All deiodinases contain a selenocysteine (Sec, indicated by a *star*) within the first thioredoxin βαβ motif. D2 also has an additional selenocysteine 7 amino acids before a TAA stop codon

form a thioredoxin (TRX)-fold, defined by βαβ and ββα motifs, which is also found in many other thiol oxidoreductases including the glutathione peroxidase selenoproteins [14]. Uniquely, the βαβ and ββα motifs within the canonical TRX-fold of the deiodinases are locally interrupted by intervening elements that are very similar (47–60% identity) to α-L-iduronidase (IDUA), a lysosomal enzyme that cleaves α-linked iduronic acid residues from glycosaminoglycans (Fig. 29.1) [13]. This structure, taken in the context of the homodimeric nature of the deiodinases, leads to a model that predicts the active center to be formed by the β1- α1- β2 motifs of the TRX-fold and one of the IDUA intervening elements, with the selenocysteine residue being contained in this pocket. Notably, mutations in the TRX-IDUA active center change kinetic properties of deiodinases, thus confirming the relevance of this model [13]. In this regard, amino acid 128 of D1 is a serine, while proline is found in the corresponding position of D2. D1 is normally PTU sensitive with ping-pong kinetics of substrate catalysis; however, a Ser128Pro modification of the D1 enzyme results in resistance to PTU and a change to sequential kinetics, making D1 more similar to D2. On the other hand, a corresponding substitution of Ser for Pro in the equivalent position of D2 made it more similar to D1, with the mutant D2 now being sensitive to PTU and displaying ping-pong kinetics [13].

29.3 Mutations in SBP-2 Result in Altered Deiodination and Thyroid Hormone Profiles Along with Other Phenotypic Defects Linked to Impaired Selenoprotein Synthesis

As with all selenoproteins, UGA encodes for the insertion of selenocysteine during translation in the deiodinases [15–17]. As detailed in many sections of this book, UGA is normally read as a signal for termination, and in order for this codon to specify selenocysteine incorporation, additional components are required to facilitate

translational read-through of selenoproteins such as the deiodinases [4, 18]. In fact, it was the expression cloning of the D1 enzyme that allowed the recognition that selenoprotein mRNAs require a stem-loop structure in their 3′ untranslated region for successful selenocysteine incorporation, termed the selenocysteine insertion sequence (SECIS) element [15, 19]. In brief, deiodinase synthesis requires the SECIS element that recruits SECIS binding protein-2 (SBP-2) [20]. SBP-2 in turn interacts with an elongation factor, EFsec, promoting the insertion of selenocysteine from a specific tRNA (Sec-tRNA^{Sec}) by the ribosome at the UGA codon [21, 22]. Selenocysteine incorporation is not a very efficient process, and studies have shown that if the UGA codon in D1 is replaced with a cysteine codon, up to 400-fold more D1-protein is produced [23]. Thus, all the factors necessary for selenoprotein production (see Chap. 3) are needed for deiodinase synthesis, and changes in their availability would be predicted to seriously disrupt thyroid physiology. This concept has recently been highlighted by the identification of patients with abnormal thyroid hormone profiles caused by defects in deiodinase synthesis as a result of mutations in SBP-2.

The first patient found with a SBP-2 mutation was identified due to growth retardation as a result of hypothyroidism, with the affected individual falling below the third percentile at 14 years of age [24]. Additional studies identified abnormal thyroid profiles in the proband and three out of seven of his siblings, with an elevated circulating TSH and T4, while serum T3 was below the normal range (Fig. 29.2) [24]. Of note, TSH is negatively regulated by T3, both at the level of transcription and secretion, and this feedback loop requires both circulating T3, as well as T3 produced by D2 [25]. T4 administration is more potent in decreasing serum TSH levels, and studies have shown that D2 is required for local conversion of T4–T3 within the thyrotropes of the pituitary to produce this effect [26]. In support of this, in D2 knockout mice with a targeted deletion of the D2 enzyme serum TSH can be suppressed by T3, but not T4, treatment [27]. With this in mind, a key piece of information in regards to discovering the basis of the deficit in thyroid hormone metabolism in the above patients was that while T3 administration suppressed TSH equally well in all family members, T4 was much less effective in affected individuals. This indicated that the affected individuals might have a deficit in D2 (and potentially D1, see below)-mediated T4–T3 conversion. Using cultured skin fibroblasts, it was determined that affected individuals had much less D2 enzyme activity [24]. Notably, Dio2 mRNA levels of these patients were not decreased in parallel, suggesting that the lower D2 activity was due to a posttranscriptional defect. While the parents of the proband were not consanguineous, they did belong to the same Bedouin tribe, and linkage analysis identified homozygosity for a missense mutation of R540Q in the RNA binding domain of SBP-2 in affected individuals. Further studies revealed that the selenoproteins glutathione peroxidase 1 and Selenoprotein P were also decreased in these individuals, suggesting a global defect in selenoprotein synthesis. The D1 enzyme also converts T4–T3, further contributing to circulating levels of T3, and thus impaired expression of this selenoprotein could also contribute to the abnormal thyroid hormone profiles found in these patients. However, because the expression

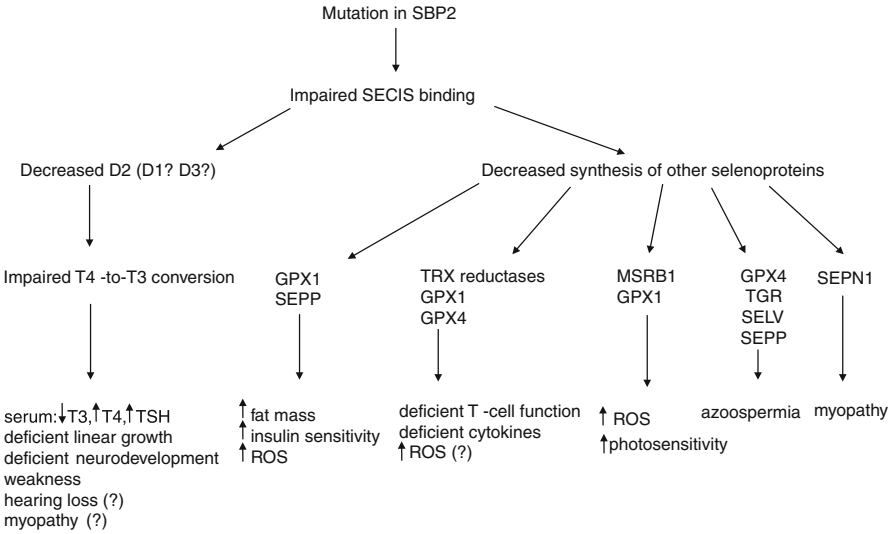

Fig. 29.2 Mutations in SBP-2 lead to impaired selenoprotein synthesis resulting in a variety of clinical disorders. Mutations in SBP-2 can result in decreased binding to SECIS elements necessary for selenocysteine incorporation into selenoproteins. D2 activity is subsequently decreased, while the effects on the other deiodinases are unknown (*left side of figure*). Decreased D2 activity (and perhaps D1) will impair T4–T3 conversion, leading to low serum T3, high serum T4, and either increased or inappropriately normal (non-suppressed) serum TSH concentrations. This in turn can lead to a variety of symptoms associated with T3-defficiency. Mutations in SBP-2 can also result in an impairment in many other selenoproteins, resulting in a variety of clinical features as detailed in Schoenmakers et al. [30]

of D1 occurs in less accessible tissues such as liver, kidney, and thyroid, it has not been possible to evaluate the expression of D1 in any of these patients.

These studies also reported another patient who was identified on the basis of growth retardation, with similar abnormal thyroid function tests [24]. This individual was found to have two different mutant alleles of SBP-2 with one allele being truncated prematurely at 438 amino acids, while the other contained an intronic mutation resulting in alternative splicing and a truncated SBP-2 protein. Notably, the linear growth of these patients was improved by T3 administration; however, not surprisingly, selenium supplementation was ineffective at normalizing serum thyroid hormone profiles [24, 28, 29].

Since the initial report, several other patients have been identified with different mutations in SBP-2 that also effect selenoprotein synthesis [29–31]. To date, all patients have had abnormal thyroid function tests, with serum T3 concentrations either low or low normal, high serum T4, and either elevated or inappropriately normal concentrations of serum TSH. Thus, an impairment of T4–T3 conversion by

D2 in the hypothalamic-pituitary axis (and potentially defects in the production of T3 by D1 containing tissues), is a hallmark of this syndrome.

Interestingly, two of these recent reports have included a more detailed characterization of the patients, thus illuminating other disorders that can be linked to defects in selenoprotein synthesis. In one report, a 12-year-old girl with a compound heterozygous mutation of the SBP-2 gene that resulted in truncated proteins (R120X and R770X) is described [29]. Along with an abnormal thyroid hormone/TSH profile, this patient had delayed motor and intellectual milestones, delayed bone maturation, congenital myopathy, peripheral neuropathy, and increased fat mass. However, many of these symptoms cannot be solely attributed to hypothyroidism caused by impaired T4 activation via the deiodinases, indicating the involvement of other selenoproteins yet to be defined. In a second detailed report, the characterizations of two other patients, including both a 2- and a 35-year-old male with distinct mutations, were described both in vitro and in vivo [30]. Both individuals were found to have defects in synthesis of most of the 25 known selenoproteins, leading to extremely complex phenotypes. Along with abnormal thyroid hormone profiles, both patients in this study had delayed developmental milestones, muscle weakness similar in nature to those described for patients with mutations in Selenoprotein N, increased body fat, and paradoxical insulin sensitivity. The adult patient also had primary infertility due to azoospermia, skin photosensitivity, impaired hearing, and rotatory vertigo. At a cellular level, both patients exhibited impaired oxidant defense in fibroblasts resulting in greater DNA damage. T-cell proliferation was also found to be impaired in the older patient, while this could not be studied in the younger patient due to treatment for eosinophilic colitis. Taken together, this work clearly illustrates that impaired selenoprotein synthesis due to mutations in SBP-2 can result in defective synthesis of many selenoproteins including the deiodinases, leading to a variety of functional abnormalities.

While some of the individuals in the above studies presented with delayed growth that is probably a manifestation of T3-deficiency, this relatively mild phenotype was surprising since mice lacking the selenocysteine tRNA die in utero [32]. This suggests that not all selenoprotein synthesis is equally affected in these patients. One reason that the production of some selenoproteins might be selectively more impaired in affected individuals can be found in studies showing that SBP-2 does not have the same affinity for all SECIS elements [33–35]. In an extension of these results, recent work has shown that a mutant SBP-2 containing the R540Q change has impaired binding to some, but not all, SECIS elements [34, 35]. Other work further suggests that the severity of the phenotype in individuals with SBP-2 mutations may be ameliorated by alternate splicing or internal initiation of transcription at a downstream AUG [31, 36]. Thus a complex set of factors, including the precise mutation in SBP-2 and the resulting affinity between the mutant SBP-2 and different SECIS elements, the amounts and types of other selenoprotein mRNAs, and balance of other factors such as L30 and eIF34a3 that can bind SECIS elements, will all play a role generating the defective pattern of selenoprotein production observed in these patients. For a further description of factors that can bind to SECIS elements and their role in selenocysteine incorporation the reader is also referred to Chap. 4 by Driscoll and Bubenik in this book.

29.4 Local Control of Thyroid Hormone Concentrations by D2 and D3 Is Critical for Tissue-Specific Regulation of Thyroid Hormone Action

It is well established that the deiodinases modulate circulating levels of thyroid hormone, with approximately 80% of the T3 produced daily in humans being derived from monodeiodination of T4 by D1 and D2 [4]. The reciprocal regulation of D2 and D3 provides a complex mechanism allowing circulating and intracellular T3 to be maintained at relatively normal levels even if serum T4 falls modestly, such as during iodine deficiency [37]. Much recent work has also allowed us to appreciate that the deiodinases allow for intricate regulation of intracellular T3 concentrations in a tissue specific fashion while circulating concentrations of both T4 and T3 remain unchanged [3]. This becomes especially important when chronologically specific changes in T3 concentrations are required in specific tissues for developmental processes or after injury, since it would be impossible to produce such subtle changes in only a single tissue through modulation of the levels of circulating T3.

D2 and D3 are the main players in the local regulation of T3 within tissue, while D1 contributes principally to circulating T3 levels, especially during hyperthyroidism [3, 4] (Fig. 29.3). As mentioned, D2 is a T4 activating enzyme, producing T3 by removing one iodine moiety from the outer ring of T4. Further, the D2 enzyme is found in the cellular endoplasmic reticulum, and thus T3 produced from D2 is thought to preferentially supply T3 to the nucleus [9, 25, 38]. Perhaps one of the best-characterized functions of D2 in local control is its essential role in mediating a full thermogenic response of brown adipose tissue (BAT) to adrenergic stimulation via increased T4–T3 conversion within this tissue [39–42]. Thus, when rats are acutely cold exposed, D2 activity increases in BAT, increasing the amount of T3 in this tissue while circulating levels remain unchanged [39]. The net result of these changes is that thyroid hormone receptor occupancy concordantly increases from approximately 50% at room temperature to 95% in cold exposed BAT, while receptor occupancy levels in other tissues remain virtually unchanged. These changes facilitate a program of gene expression in BAT that allows the animal to adapt to cold exposure [43]. In line with this, D2 knockout mice have an impaired thermogenic response to cold exposure, only surviving by compensatory shivering [44].

Conversely, the thyroid hormone signal can be reduced at the tissue/cellular level via inactivation of T4 and T3 by D3. D3 preferentially removes an iodine from the inner ring of thyroid hormones, thus converting T4 and T3 to the biologically inactive reverse T3 (rT3) and 3, 3′ T2, respectively. The D3 enzyme is located in the plasma membrane and is recycled through the early endosomes, inactivating thyroid hormones before they are able to access the nucleus and occupy thyroid hormone receptors due to its location [10]. A striking example of the downstream effects of D3 can be found in basal cell carcinomas (BCCs) [45]. In these tumors, D3 overexpression is driven by aberrant activation of the sonic hedgehog pathway, leading to

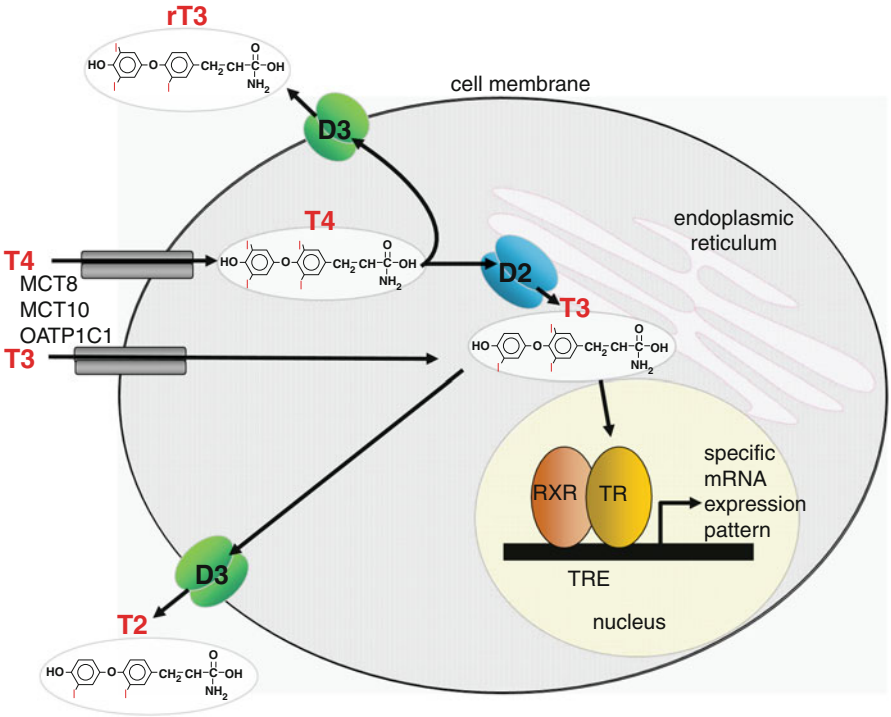

Fig. 29.3 D2 and D3 can fine tune intracellular/local thyroid hormone concentrations. T3 or T4 enters the cell via specific thyroid hormone transporters such as MCT8, MCT10, or OATP1C1. T4 can then be activated by D2 to produce T3, or can be inactivated by D3 to rT3. When T3 enters the cell it can also be inactivated to 3, 3′T2 if D3 is present. In this manner, cells that express D2 in an excess will have increased T3, leading to a relatively "hyperthyroid" pattern of gene expression. In contrast, D3-expressing cells will have a relatively "hypothyroid" pattern of mRNA expression due to thyroid hormone inactivation by this enzyme

increased Gli2, which causes direct transcriptional stimulation of the *Dio3* gene. Notably, when D3 expression is blocked or an excess of T3 is provided, BCC cell proliferation rates are greatly reduced. Remarkably, these same studies showed that elimination of D3 activity in BCC tumors using an RNAi strategy in mice abolishes tumor formation (Fig. 29.4).

The use of D2 knockout mice has uncovered many physiological roles of local T3 production by D2. The importance of D2-generated T3 has been established in many diverse processes including brown adipose activation during cold exposure, chondrocyte differentiation, cochlear development, optimal bone strength and mineralization, and muscle regeneration after wounding [44, 46–49]. To further illustrate the importance of D2 in generating a local supply of T3 we will discuss one recent example, the role of D2 during muscle regeneration after injury, in detail, below.

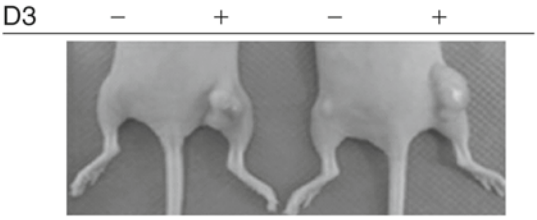

Fig. 29.4 Elimination of D3 decreases basal cell carcinoma (BCC) growth. Nude mice were injected with BCC cells either with high D3 expression (left side of mouse, indicated by +) or in which D3 expression had been greatly reduced by RNAi (right side of mouse, indicated by −). Elimination of D3 blocks tumor growth due to the suppressive effect of endogenous circulating T3. (Modified from Dentice et al. [45])

29.5 D2 Plays a Key Role in Skeletal Muscle Regeneration During Injury

Skeletal muscle is a major target of thyroid hormone action, and expresses D2 [16, 50, 51]. Recent advances in the technique used to measure D2 in this tissue indicate that D2 activity is higher in slow-twitch-oxidative vs. fast-twitch-glycolytic muscles in adult mice, and further, that D2 activity increases in hypothyroidism due to a posttranslational upregulation as expected due to a decrease in the substrate-mediated ubiquitination and degradation of D2 [52, 53]. In mouse skeletal muscle, D2 is high in early postnatal life then decreases rapidly there after. This expression parallels the pattern of Pax7, a marker of satellite cells which are the skeletal muscle equivalent of the stem cell, suggesting a link between D2 activity and myogenesis [49].

D2 mRNA and activity are higher in differentiating vs. proliferating cultures of mouse primary muscle precursor cells [49]. Additionally, when the muscle cell line, C2C12, was induced to differentiate in myotubes, there was also a corresponding increase in D2. This increase in D2 was marked by a subsequent increase in the T3-responsive gene MyoD, the master regulator of the myogenic developmental and regeneration program [54]. Further studies also showed that blocking D2 induction via RNAi could inhibit differentiation of either primary myogenic precursor cells or of C2C12 cells. This blockade could be partially overcome by supplementation with supraphysiologic amounts of T3, confirming a requirement for D2-mediated intracellular T4–T3 conversion. In muscle, the differentiation-dependent induction of the *Dio2* gene was found to be under the control of the developmentally expressed transcription factor Forkhead box O3 (FoxO3), with the *Dio2* gene containing a FoxO3 binding site in its promoter [49]. As expected, inhibition of FoxO3 blocked *Dio2* induction in primary myogenic precursor cells and their subsequent differentiation into myotubes.

In an extension of these results, our studies indicated that muscles of D2 knockout mice presented a phenotype of mild hypothyroidism, with the expression of many relevant T3-responsive muscle marker genes such as MyoD, Myogenin,

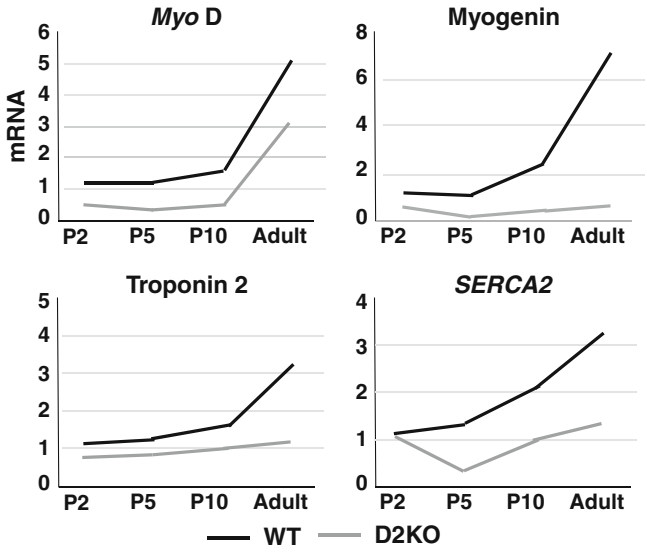

Fig. 29.5 D2 knockout (D2KO) mouse muscle exhibits a relatively hypothyroid phenotype. Expression levels of T3-responsive muscle marker genes were measured at P2, P5, P10 and in adult wild type and D2KO mice by qRT-PCR. (Modified from Dentice et al. [49])

Tropinin 2, and SERCA2 all being decreased (Fig. 29.5) [49]. We further evaluated the regeneration potential of muscle of D2 knockout mice using a model where muscle was wounded by an injection of cardiotoxin into the anterior tibialis muscle. Cardiotoxin injection caused localized muscle damage, which was followed by a robust regenerative response in wild type animals, while circulating levels of T3 remained unaltered. D2 activity was also found to increase after muscle injection, peaking at 8–11 days after injury. However, by day 15 when regeneration was almost complete, as evidenced by a nearly complete localization of myonuclei at the periphery of the myofibers (Fig. 29.6), D2 activity was close to background levels. Notably, wild type and D2 knockout mice displayed no difference in the extent of injury at 4 days after injection, with a similar amount of fiber necrosis and number of non-myogenic cells within the damaged site (Fig. 29.6). However, D2 knockout mice displayed a significantly delayed regeneration response that was clearly evident by 15 days post-injury (Fig. 29.6). While regeneration was almost complete by this time in wild type animals, D2 knockout mice still showed a greater number of newly formed centrally nucleated immature myofibers, indicating a delay in differentiation. Cellular proliferation rates were also assessed by injecting injured animals with Bromodeoxy Uridine (BrdU), a marker for DNA synthesis. Fifteen days post-injury, the number of BrdU labeled nuclei found in D2 knockout muscle was twice that of wild type mice, indicating a greater percentage of cells still replicating. Taken together, these experiments indicate the local increase of T3 production mediated by

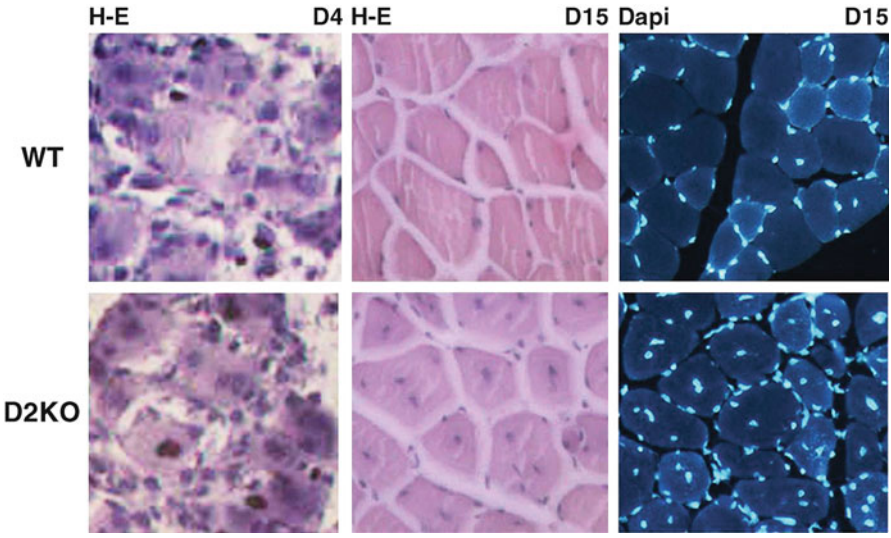

Fig. 29.6 D2 knockout (D2KO) mice exhibit impaired muscle regeneration after injury. Mice were injured by cardiotoxin injection of their anterior tibialis muscle at day 0. No difference can be observed between wild type and D2KO mice by H&E staining 4 days after injury. However, at 15 days post-injury D2KO mice clearly exhibit a significant delay in regeneration compared to wild type mice, as shown by the increased numbers of centrally localized nuclei characteristic of immature myofibrils visualized by either H&E staining or with the nuclear stain DAPI (Modified from Dentice et al. [49])

D2 in satellite cells is critical for proper muscle regeneration. These observations are very exciting and suggest that modulation of the thyroid status of muscle precursor cells by manipulation of deiodinases could have therapeutic application for patients with muscle disease.

29.6 Concluding Remarks

One key feature of all three deiodinase enzymes is the presence of selenocysteine in their active center. Thus, deiodinases are subject to all the constraints that will also regulate selenoprotein synthesis. An example of this can be found in newly identified patients with mutations in SBP-2 that exhibit abnormal serum thyroid hormone profiles due to deficits in synthesis of D2. Another important role of D2 is activation of T4–T3 at the local level, within a specific tissue. One newly identified example of this can be found in the crucial role of D2 in muscle differentiation and regeneration. These examples further highlight the permissive role of selenium in normal thyroid hormone physiology.

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Chapter 30
Role of Selenium in HIV/AIDS

Adriana Campa and Marianna K. Baum

Abstract HIV/AIDS continues to be a major health priority worldwide as the number of people living with HIV grows due to the life-prolonging effects of antiretroviral therapy. Nutrient deficits, however, may interfere with the effectiveness of antiretroviral therapy by delaying the recuperation of the immune system and aggravating side-effects, such as oxidative damage, which have been associated with treatment. Selenium status influences HIV disease progression through its role in cytokine modulation and antioxidant systems. Selenium supplementation in HIV-positive patients has shown benefits on biomarkers of disease progression, morbidity and mortality. Further research is needed to elucidate its effect on other aspects of the disease such as HIV shedding, mitochondrial damage, and HIV transmission.

30.1 Introduction: HIV Epidemic

HIV/AIDS continues to be a major health priority worldwide. The absolute number of people living with HIV has grown due to the life-prolonging effects of antiretroviral therapy (ART) [1]. Despite limitations in the reporting system, the Centers for Disease Control and Prevention (CDC) reported an incidence of approximately 35,000 new cases of HIV infection in the United States in 2008, and currently there are approximately half a million people living with HIV/AIDS in this country [2].

The latest national estimates suggest that the number of AIDS cases remained stable and that the number of deaths is decreasing. In developed countries, antiretrovirals and behavioral prevention interventions have contributed to abating

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DOI 10.1007/978-1-4614-1025-6_30,

the incidence of adult cases. In addition, maternal HIV testing, coupled with the introduction of successful interventions during prenatal, perinatal, and postnatal care have dramatically reduced the incidence of pediatric AIDS [3].

Worldwide, the situation is different. The number of people living with HIV were 33.3 million at the end of 2009 with 2.6 million newly infected in this year [1]. Despite the success of ART in the majority of countries around the world, including Africa, the gap between developed and developing countries in the control of the pandemic and treatment of infected persons continues to grow, and one of the factors that increases case fatality in limited-resource countries is malnutrition. Poor nutritional status can affect immune function independent of HIV infection [4, 5]. Death rates appear to be higher among HIV-infected persons with malnutrition, including those who already were started on ART [6, 7]. Numerous studies have demonstrated that nutritional deficiencies accelerate HIV disease progression and decrease survival [8–17]. Moreover, nutrient deficits interfere with the effectiveness of ART by delaying the recuperation of the immune system and aggravating side-effects, such as oxidative damage that appears to be one of the side-effects of HIV treatment [18–22].

30.2 Selenium and Immunity

The relationship between selenium and immunity might be derived from its role as an essential nutrient. Selenium deficiency produces changes in several metabolic functions, including the immune system. Among several potential mechanisms, selenium status influences the immune response through its role in cytokine modulation [23]. In an in vitro model, the addition of selenium regulated and enhanced the production of interleukin-2 through the increased expansion of high-affinity cytokine receptors in a dose-dependent manner [24]. In animal models, phagocytic neutrophils and macrophages exposed to selenium deficiency had reduced ability to destroy antigens. The immunostimulatory properties of selenium have been documented in animal supplementation studies [25], and in elderly subjects [26], as well as in patients with chronic uremia, psoriatic lesions, and gastrointestinal failure syndrome [27–29].

In HIV-1 infected patients, plasma selenium levels have been associated with markers of immune parameters. Plasma selenium levels were positively related with CD4 cell counts and CD4/CD8 ratio, and inversely correlated with β_2-microglobulin, a marker of CD4 depletion and HIV disease progression, and with thymidine-kinase activity, which seems to have a role in nucleoside analog activation and toxicity [30]. Selenium status was also shown to affect production of TNF-α, a cytokine related to anorexia, wasting and Kaposi's sarcoma [31]. Look et al. [30] demonstrated that plasma selenium levels were inversely associated with TNF type II receptors in HIV-positive patients. Hori et al. [32] showed that selenium supplementation reduced viral replication and suggested that this effect was through the synthesis

of selenoprotein in the glutathione and thioredoxin systems. In addition, several in vitro and in vivo reports provided evidence that adequate selenium status decreases neuropathogenesis, and that selenium appears to act through suppression of interleukin-induced HIV-1 replication, neuronal apoptosis, reduction of blood brain barrier damage, and of the potential interactions between selenium and cytokines [30, 32–34].

30.3 HIV, Antiretroviral Treatment, Oxidative Stress, and Selenium

HIV infection has been characterized by increased oxidative stress [35–40], and a decrease in the levels of major antioxidant nutrients, most notably vitamins E and C, carotenoids, and zinc and selenium [41–45]. The mechanism appears to be through increased chronic immune activation by HIV, which increases the production of reactive oxygen species (ROS). In healthy persons, ROS are continually produced in tissues as a consequence of substrate oxidation, aerobic respiration, and immune activation. These ROS are useful to many of the processes of the cell including cell growth, apoptosis, immunity, and microbial defense [46–48]. Because excessive oxidative products, such as the one observed in HIV infection, can be damaging to tissues, multiple enzymatic and nonenzymatic antioxidant defense systems exist to prevent damage by oxygen radicals.

Among the major antioxidant micronutrients, selenium is critical due to its role in the synthesis of glutathione peroxidase and other selenoproteins [49]. Selenium supplementation to increase the effectiveness of the enzymatic antioxidant defense systems has been investigated for the prevention and treatment of cancer [50]. In HIV-infected patients, supplementation with 100 μg of selenium daily for an year has been demonstrated to increase glutathione peroxidase activity in latently HIV-infected T-lymphocytes [34, 51]. The antioxidant demand in HIV infection is also reflected in declining total glutathione levels with HIV disease progression [52]. In addition, the major antioxidant defense enzymes are also altered, including superoxide dismutase, catalase, and glutathione peroxidase [38, 39, 43]. Gil et al. [40] reported that, compared to HIV-negative patients, HIV-positive patients have shown a reduction in glutathione and glutathione peroxidase, an increase in malonaldehyde (MDA – a marker of lipid peroxidation) and lymphocyte DNA fragmentation, as well as increasing superoxide dismutase activity. The total antioxidant status of the HIV-infected group was also significantly lower than that of the HIV-negative group in this study [40].

Antiretroviral therapy, rather than decreasing the importance of antioxidant supplementation, has created new research challenges for the role of selenium in HIV-1 disease. Antiretrovirals have been associated with increased oxidative stress and oxidative damage [53–56]. However, some studies have found increased antioxidant capacity

and DNA damage repair with the use of ART [57– 59]. Although the effect of different types of antiretrovirals on oxidative stress may vary, protease inhibitors (PIs) have generally been found to increase the production of ROS including peroxides, which are associated with endothelial dysfunction and dyslipidemias leading to increased cardiovascular risk [54, 60]. Nucleotide reverse transcriptase inhibitors have a well-established effect on mitochondria which results in increased measures of oxidative damage including lipid peroxidation products, protein carbonyls, and mitochondrial damage [55, 56]. Studies that combine several types of antiretrovirals have also been shown to produce increased oxidative stress. A study of oxidative stress in 85 HIV-infected patients who were either ART-naïve or on three different ART regimens showed increased lipid peroxidation measured by MDA in the HIV-infected patients vs. healthy controls, and in the ART treated groups compared to the ART-naïve group [61]. Exposure to ART has also been found to increase the generation of ROS in human aortic endothelial cells [62]. Increasing oxidative stress due to mitochondrial toxicity may affect the pathophysiology of HIV disease and the cellular damage seen in AIDS [63].

Low plasma selenium levels have been associated with hyperglycemia, and thrompocytopenia in HIV chronic drug users on ART [64]. No association was found, however, between these two conditions and ART [65]. Lipodystrophy, hyperlipidemias, and insulin resistance in patients receiving PIs [66] may increase the long-term risk of oxidative damage associated with development of atherosclerosis and coronary heart disease [67]. Supplementation of antioxidants, including selenium, may prove to be an important part of the therapy used to fight the sequelae of HIV disease and its treatment.

30.4 Observational Studies of Selenium Deficiency and HIV

Selenium deficiency has been associated with HIV disease progression and mortality [15, 16, 68–70]. In Africa, lower levels of selenium in pregnant women has been found to be predictive of higher risk of intrapartum transmission, and fetal and child death [71]. Several observational studies have reported prevalence of selenium deficiency between 7 and 33% among various HIV-1 infected cohorts, with increasing prevalence as the disease advances to AIDS [45, 68]. Similar findings have been observed in simian immunodeficiency virus models [72].

Before the advent of antiretrovirals, in a study of HIV-1 infected chronic drug users, selenium deficiency was an independent predictor of survival (relative risk 10.8; 95%CI [2.37–49.2], $p<0.002$) after controlling for the joint effects of nutritional deficiencies associated with mortality. This significant effect of selenium deficiency was evident when controlling for CD4 cell count <200 cells/mm^3 at baseline and CD4 cell count over time [15]. When similar analyses were conducted in a cohort of HIV-infected men who had sex with men (MSM) the odds ratio (OR) was

7.2 for mortality in those with low plasma selenium compared to those with normal selenium levels, after controlling for age, race, and CD4 cell count <200 cells/mm^3 at baseline. In this cohort, selenium deficiency was also associated with decreased survival; patients with selenium deficiency lived 31.4 months, compared with 57.4 months for those with normal plasma selenium levels after controlling for CD4 cell levels, viral load, and antiretroviral medications [45]. In HIV-infected persons, adequate dietary selenium intake was strongly associated with reduced measures of oxidative stress [73].

In HIV-infected children, selenium deficiency has been associated with advanced immune-deficiency [74] and mortality [68]. In agreement with the previous findings, a two-year study of 610 children born to HIV-infected women in Tanzania showed that the children's plasma selenium levels were inversely associated with risk of mortality for all causes [16]. In addition, depressed maternal plasma selenium levels significantly predicted risks of fetal death, child death, and intrapartum HIV transmission, but were not associated with risk of delivering a small for gestational age child [71].

Genital HIV shedding, a marker of risk of HIV transmission, has been associated with selenium deficiency. Baeten el al. [75] showed that selenium deficiency was associated with increased vaginal HIV-RNA shedding in Kenyan women. However, higher levels of plasma selenium levels (≥114 μg/L) reported by Kupka et al. [76] were also significantly associated with increased risk of genital shedding of HIV-RNA in Tanzanian HIV + pregnant women. After excluding women with genital infections, this association was strengthened (RR tertile 2 = 1.46, 95% CI = 1.10, 1.92; RR tertile 3 = 1.39, 95% CI = 1.05, 1.84). Consistent with Kupka's findings, a short-term (6 weeks) randomized clinical trial in Kenya that supplemented a multivitamin formula that included 200 μg of selenium, compared to placebo, reported an increase in genital HIV shedding (OR = 2.5, 95% CI (1.4–4.4), $p = 0.001$), after adjusting for baseline $\log_{10}$ vaginal HIV-1 RNA, and body mass index [77]. On the positive side, the report from this trial showed that the parameters for disease progression, CD4 (+23 cells/mm^3, $p = 0.03$) and CD8 cell counts (+74 cells/mm^3, $p = 0.005$) significantly increased with selenium supplementation when compared to placebo, with no effect on serum HIV viral load [77] (Table 30.2).

Selenium deficiency has shown significant association with herpes and candida infections in HIV-infected drug users in Miami [78]. Furthermore, participants with low plasma selenium levels were at a significantly higher risk for mycobacterial disease, both TB and mycobacterium avium ($RR = 3$, $p = 0.015$), after controlling for ART and CD4 cell count [79].

The significant association of selenium status with HIV-related morbidity and mortality may be related not only to selenium's role in maintaining immune competence, but also to its activity in modulating viral expression and protection against oxidative damage caused by the chronic infection and its treatment [80–84].

30.5 Selenium Supplementation in HIV

Selenium supplementation for the treatment of other conditions besides HIV has shown mixed results. A long-term clinical trial of selenium supplementation as a chemopreventive agent in cancer [85] demonstrated safety and efficacy at nutritional doses (200 μg of selenium) [85]. In contrast, a recent report on the preliminary findings of the Selenium and Vitamin E Cancer Prevention Trial (SELECT) demonstrated no effect of selenium or vitamin E alone, or in combination, on the risk of prostate cancer [86, 87]. Other studies, however, have shown that nutritional supplementation of selenium significantly reduced the incidence of primary liver cancer in China [88], and provided significantly greater resistance to aflatoxin B1-induced carcinogenic damage in lymphocytes from healthy human subjects administered daily selenium [88].

In an early case study of a child with HIV/AIDS, Kavanaugh-McHugh et al. [89] described complications with features of Keshan disease, a disease associated with selenium deficiency [90]. Upon supplementation of the child with selenium (4 μg/kg), the deficiency symptoms improved [89]. An improvement in general health has been described after daily selenium supplementation [44, 91, 92], without apparent adverse effects in HIV-positive patients [44, 91]. The association of high risk of HIV-related mortality with selenium deficiency highlights the importance of maintaining adequate selenium status in HIV infection [15].

In two early reports from a small one-year study, French researchers [51, 93] reported benefits from supplementing HIV-positive patients with 100 μg of selenium daily, compared to 30 mg of beta-carotene twice daily, and also compared to a control group without supplementation. The control group increased markers of endothelial damage at the end of the first year while those in the supplementation groups were unchanged [93]. Glutathione peroxidase activity increased significantly ($p=0.04$) in the selenium group between 3 and 6 months of supplementation compared to those receiving beta-carotene or no supplements [51].

In Miami, 186 HIV-positive adults, some of whom were already on ART or started on ART during the study, were randomized into receiving 200 μg of selenomethionine or placebo in a randomized, double-blind, placebo-control trial. Those supplemented with selenium had a reduced cost of health care and were 60% significantly less likely ($p=0.01$) to be hospitalized during the two-year follow-up [94]. In a 9-month, randomized clinical trial of selenium supplementation in HIV-positive adults, also in Miami, Hurwitz et al. [95] demonstrated that those supplemented with 200 μg of selenium, whose serum selenium increased as evidence of treatment adherence, maintained their HIV-1 viral load (□ $=-0.04\pm0.7$ $\log_1 0$units), and increased CD4 cell count (□ $=+27.9\pm150.2$ cells/p□L) over time. A greater increase in plasma selenium concentration predicted a decrease in viral load ($\beta=-0.14$) ($z=-2.2$; $\beta=0.09$; $p<0.03$), and their models showed that the effect of selenium supplementation on CD4 cell count was secondary to the effect on viral load ($\beta=-0.29$), ($z=2.3$; $\beta=0.06$; $p=0.03$).

In a large randomized trial of supplementation with selenium (200 μg in the form of selenomethionine) in 915 HIV-infected pregnant women in Tanzania, who were supplemented from the 12–27th week of gestation until 6 months after delivery, Kupka et al. [96, 97] reported a reduction of 40% of diarrhea without significantly increasing the risk for anemia in the women, and a reduction in risk of child mortality after 6 weeks postdelivery.

30.6 Clinical Trials of Supplementation in HIV-Positive Patients that Included Selenium in the Experimental Formula

Selenium doses have been tested in several clinical trials in HIV-positive patients. Trials of selenium alone [51, 93–97] (Table 30.1), or in combination with other antioxidants, vitamins and minerals in the experimental formula (Table 30.2) [77, 98–102] have provided evidence of beneficial outcomes. In those trials in which selenium was part of a formula with other antioxidants and micronutrients, it is not possible to separate the benefits of selenium from those of the rest of the components of the intervention. Moreover, separating this effect may not be desirable, because the benefits of supplementation might be magnified by the interactive and synergistic character of nutrients and antioxidants.

Formulas with selenium have been tested as experimental or standard-of-care formulas with other medications in Africa [98, 103]. Kelly et al. [98], in a short-term 2 week randomized clinical trial of supplementation with a micronutrient formula that contained selenium, compared to placebo, explored the effect of supplementation on enhancing the effect of 800 mg of Albendazole, an anthelmintic, in Zambia. The trial randomized 106 HIV-positive adults with diarrhea-related wasting, who were not on ART, into a micronutrient formula with vitamins A, C, and E, zinc and selenium plus Albendazole or into the anthelmintic and placebo. After 2 weeks of supplementation, the addition of the nutrient formula to albendazole did not improve outcomes. In a recent pilot study in Nigeria involving the advantages of adding daily aspirin to a nutrient formula, 32 HIV-positive, ART-naïve patients were supplemented with 200 μg of selenium, vitamin A, B-complex, C, and D. Twenty-three patients were randomized into the multivitamin/mineral formula with 300 mg of aspirin 4–6 times daily, and a second group that included nine patients, into the multivitamin/mineral formula alone without aspirin. After 6 months of supplementation, the post-therapy mean weight was significantly higher (61.6 ± 15.2 kg vs. 60.0 ± 14.3 kg, $p = 0.015$) in the experimental arm with aspirin compared to the micronutrient formula alone, and CD4 cell count increased by an average of 36.2 cells/mm^3, showing a strong trend towards improvement ($p = 0.059$), albeit not significant [103].

Kaiser et al. [99] supplemented 40 HIV-positive adults on ART with controlled viral load and a combined formula of antioxidants, minerals and vitamins that contained 200 μg of selenium for 12 weeks that resulted in an improvement in their

Table 30.1 Summary of clinical trials that supplemented selenium in HIV-positive patients and findings

Authors	Population	Methods	Formula composition and selenium doses	Findings
Kupka et al. 2009 [96]	915 HIV+pregnant Tanzanian women Participants were not on ART at baseline	Randomized, double-blind, placebo-controlled trial of supplementation to women who were recruited between 12 and 27 weeks of gestation and followed until 6 months after delivery	*Supplement:* selenomethionine, 200 μg daily, compared to placebo *All women received*: antenatal ferrous iron (60 mg/day), and 20 mg riboflavin, 110 mg niacin, 25 mg B_6, 50 μg B_{12}, 500 mg C, 30 mg E, and 0.8 mg folic acid	Supplementation with selenium during pregnancy and postpartum reduced diarrheal morbidity risk by 40% with no significant risk for anemia. No effect on morbidity endpoints
Kupka et al. 2008 [97]	913 HIV-infected pregnant women in Tanzania and their children Participants were not receiving ART at baseline	Randomized, double-blind, placebo-controlled trial of supplementation to women who were recruited between 12 and 27 weeks of gestation and followed until 6 months after delivery	*Supplement*: selenomethionine, 200 μg daily, compared to placebo *All women received*: antenatal ferrous iron (60 mg/day), and 20 mg riboflavin, 110 mg niacin, 25 mg B_6, 50 μg B_{12}, 500 mg C, 30 mg E, and 0.8 mg folic acid	Maternal supplementation with selenium during pregnancy and postpartum reduced risk of child mortality after 6 weeks of delivery
Hurwitz et al. 2007 [95]	262 HIV-infected adults in Miami, Florida, USA 73.5% on ART at baseline	Randomized, double-blind, placebo-controlled trial of supplementation for 9 months	*Supplement*: selenium yeast providing 200 μg elemental selenium daily, compared to placebo	Daily selenium supplementation suppressed the progression of HIV-1 viral burden and provided indirect improvement of CD4 cell count

Burbano et al. 2002 [94]	186 HIV-positive adults with some of them on ART at baseline	Randomized, double-blind, placebo-controlled trial of supplementation for two years in Miami, USA	*Supplement:* selenium yeast providing, 200 μg daily, compared to placebo	Daily selenium supplementation reduced the rates of hospitalization significantly ($p=0.01$) and health-related costs
Constans et al. 1998 [93]	36 HIV-seropositive subjects supplemented for one year. Study before the ART era	10 subjects supplemented with selenium, 11 supplemented with beta-carotene and 15 controls who were not supplemented for one year	*Supplement:* 100 μg of selenium daily *or* 30 mg beta-carotene twice daily compared to no supplements for the control group	The comparison group experienced increased von Willebrand factor and soluble thrombomodulin implying increased damage to the endothelium over an year of the study. Both supplemented groups had no change in these parameters
Delmas-Beauvieux et al. 1996 [51]	36 HIV-seropositive subjects supplemented for one year. Study before the ART era	14 subjects supplemented with selenium, 13 subjects with beta-carotene and 18 controls not supplemented	*Supplement:* 100 μg of selenium daily *or* 30 mg beta-carotene twice daily compared to no supplements for the control group	GPX activity increased significantly after selenium treatment ($p=0.04$ between 3 and 6 months)

Table 30.2 Summary of randomized clinical trials that included selenium in the experimental formula in HIV-positive patients

Authors	Population	Methods	Formula composition and selenium doses	Findings
Villamor et al. 2008 [101]	471 HIV-positive and 416 HIV-negative adults with pulmonary TB in Tanzania Participants were not receiving ART	Randomized clinical trial of multivitamins and selenium compared to placebo and followed from initiation of TB therapy for a median of 43 months	*Multivitamin supplement*: 20 mg thiamin, 20 mg riboflavin, 100 mg niacin, 25 mg B_6, 50 μg. B_{12}, 0.8 mg folic acid, 500 mg C, 30 mg E, and 200 μg of selenium, compared to placebo	Multivitamin supplementation with the multivitamin increased CD3+ and CD4+ cell counts and decreased the incidence of extrapulmonary TB and genital ulcers in HIV-negative patients. Reduced the incidence of peripheral neuropathy by 57%, irrespective of HIV status
Range et al. 2006 [102]	213 patients with TB + HIV, 286 patients with TB and HIV-negative Participants were not receiving ART at the onset of the study	Randomized, factorial design 2×2 to Zn (45 mg), or Multivitamins with minerals (including selenium), or multivitamins and minerals + zinc or placebo. Participants were supplemented for 8 months	*Multivitamin and mineral supplement:* 5,000 IU Vitamin A, 20 mg thiamin, 20 mg riboflavin, 25 mg B_6, 40 mg niacin, 50 μg. B_{12}, 0.8 mg folic acid, 200 mg vitamin C, 60 mg vitamin E, 200 IU vitamin D3, 200 μg selenium, 5 mg copper, and 45 mg zinc, compared to placebo	Supplementation with multivitamins and minerals, including Zn, Cu, and Se during treatment of pulmonary TB reduced mortality in those coinfected with HIV and tuberculosis

Kaiser et al. 2006 [99]	40 HIV-infected participants on stable ART	Randomized, double-blind, placebo-controlled trial comparing a supplementation formula to placebo for 12 weeks	*Supplement*: 1,200 mg *N*-Acetyl cysteine, 100 mg Acetyl L-carnitine, and 400 mg α-lipoic acid. *Vitamins*: A 8000 IU, β-carotene 20,000 IU, C 1800 mg, 60 mg thiamin, 60 mg riboflavin, 60 mg pantothenic acid, 60 mg niacinamide, 60 mg Inositol, 50 µg. Biotin, 260 mg B_6, 2.5 µg. B_{12}, 400 IU vitamin D, 800 IU vitamin E, 300 mg Bioflavonoid complex, 800 µg folic acid, and 60 mg Choline. *Minerals*: 800 mg Ca, 18 mg Fe, 30 mg Zn, 400 mg Mg, 200 µg Se, 150 µg iodine, 100 µg Cr, 10 mg Mn, 2.0 mg Cu, 300 µg Molybdenum, 2.0 mg Boron, 99 mg Potassium, and 150 mg Betaine HCL, compared to placebo	Micronutrient supplement administered to HIV-infected patients taking stable ART significantly enhanced CD4 lymphocyte reconstitution
McClelland et al. 2004 [77]	400 HIV-infected women in Kenya Participants were not receiving ART	A double-blind, randomized, placebo-controlled trial of 6 weeks of multi vitamins plus selenium supplementation or placebo	*Supplement*: 20 mg thiamin, 20 mg riboflavin, 25 mg B_6, 100 mg niacin, 50 µg B_{12}, 500 mg vitamin C, 30 mg vitamin E, 0.8 mg folic acid, and 200 µg selenium, compared to placebo	Supplementation resulted in higher CD4 (+23 cells/µL, $p=0.03$) and CD8 cell counts (+74 cells/µL, $p=0.005$) than placebo, but increased genital shedding

(continued)

Table 30.2 (continued)

Authors	Population	Methods	Formula composition and selenium doses	Findings
Jiamton et al. 2003 [100]	481 HIV-infected men and women in Thailand with CD4 cell counts in the range of 50–550 cells/mm^3 Participants were not receiving ART	Randomized, double-blinded controlled clinical trial of micronutrient supplementation compared to placebo and followed for 48 weeks	*Supplement: Vitamins:* 3,000 μg vitamin A, 6 mg β-carotene, 20 μg or 800 IU vitamin D_3, 80 mg vitamin E, 180 μg vitamin K, 400 mg vitamin C, 24 mg thiamin, 15 mg riboflavin, 40 mg vitamin B_6, 30 μg vitamin B_{12}, 100 μg folacin, and 40 mg panthothenic acid; and *Minerals*: 10 mg Fe, 200 mg Mg, 8 mg Mn, 30 mg Zn, 300 μg Iodine, 3 mg Cu, 400 μg selenium, 150 μg Cr, and 66 mg cystine (Immunace®, Vitabiotics Ltd, London, UK), compared to placebo	Multiple micronutrient supplementation improved the survival of HIV-infected individuals with CD4 cell counts <200
Kelly et al. 1999 [98]	106 HIV-positive adults in Zambia with diarrhea-related wasting syndrome who were not on ART	Randomized, placebo-controlled clinical trial of micronutrient supplementation and 800 mg Albendazole compared to Albendazole alone	*Supplement:* 10,500 IU of vitamin A, 300 mg vitamin C, 300 mg vitamin E, 200 mg zinc, and 150 μg selenium given as three tablets of Selenium-ACE (Boots Co plc, Nottingham, UK) plus 800 mg of Albendazole, compared to Albendazole alone	Adding micronutrients to Albendazole did not significantly affect outcomes

CD4 cell counts. Jiamton et al. [100] supplemented a complex formula containing selenium that resulted in improved survival in those with advanced HIV disease (CD4 cell count<200 cells/mm^3) [100].

HIV/TB coinfection is one of the main causes of mortality in resource-limited countries [104, 105]. In a randomized controlled clinical trial in Tanzania, Villamor et al. [101] randomized 887 TB patients, of whom 471 were also HIV-positive, into a micronutrient formula containing selenium or into placebo. Supplementation increased CD3 and CD4 cell counts, decreased the incidence of extrapulmonary TB and genital ulcers in those who were HIV-negative, and reduced peripheral neuropathy by 57% irrespective of HIV status [101]. In addition, participants in a cohort study, that included patients infected with TB alone and patients coinfected with HIV and TB, were provided with a multivitamin/mineral supplement that contained 200 μg of selenium in their daily dose. This trial reported significant reduction in mortality in those supplemented who were coinfected with HIV and TB [102].

In summary, randomized clinical trials of selenium (using doses ≤tolerable upper intake of 400 μg/day for adults) [106] either with selenium alone (Table 30.1) or with multivitamin/mineral formulas that included selenium in the experimental intervention (Table 30.2) in HIV-positive patients have shown benefits on biomarkers of disease progression, morbidity and mortality. Further research is needed on the effect of selenium on other aspects of the disease such as HIV shedding, mitochondrial damage, and HIV transmission.

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Chapter 31
Seafood Selenium in Relation to Assessments of Methylmercury Exposure Risks

Laura J. Raymond, Lucia A. Seale, and Nicholas V.C. Ralston

Abstract Selenium, an important nutrient present in many foods and especially abundant in ocean fish, is known to counteract mercury toxicity. This effect has been attributed to the high-binding affinities between mercury and selenium, whereby selenium was assumed to sequester mercury and thus prevent its harmful effects. However, recent research indicates that methylmercury is a highly specific irreversible inhibitor of selenium-dependent enzymes (selenoenzymes). Therefore, selenium may not act as a "tonic" that sequesters mercury, but may instead be the "target" of mercury binding which inhibits essential selenoenzyme functions. Since methylmercury readily crosses the placental and blood–brain barriers, its affinity for selenium enables it to impair synthesis and activities of selenoenzymes that are required for healthy fetal brain development. Effects of high methylmercury exposures depend on dietary selenium intakes and selenium status.

31.1 Introduction

Fish consumption during pregnancy exposes the mother and her developing fetus to methylmercury (MeHg), a readily absorbed soft electrophile that is neurotoxic at high tissue concentrations [1]. However, ocean fish are also rich in nutrients such as selenium (Se), vitamin D, and long-chain polyunsaturated fatty acids such as docosahexaenoic and eicosapentaenoic fatty acid (DHA and EPA) that are required for

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brain development and health [2]. Therefore, the risks and benefits of ocean fish consumption are mutually confounding in statistical assessments, and neither should be considered in isolation.

Since all forms of fish contain at least traces of MeHg, low-level exposures from fish consumption are nearly universal. Although low MeHg exposures have not been associated with neurodevelopmental harm, potential risks from consuming varieties of fish with higher MeHg levels continue to be a concern. Ocean fish generally contain less than 0.5 ppm MeHg, although levels over 1 ppm (~5 μmol/kg) do occur in large ocean fish of certain species. However, in two mass-poisoning incidents that occurred in Japan [3, 4], fish from polluted waters that had MeHg levels exceeding 50 ppm and up to 200 ppm in fish livers were consumed [5]. The consequences of exposure to high doses of MeHg are well characterized, but cause concerns regarding low-level MeHg exposures from fish consumption. Although there were uncertainties regarding effects of MeHg exposure from eating ocean fish, federal and state agencies recommended in 2004 that women restrict fish consumption during pregnancy to no more than two meals a week. The advice to limit maternal MeHg exposure consequently restricts the mother's intakes of beneficial nutrients from seafood and thereby impairs her child's development. Ocean fish are also great sources of Se, which is not only nutritious, it counteracts Hg toxicity (see reviews in [6, 7]).

In order to quantify health risks associated with MeHg exposure and define the molecular mechanism of its toxic effects, fish consumption studies have been conducted around the world. Several major studies have examined the effects of maternal fish consumption on the subsequent neurodevelopment of children exposed to MeHg in utero. The overall conclusions from these studies report what initially seem to be conflicting results, but are consistent when considering the importance of physiological Hg–Se interactions in these assessments.

Studies that examined effects of exposure to MeHg from typical varieties of ocean fish [8–12] have not found adverse effects on child outcomes, but have instead found substantial benefits accompanying increasing maternal seafood consumption [8–12]. The ocean fish consumed by these populations all contain a molar excess of Se relative to Hg. In contrast, the studies from the Faroe Islands and New Zealand reported subtle neurodevelopmental impairments from seafood consumption [13–15], but these studies involved maternal consumption of food that contained Hg in molar excess of Se such as shark >2:1 [16] or pilot whale: >4:1 [17].

Since diminished seafood intakes during pregnancy increase risks of poor developmental outcomes in their children [8–12], maternal avoidance of fish consumption causes worse outcomes in neurodevelopmental domains where benefits from avoidance of MeHg was intended. Children of mothers who avoided fish consumption during pregnancy showed developmental impairments of a magnitude approximately 60 times greater than the worst case effects associated with the highest pilot whale consumption in the Faroes. Children of mothers who complied with the US Environmental Protection Agency reference dose (RfD) had an increased risk of scoring in the lowest quartile for verbal intellectual quotients (IQ), compared to children of mothers exceeding the recommended fish intake [10]. Maternal compliance

with diminished fish consumption also increased risks for pathological scores in fine motor, communication, and social skills. Conversely, increasing maternal fish consumption during pregnancy is associated with improved child performance in a number of indicators of neurological health.

31.2 Mercury and Selenium-Binding Affinities

The Se of selenocysteine (Sec) (pK_a 5.5) is predominantly ionized at physiological pH, while the sulfur of cysteine (Cys) (pK_a 8.3) is largely protonated. Therefore, being the strongest intracellular nucleophile, the Se of Sec is an attractive target for binding by the electrophilic Hg of MeHg. The high affinity between Hg and Se results in formation of HgSe, thereby compromising Se's biological availability. In comparison to Hg's affinity for the sulfur of Cys, 10^{14}, the affinity constant between Hg and the Se of Sec is estimated to be ~10^{22}. The selenides that are formed during each cycle of Sec synthesis have an exceptionally high affinity for Hg (10^{45}), which is a million times higher than that of sulfide (10^{39}), Hg's second best binding partner. Mercury selenide (HgSe) precipitates have extremely low solubility, 10^{-58} to 10^{-65} [18], and therefore are metabolically inert.

The extremely high affinity between Hg and Se, therefore, allows Hg to sequester Se and reduce its biological availability. Exposure to disproportionate amounts of Hg has been shown to limit the activity of numerous Se-dependent enzymes, many of which are essential for development (Table 31.1). Consequently, high Hg exposures impair Se transport from maternal blood across the placenta to the fetus [19, 20]. High Hg exposures can also inhibit the activities of Se-dependent glutathione peroxidase in placenta [19] and brain [19, 21]. Likewise, MeHg has been shown to inhibit the activity of glutathione peroxidase and thioredoxin reductase (Table 31.1). Since MeHg binds covalently with the Se of Sec at the active sites of these enzymes, it is by biochemical definition a highly specific irreversible selenoenzyme inhibitor. This enzyme inhibition is exacerbated under low Se conditions, but selenoenzyme activities can be maintained by supplemental dietary Se [22].

Methylmercury readily crosses placental and blood–brain barriers in the form of a cysteine adduct (MeHg-Cys) that apparently resembles methionine (Met) [23]. Because MeHg-Cys is a molecular mimic of Met [24, 25], it is taken up by the LAT1 amino acid transporter which tends to be nonspecific in its transport activities [26]. At low levels, MeHg-Cys occurs in proteins without apparent pathological consequence. MeHg readily exchanges covalent associations with binding partners of equal or greater affinities. This is succinctly summed up by the observation that "mercury is thermodynamically stable, but kinetically promiscuous." Mass action effects drive the slow, but inexorable and highly selective, sequestration of Se in association with MeHg.

As intracellular concentrations of MeHg approach or exceed 1:1 stoichiometries with Se, selenoenzyme activities will be increasingly inhibited and formation of insoluble HgSe will deplete intracellular Se for subsequent cycles of selenoprotein

Table 31.1 Selenoprotein relationships with mercury

Selenoprotein	Functional effects of mercury or methylmercury	References
Cytosolic glutathione peroxidase (GPx1)	Hg binds directly glutathione and Sec of GPx, which may decrease enzyme activity overall, increasing oxidative stress.	[19, 21, 54–58]
Gastrointestinal glutathione peroxidase (GPx2)	Hg binds directly glutathione and Sec of GPx, which may decrease enzyme activity overall, increasing oxidative stress.	[59–64]
Plasma glutathione peroxidase (GPx3)	Implicated in Hg detoxification on occupational and environmental exposure to Hg vapor	[65–70]
Phospholipid glutathione peroxidase (GPx4)	Reduced activity in testis of $HgCl_2$-exposed rats, which leads to decreased sperm count and motility.	[71–76]
Thioredoxin reductase Type I (TrxR1)	Hg from $HgCl_2$ and MeHg directly binds to thiols that react with TrxRs and to Sec in the active site of TrxRs, directly inhibiting activity. Se sequestered by Hg would impair Sec insertion into protein, yielding GRIM-12, a potent apoptosis initiator.	[73, 77–82]
Thioredoxin reductase Type II (TrxR2)	Hg from $HgCl_2$ and MeHg directly binds to thiols that react with TrxRs and to Sec in the active site of TrxRs, directly inhibiting activity	[57, 73, 78–80]
Thioredoxin reductase Type III (TrxR3, TGR)	Hg from $HgCl_2$ and MeHg directly binds to thiols that react with TrxRs and to Sec in the active site of TrxRs, directly inhibiting activity	[77, 78, 83]
Deiodinase Type I (Dio1)	Inhibitory effect on thyroid hormone synthesis, secretion, and metabolism. Hg vapor from occupational exposure shown to decrease T_4/T_3 plasma ratio.	[84–88]
Deiodinase Type II (Dio2)	Inhibitory effect on thyroid hormone synthesis, secretion, and metabolism. Hg vapor from occupational exposure shown to decrease T_4/T_3 plasma ratio.	[84, 88]
Deiodinase Type III (Dio3)	Inhibitory effect on thyroid hormone synthesis, secretion, and metabolism. Hg vapor from occupational exposure shown to decrease T_4/T_3 plasma ratio.	[78, 83]
Selenoprotein P (SelP)	Directly binds to Hg. Implicated in Hg detoxification in the plasma upon exposure to Hg vapor. Decreased Se levels bound to SelP in Hg-exposed subjects. Sequestering of Se bound to SelP by Hg potentially related to Alzheimer's Disease development	[32, 70, 89–93]
Selenoprotein W (SelW)	Molecular target of MeHg in human neuronal cells	[80, 94, 95]

synthesis. Selenoenzymes are vital for redox control that prevents and reverses oxidative damage in the brain and neuroendocrine tissues, as well as for other important biological functions. This is supported by studies showing MeHg inhibits the activity of glutathione peroxidase and thioredoxin reductase (Table 31.1). Therefore, loss of these enzymes can have severe and potentially lethal consequences. Additional Se in the presence of MeHg ensures adequate Se is available to replace the Se that is lost because of Hg sequestration and thereby maintains normal selenoprotein synthesis.

31.3 Importance of Selenoproteins in Development

Selenium is recognized for its importance in neurophysiology [27–29]. Many metabolic processes depend upon Se physiology [30, 31], and increasing numbers of diseases and clinical conditions are recognized to involve disruptions of Se-enzyme metabolism. Although selenoenzyme levels are homeostatically regulated in neuroendocrine tissues, selenoprotein concentrations in other tissues reflect dietary Se intakes. Selenoprotein functions and tissue distributions demonstrate high homologies in all vertebrates and throughout most of the animal kingdom. The enzyme activities of the gene products of the 25 human selenoprotein genes identified have become increasingly well defined, and many employ Sec in their active sites to perform their catalytic functions. The functions of certain selenoproteins remain inadequately described, although structural resemblances with other selenoproteins suggest functional similarities.

Selenoproteins regulate and have pivotal functions in several important cell pathways, which may explain why Se is highly regulated and homeostatically conserved in most tissues. The three main families of characterized selenoproteins (the iodothyronine deiodinases, thioredoxin reductases, and glutathione peroxidases) all have critical roles in fetal development, growth, hormone metabolism, and oxidative stress detoxification in a variety of tissues, particularly endocrine and brain tissues. The importance of Se in these tissues is further emphasized by the fact that mechanisms have evolved to maintain normal concentrations of Se even when severe dietary Se deficiency is present. This is especially exemplified by the brain, which retains approximately 60% of its normal Se concentration even when fed a Se-deficient diet [30, 31]. Studies with SelP-deficient mice indicate that a moderate reduction of brain Se content impairs brain function [32], confirming Se's crucial role in this organ.

31.4 Selenium and Mercury–Selenium Molar Ratios in Fish

The molecular forms of Se present in most food are the amino acids selenomethionine (SeMet) produced by plants nonspecifically during Met synthesis and Sec that is synthesized de novo in selenoproteins expressed in tissue-specific distributions.

In most forms of animal life, SeMet and Sec make up the bulk of tissue Se. However, a novel low-molecular weight form of Se has recently been found to predominate in meats of ocean fish [33]. Known as selenoneine, this low-molecular weight form comprises up to 95% of the total amount of Se present in red muscle and over 60% of the total Se in white muscle of blue fin tuna. It is not yet known how biologically available selenoneine is or whether it has specific functions that it may uniquely support.

Since Se is homeostatically regulated in tissues while MeHg accumulation is uncontrolled, the Hg:Se molar ratios of seafood tend to vary in direct proportion to MeHg, which is directly related to food chain status. Seafood typically contain far more Se than Hg [16, 34]. However, pilot whale and certain varieties of shark are unusual in that they contain Hg in molar excess of Se [16]. This is in stark contrast to most other varieties of ocean fish and seafood, which are among the richest sources of dietary Se. In a survey of 1,100 food consumed in the US [35], 18 of the top 25 dietary sources of Se were seafood.

31.5 Animal Studies Examining MeHg–Se Interactions

Selenium's ability to counteract toxic effects of high Hg exposures has been recognized since 1967 when Parizek and Ostadalova first reported that lethal toxicity of mercuric chloride was alleviated by simultaneously administering sodium selenite to rats [36]. Subsequent studies have confirmed that supplemental Se counteracts motor function, growth impairments (see Fig. 31.1), and lethality that otherwise accompany high Hg/MeHg exposures [37–48]. In 1972, Ganther et al. showed that

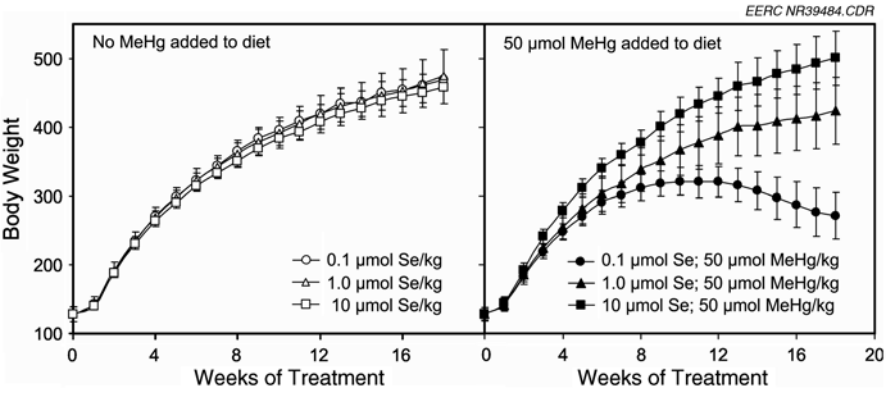

Fig. 31.1 Effects of dietary Se intakes and MeHg exposure on growth. Growth of groups of rats fed low, normal, or enriched (0.1, 1.0, or 10.0 µmol Se/kg) dietary Se with MeHg at 0.5 µmol (*left*) or 50 µmol MeHg/kg (*right*). Data depict means ± SD for group ($n = 10$) body weights (in grams) at the times indicated (Adapted from Ralston et al. [45])

Se diminished the toxicity of MeHg, reduced MeHg-induced mortality, and restored weight gain in MeHg-affected rats [47]. Friedman et al. found that the Se present in freeze-dried swordfish counteracted MeHg toxicity [48]. In that study, the rats that were fed MeHg along with swordfish showed no signs of Hg poisoning, while rats fed MeHg fed diets without Se from fish exhibited symptoms of neurotoxicity.

Animal studies indicate that maternal dietary Hg:Se ratios need to be lower than 1:1 in order to maintain maternal supply of Se to the fetus and prevent loss of selenoenzyme activities. High MeHg exposures result in dose-dependent diminishments in brain selenoenzyme activities [19, 21, 49] and increase oxidative damage as measured by F_2-isoprostane levels in fetal brain [49]. When mice were exposed prenatally to high MeHg, they initially had high Hg:Se molar ratios, but this ratio diminished to near basal levels by postnatal day 21. In this study, brain selenoenzyme activities remained significantly diminished, indicating prenatal exposure to MeHg can have lasting effects [49]. Adverse neurodevelopmental outcomes have been uniformly observed in offspring of mothers who were fed MeHg in stoichiometric excess of Se [50–52]. Selenoenzyme activities have not yet been assessed in that model system, but it appears that maternal Se intakes may need to significantly exceed MeHg exposures to prevent interruptions of fetal brain selenoenzyme activities.

Although brain Se concentrations in Se-deficient animals are nearly impossible to reduce to less than 60% of normal, feeding diets containing less than 0.1 ppm Se to SelP knockout mice reduced their brain Se concentrations to 43% of normal, the lowest brain Se concentration achieved in any experimental animal model not involving high MeHg [44, 45]. While rats with brain Se at 60% of normal appear asymptomatic, the SelP knockout mice demonstrated pronounced loss of motor coordination. However, the motor coordination could be restored and brain Se replenished by feeding them diets containing 2 mg Se/kg food. When the SelP knockout mice were fed Se-deficient diets, neurological dysfunction and death resulted within weeks [32]. Feeding them a Se-rich diet, on the other hand, prevented all but minor neurological deficits.

Consequently, any substance that can enter the brain and disrupt selenoprotein synthesis will accomplish what multigenerational Se deficiency cannot. Mercury not only has the ability to cross the placental and blood–brain barrier, but its high Se affinity also enables it to specifically and irreversibly sequester the brain's Se by forming insoluble HgSe, thereby diminishing Se bioavailability for selenoprotein synthesis in these otherwise protected tissues.

Methylmercury toxicity in experimental animals is counteracted by Se supplied when ocean fish is added to their diets [47, 48, 53]. Likewise, the Se supplied from delipidated proteins of yellowfin tuna, swordfish, and mako shark was all effective in preventing the onset of growth inhibition and neurotoxic effects from high (~10 ppm) dietary MeHg exposures [22]. Although the fish protein increased total MeHg in these diets by ~10%, these ocean fish diets did not accentuate symptoms of MeHg toxicity but prevented them instead. Hence, the organic forms of Se present in ocean fish are bioavailable and effective in counteracting MeHg toxicity.

31.6 Concluding Remarks

Recognizing the effect of Hg on Se physiology is of pivotal importance in understanding Hg toxicity, yet it is overlooked. The extremely high affinity between Hg and Se allows Hg to sequester intracellular Se, thereby abolishing its availability for Sec synthesis. Exposure to MeHg not only directly inhibits the activity of numerous Se-dependent enzymes, disproportionate MeHg-Se exposures also terminally sequester Se, preventing synthesis of selenoenzymes that are essential for healthy fetal development. Therefore, studying the pathology of Hg toxicity requires a more insightful question than simply, "How much Hg was consumed?" Individual sensitivities to Hg depend upon the amount of Se in body tissues available to create selenoenzymes. In this regard, the amount of Se lost by binding to Hg, as well as the timing and duration of this transient limitation of Se availability, will be important determinants in the toxicity risk from Hg exposure.

Expectations of risks associated with consumption of food with high Hg contents have resulted in regulatory advisories designed to limit maternal MeHg exposures. However, current seafood advisories fail to consider the effects of MeHg:Se molar ratios and the beneficial effects of seafood on maternal nutritional status and child health outcomes. Although ocean fish consumption has been found to benefit rather than harm children, many pregnant women and new mothers currently avoid eating fish. These individuals lose the benefits of improved nutritional Se, omega-3, and vitamin D status that could improve their own health as well as that of their children. Future seafood safety assessments need to assess Hg:Se molar ratios when evaluating Hg-related risks and employ balanced equations that include consideration of the benefits of improved maternal/fetal nutritional status that accompany ocean fish consumption.

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Chapter 32
Selenium and Male Reproduction

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Abstract Selenium (Se) has long been known to be important for male reproduction as severe Se deficiency causes impaired male fertility in livestock, laboratory animals, and humans. In the last decade, the role of Se in male reproduction was elucidated at the molecular level, establishing the roles of specific selenoproteins in this process. Using protein- and isoform-specific knockout mice, it was found that at least two selenoproteins are responsible for the effect of Se: Selenoprotein P, a protein secreted from the liver and serving as the main source of Se for testes, and a mitochondrial form of glutathione peroxidase 4 that has two functions: a peroxidase specific for phospholipid hydroperoxides and a structural component in the midpiece of sperm. Clinical studies further showed that the compromised glutathione peroxidase 4 function in testes is associated with male infertility. In addition, application of X-ray fluorescent microscopy allowed direct visualization of Se distribution in testis and sperm, defining the roles of individual selenoproteins during spermatogenesis. Finally, recent identification of individuals with *SBP2* mutations characterized by impaired fertility and azoospermia provided further evidence for importance of Se and selenoproteins in male reproduction.

32.1 Introduction

Selenium (Se) was found to be an essential trace element in mammals in the 1950s, and further characterization revealed its importance for male reproduction [1, 2]. In the original experiments, moderate or severe Se deficiency resulted in phenotypes that ranged from impaired sperm motility to morphological alterations of

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sperm head and tail to infertility [3–7]. Administration of the radioactive Se tracer, ^{75}Se, to rodents showed that Se accumulated in testis and epididymis [8–10]. In addition, metabolic ^{75}Se labeling revealed that high Se levels were present within the midpiece of spermatozoa and were associated with a structural cysteine-rich protein of the mitochondrial sheath. Initially, this protein was designated as MCS for *m*itochondrial *c*apsule selenoprotein [11–15]. However, cloning of the MCS gene revealed its nonselenoprotein nature and the protein was renamed sperm mitochondrion-associated cysteine-rich protein (SMCP) [16, 17]. Finally, Ursini and collaborators identified the major testis selenoprotein as phospholipid hydroperoxide glutathione peroxidase (PHGPX or GPx4) that was a component of the midpiece structure of rodent spermatozoa [18, 19]. Further ^{75}Se labeling experiments involving Se-deprived animals demonstrated specific Se incorporation into several other testicular and epididymal proteins [20, 21]. An initial observation of significant testicular accumulation of Se resulted in the identification of SelP as an indispensible source of this element for testes [22, 23]. Another abundant testis-specific, selenocysteine-containing protein, thioredoxin-glutathione reductase (TGR or TxnRd3), was also identified and was suggested to participate in disulfide bond isomerization during sperm maturation; accordingly, it could also play a role in male reproduction [24–26]. Additionally, a recently identified selenoprotein V (SelV) showed testis-specific expression in rodents. In situ hybridization experiments revealed high levels of SelV mRNA in seminiferous tubules in mouse testes, but the exact function of this selenoprotein in spermatogenesis remains unclear [27].

32.2 Identification of Selenoproteins Involved in Spermatogenesis

Among several selenoprotein glutathione peroxidases (GPxs), one enzyme was particularly interesting because of its high expression in testis [28]. This protein, GPx4, was found to be highly active in spermatids, but was inactivated in mature sperm [29]. Based on the enzymatic properties of GPxs, it was proposed that GPx4 protects sperm cells from oxidative damage during maturation [30]. Further analyses showed that the enzymatically inactive GPx4 localized to mitochondria in the midpiece of spermatozoa. It was isolated as mostly insoluble protein due to oxidative cross-linking with itself and certain structural proteins [19, 31]. Much of the testis-specific GPx4 was found to occur in keratin-like capsules in the sperm mitochondrial sheath. After reduction with GSH or other thiol reductants, GPx4 became soluble and regained activity. Mass spectrometry analyses revealed that GPx4 accounted for about 50% of the structural material in the sperm mitochondrial sheath. Based on the observations of GPx4 localization, solubility, and rapid inactivation in late spermiogenesis, an additional structural function was assigned to this moonlighting protein [19].

Mouse GPx4 gene has a complex structure, generating three different transcripts that utilize alternative transcription start sites. One GPx4 transcript has two translation

initiation codons. Initiation at the first AUG leads to a protein of 197 amino acids, while initiation from a downstream AUG makes a protein of 170 residues [32]. Analysis of the longer isoform (mGPx4) revealed a 27-amino-acid-long mitochondrial signal peptide, consistent with the previously observed mitochondrial localization of the enzyme. However, mature mGPx4, after signal peptide cleavage, is identical to the shorter cytosolic form (cGPx4) [33]. The third form of GPx4 was first identified in testis as an alternative form that localized to the nucleus and named nGPx4 [34, 35]. It was shown that this form arose from transcription initiation from an alternative promoter and that its start codon was located within the first intron of the GPx4 gene [36, 37]. Such expression/localization pattern of GPx4 in testis made identification of function of each isoform difficult. In particular, it was not clear which form was responsible for the role of Se in male reproduction. Moreover, the whole-body knockout of *GPx4* gene was embryonic lethal, leaving the question about the exact role of GPx4 in male reproduction open [38, 39]. However, knockout studies that specifically disrupted nGPx4 showed that the nGPx4-null mice were viable and developed normally, although they had minor phenotypes associated with DNA condensation [40]. Recently, two additional GPx4 knockout mouse models were reported. First, it found that depletion of GPx4 specifically in spermatocytes resulted in male infertility [41]. These mice were characterized by decreased testis size and weight as well as very low spermatozoa counts in the epididymis. The GPx4-null spermatozoa also had severe morphological and functional defects: damaged mitochondria in the midpiece, and loss of the mitochondrial membrane potential and flagellar bending, leading to decreased forward sperm motility and ultimately to infertility [41]. The second GPx4 knockout mouse model provided evidence that deletion of mGPx4 did not affect embryogenesis and normal postnatal development, but caused male infertility [42]. In this study, in order to specifically disrupt the expression of the mitochondrial form of GPx4, a stop signal was introduced in the mitochondrial signal sequence. This strategy allowed disruption of the mitochondrial form while not affecting expression of the cytosolic form. Analysis of embryonic and adult tissues revealed that the mGPx4-null males had low GPx4 expression in testes, particularly in the midpiece of isolated spermatozoa, but not in the sperm head region. In addition, GPx4 expression in somatic tissues was unchanged. These knockout males had no offspring from plug-positive females and showed the loss of fertility, while the female fertility was not affected. In addition, sperm motility was significantly lower in the mGPx4-null mice, and spermatozoa were characterized by multiple sperm abnormalities, such as bending, detached heads, and broken midpiece region. Interestingly, mGPx4-dependent infertility could be overcome by intracytoplasmic sperm injection that resulted in viable offspring [42]. All these observations were in perfect agreement with the findings and suggested that only the mitochondrial form of GPx4 is important for male reproduction. Taken together, these data finally linked the importance of Se for male fertility to the specific isoform of a selenoprotein located in sperm mitochondria, whose function changed completely during spermatogenesis.

Selenoprotein P (SelP) was originally identified as a plasma selenoprotein. It is relatively abundant and accounts for most of Se in plasma. In addition, this protein

is the only mammalian selenoprotein with more than one selenocysteine (e.g., 10 selenocysteines in human, mouse, and rat SelPs) [43]. SelP is a glycoprotein that is synthesized mostly in the liver and secreted into the bloodstream [44, 45]. Based on the high selenocysteine content and rapidly increased expression in response to Se supplementation, it was proposed that the function of SelP is to protect cells from oxidative stress [46, 47]. The Se transport function was also proposed for this protein [48], and it was found that SelP could serve as a plasma biomarker of Se status in humans [49]. More recently, the SelP function was defined by using knockout mouse models [22, 23]. Deletion of this gene resulted in disruption of Se homeostasis. For example, the SelP-null mice were characterized by a significant decrease in Se levels and selenoprotein activities, which are features that resembled Se deficiency. Interestingly, SelP knockout mice displayed male infertility and showed biochemical, physiological, and phenotypical changes in testes and sperm similar to those observed in Se-deficient mice. Moreover, despite the fact that SelP is ubiquitously expressed, SelP secreted from the liver is responsible for Se transport to testes since the SelP-null mice with transgenic expression of human SelP under control of the hepatocyte-specific promoter restored both Se levels and male fertility [50]. These findings clearly showed that SelP plays an important role in Se transport from liver to testis, delivering Se in the form of Sec and supporting selenoprotein synthesis in testes. A recent study identified a SelP-binding protein in testes as apolipoprotein E receptor-2 (ApoER2) located in Sertoli cell membranes [51]. Analysis of the ApoER2-null mice revealed a very low Se content in testes, and these mice also showed reduction in fertility suggesting that ApoER2 is the SelP receptor in testis [52]. Thus, Se is delivered to testis in the form of SelP through ApoER2-mediated uptake in Sertoli cells.

32.3 Quantitative Imaging of Se During Male Reproduction

A complete understanding of Se transport, utilization, and distribution during spermatogenesis requires information on biochemical processes and selenoproteins involved as well as the availability of approaches for accurate detection and mapping of this trace element. Despite the identification of testis-specific selenoproteins, the issues of distribution and relocalization of protein-bound or elemental Se have only recently been addressed. The precise imaging of Se distribution in testes and the quantitative elemental analysis nicely complement the biochemical and histological data and provide a more complete picture of the role of Se in male reproduction. Recent developments in metallomics focused on two main goals: reliable, reproducible imaging of trace elements and identification of changes in trace elements in response to various treatments. Several methods are now available to quantify and image Se in biological samples. Bulk concentrations of Se and other trace elements in cells and tissues can be determined by inductively coupled plasma mass spectrometry (ICP-MS) or atomic absorption spectroscopy (AAS). These methods are capable of simultaneously quantifying multiple elements with high sensitivity. However, all

spatial information is lost during homogenization and digestion steps. In some cases, spatial distribution of abundant trace elements (such as copper, zinc, iron) in cells and tissues can be determined using fluorescent probes which specifically recognize a trace element of interest. However, chemical sensitivity and specificity for many such probes is low or unknown. In addition, there are no reliable fluorescent probes for detection of Se in cells and tissues.

Several elemental imaging techniques were developed to complement bulk analyses by ICP-MS and AAS. Electron microprobe uses an energy-dispersive detector coupled with a transmission electron microscope. In this method, an electron beam excites outer shell electrons in atoms, and the X-rays generated are measured by the detector. However, due to low sensitivity (100 μg/g), this method has a limited application for Se imaging. A more advanced method, laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) can be used to image distribution of elements in samples. It employs lasers to excavate samples (e.g., tissue sections) for further analysis by ICP-MS. LA-ICP-MS was successfully used for Se imaging in large sections of mouse and rat brains [53]. It was found that Se is mainly localized in the olfactory bulb. However, the resolution of this method is limited to 20–50 μm, making it impossible to analyze cellular distribution of Se and other trace elements.

A great new method for high resolution and sensitive elemental imaging is X-ray fluorescence microscopy (XFM). In this method, high-energy X-rays are used to excite inner shell electrons in atoms; outer shell electrons then fill the inner shell vacancies emitting fluorescence. Each chemical element has its own characteristic fluorescence emission energy allowing simultaneous detection of several elements. Submicron XFM imaging is possible using high-energy X-rays (greater than 10 keV) at the third generation synchrotron sources, such as the Advance Photon Source, European Synchrotron Radiation Facility, and Spring 8 [54]. High-energy synchrotron X-rays have been used for imaging several trace elements in biological samples with great sensitivity [55]. However, a disadvantage of this method is that it requires availability of high-energy X-rays (i.e., access to a synchrotron).

Recently, we applied XFM to characterize Se delivery and utilization in testes and sperm [56]. Imaging seminiferous tubules in mouse testes showed an uneven distribution of Se, wherein this element was enriched specifically in late spermatids. Such a pattern of Se distribution was unique (among other elements imaged). In order to characterize the origin of Se in testes, samples were imaged from various knockout mouse models characterized by deficiency in individual selenoproteins [56]. As discussed above, plasma SelP supplies testes with Se [49], and the ApoER2 receptor is responsible for SelP uptake in testes through Sertoli cells [51]. XFM imaging of testes from SelP knockout mice showed that spermatids had a significantly reduced Se content (77% decrease). The remaining Se was uniformly distributed in various cells in the seminiferous tubules. These data clearly showed that SelP is the major Se delivery protein for testes. However, since this protein is localized to the basal membranes in seminiferous tubules, SelP is not the protein enriched in the elongating spermatids. Se was further imaged in various GPx4 knockout mice. Knockout of nGPx4 led to minor changes in Se distribution (Fig. 32.1).

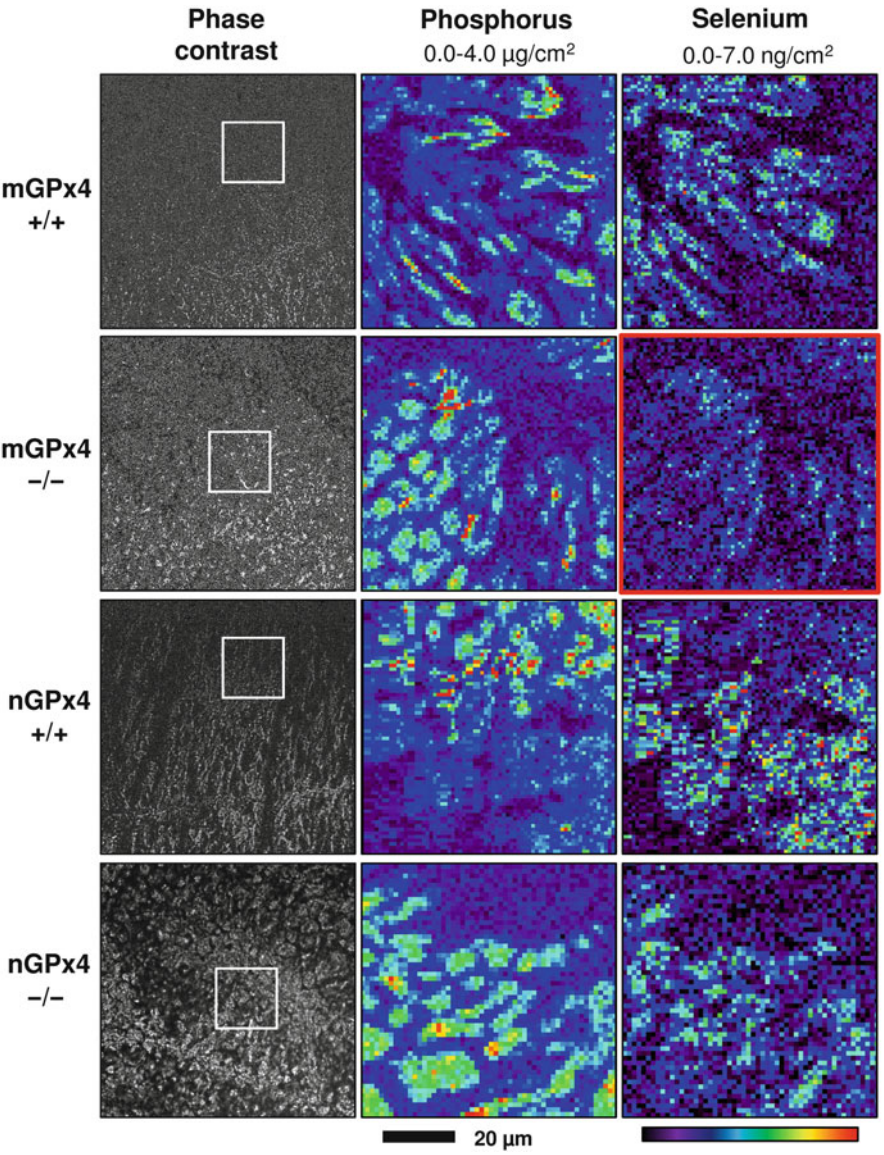

Fig. 32.1 Selenium imaging in testes of various GPx4 knockout mice. Testes from mitochondrial (mGPx4) and nuclear (nGPx4) GPx4 knockout mice and from wild-type mice were imaged using synchrotron X-ray fluorescence microscopy. Images were obtained with a 1.7 s dwell time and 1 µm steps at the incident energy 12 keV. Left panels were acquired using light microscopy and represent morphology of tissue samples. The *red squared* panel shows a decrease in Se in mGPx4 KO testes

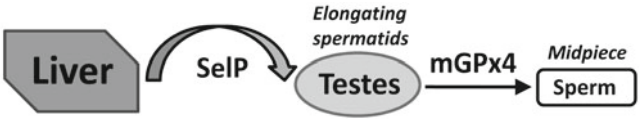

Fig. 32.2 Se transport and distribution from liver to sperm cells. SelP is synthesized in the liver and secreted to plasma. It is taken up by ApoER2 receptor in Sertoli cells in testes. Se is then used for expression of the mitochondrial GPx4 in elongating spermatids, and this selenoprotein is then preserved in the midpiece of sperm cells as a structural protein. Whether other testis-specific selenoproteins, TGR and SelV, are essential for male reproduction remains unknown

However, mGPx4 knockout resulted in a 60% decrease in Se in testes and largely abrogated the enrichment of this element in spermatids (Fig. 32.1).

Further XFM analyses showed that Se, enriched in the elongating spermatids in mouse testes, was preserved in the midpiece region of mature sperm [56]. Based on XFM imaging and the information on the expression of selenoproteins, we suggest a model of Se transfer and distribution during spermatogenesis (Fig. 32.2). ApoER2 in Sertoli cells senses and takes up SelP from plasma. Much of this Se is used in spermatids for mGPx4 expression. During maturation, chromatin becomes condensed and tightly packed through cross-links between protamine molecules. At the same time, structural components of the midpiece and tail are formed [34]. GPx4 contributes to both processes, with its nuclear form serving a role in the sperm head and the mitochondrial GPx4 in the midpiece [42].

32.4 Mutations in the *SBP2* Gene and Their Impact on Male Fertility

With regard to identification of factors that established the role of Se in male reproduction, much of the previous research was done in laboratory animals and clinical trials with healthy adults. In order to decipher the mechanisms of Se and selenoprotein actions in human reproduction and to provide better treatment to patients diagnosed with infertility, additional information is required. The discovery of the role of GPx4 in male fertility led to the analyses of this protein in men with impaired fertility and infertile individuals. It was found that GPx4 expression is decreased in about 30% infertile men diagnosed with oligoastyhenozoospermia [57]. A recent study examined men with heterozygous mutations in the *SBP2* gene [58]. SBP2 is an essential RNA-binding protein required for insertion of Sec into selenoproteins [59, 60]. Mutations identified in the *SBP2* gene led to a lower expression of SBP2 and, as a consequence, reduced expression of selenoproteins. Interestingly, analysis of one adult subject showed complete azoospermia and histological examination of testes (removed in adolescence) revealed maturation arrest in spermatogenesis, preservation of spermatogonia, and spermatocytes in the germline, and deficiency in testis-expressed selenoproteins [58]. These severe phenotypes due to *SBP2* mutations highlight the importance of Se for reproduction in men.

32.5 Concluding Remarks

The identification of Se as an important trace element for male reproduction led to a series of clinical and interventional studies involving Se supplementation as treatment to improve fertility. Unfortunately, these studies that involved either supplementation with different forms of Se or a combination of Se and certain antioxidants such as *N*-acetylcysteine and vitamin E yielded mixed results (reviewed in ref. [61]). In most cases, an increase in sperm quality was moderate or insignificant and depended greatly on the chemical form of Se, dosage, and experimental and control cohorts. In the last decade, significant progress was made with regard to characterization of molecular mechanisms of Se action in the male reproductive system. Deciphering the Se delivery pathway, identification of molecular targets of dietary Se as testis-specific selenoproteins and precise visualization and quantification of Se in testis and sperm provided a foundation for future studies. On the other hand, inconsistencies in the outcomes of clinical trials made it difficult to interpret the relationship between dietary Se and male reproduction. A thorough analysis of current data, additional studies with model systems, and further combined efforts of biochemists, nutritionists, geneticists, and clinicians are needed to improve current models for the role of Se in male reproduction.

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Chapter 33
Variations in Selenium Metabolism in Males and Females

Lutz Schomburg

Abstract Selenium elicits its effects on human health mainly in the form of selenoproteins, even though some selenocompound-specific effects have been described. The physiological roles of certain selenoproteins have been characterized in transgenic mice, and epidemiological analyses have indicated associations of selenoprotein genotypes with common pathologies. Supplementation studies yielded promising results indicating that selenium can reduce cancer risk, autoimmune disease, subfertility, or mortality risk in severe illness. General conclusions are drawn and discussed vividly in science, health politics, and elsewhere. But studies in experimental rodents indicate that selenium metabolism and selenoprotein expression patterns differ between the sexes. Similarly, the selenium-dependent reduction of cancer risk, subfertility, or mortality in sepsis is mainly observed in males but not in females. Selenium-dependent health effects in thyroiditis are described in females only, and associations of selenium status and goiter, thyroid nodules or cardiovascular disease are sexually dimorphic. Even the major side effect, i.e., increased diabetes risk, appears to be male-specific. Therefore, selenium metabolism and selenium health effects differ between females and males, and generalizations should not be made across the sexes.

33.1 Introduction

Women and men differ. This notion which is well known, appreciated, and savored in everyday's life becomes underestimated and ignored all too often in medicine and medical sciences. Women are underrepresented in most clinical studies [1, 2],

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and suffer in general a higher frequency of adverse drug reactions [3]. Moreover, pharmacodynamics and pharmacokinetics differ considerably between the sexes [4, 5]. Although the varying body sizes and body compositions are obvious and sometimes taken into account when dosages are determined in a personalized manner, other sex-specific characteristics are less-well considered including differences in intermediary metabolism, e.g., renal glomerular filtration and secretion or hepatic phase I metabolism and phase II conjugation rates. On top of these physiological characteristics, the way of living has gender-specific aspects including differences in physical activities, eating habits, and even frequency, choice and extent of dietary supplement intake with respect to vitamins, minerals, or secondary plant metabolites [6, 7].

Accordingly, the health outcome of a chosen personalized self-medication can not be predicted for a given individual but general trends have been deduced [8] including the disturbing notion that many antioxidant supplements are having an adverse effect on health instead of prolonging life expectancy [9]. Consequently, the current picture of nutritional supplements is changing from sheer enthusiasm to general skepticism and selenium is no exception to this trend. But this verdict is not justified in general, and we need to more carefully analyze out study results since many aspects of metabolism, regulation, and health effects of the essential micronutrient selenium display strong sex-specific differences. This section tries to summarize the respective state of research and knowledge on this emerging issue in selenium biology [10, 11].

33.2 Selenium Metabolism in Female and Male Animals

During the initial phases of selenium research, when a rat model of vitamin E deficiency-induced necrotic liver degeneration was studied, an emphasis was put on the characterization of different selenocompounds and their relative characteristics, bioavailabilities, and protective effects [12]. In these pioneering studies, female and male rats were used without discrimination and results were generalized since the selenocompounds afforded a comparable degree of protection in both sexes. But soon thereafter, samples of males and females were separately analyzed and sex-specific differences were observed, e.g., when growth of second generation selenium-deficient animals was compared [13], when plasma and cell selenium concentrations were determined in adult humans [14], or when retention of selenium isotopes was analyzed in the tissues of male and female rats (Fig. 33.1) [15].

Of all the human genes encoding selenoproteins [16], none is located on the Y- or X-chromosome. This notion excludes that number of selenoprotein genes is a major reason for sexual dimorphic selenium metabolism. Selenium is essential for reproduction, especially in males by affecting testes development and spermiogenesis [17]. When comparing selenium effects on the gonads, profound differences with respect

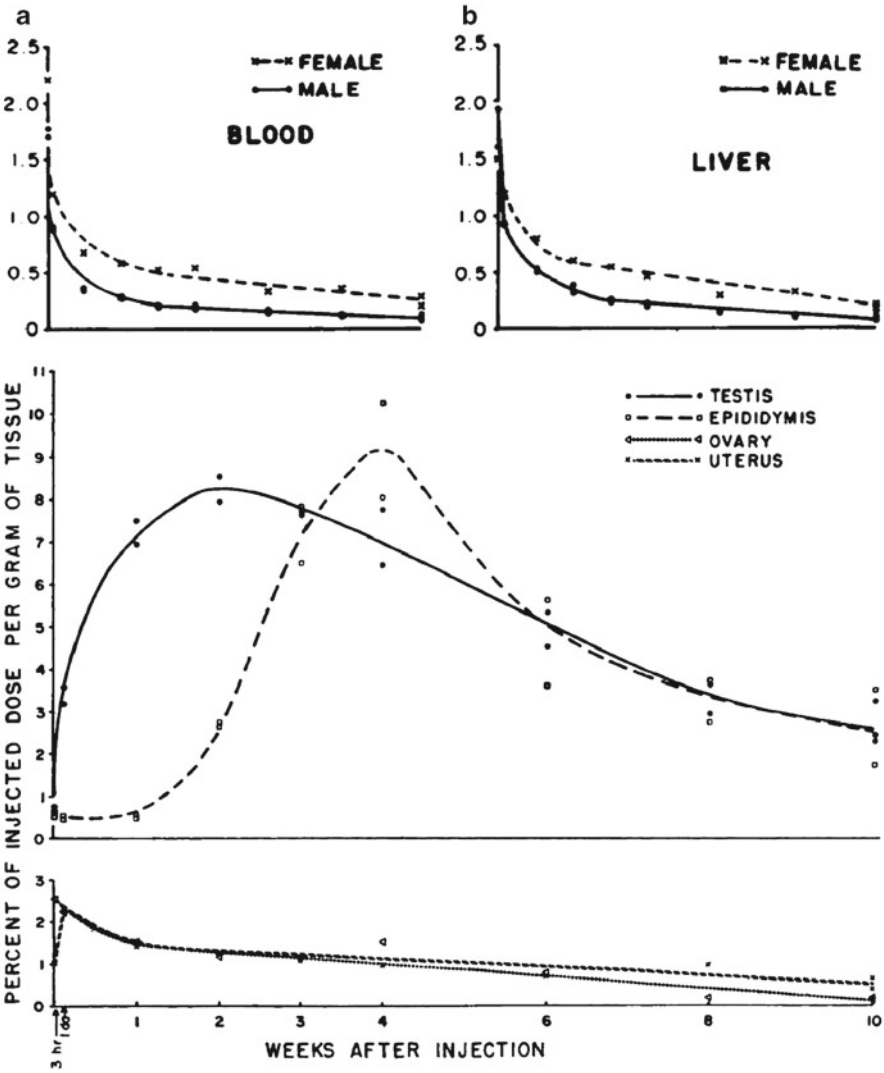

Fig. 33.1 Comparison of the retention of 75-selenium labeled selenite in male and female rats. Retention of 75-selenium was compared over 10 weeks after injection (i.p.) (reproduced from Brown and Burk [15] with permission from the "American Society for Nutrition"). Labeled selenium decreases faster in blood (**a**) and liver (**b**) of male compared to female animals. Gonads yielded a very dimorphic picture (*bottom*); the testes accumulated the radioactively-labeled selenium over an extended period of time, and apparently transferred the isotope to the epididymis, whereas the ovaries and uterus took up the 75-selenium selenite rapidly, and then lost the label as constantly as observed above in blood and liver. The underlying molecular pathways controlling this sexually dimorphic selenium metabolism involve transport via SePP from liver to the testes, specific uptake of selenium and use for GPx4 biosynthesis which becomes irreversibly converted into a structural component of the developing spermatids during spermiogenesis

to retention are observed (Fig. 33.1). Testes belong to the preferentially supplied organs residing high in the hierarchy of selenium supply among all the mammalian tissues [11, 18]. The kinetic profile of injected 75Se-SeO_3^{2-} highlights that selenium is taken up by the testes with a certain delay and then becoming transferred to the epididymis while the ovaries show a fast accumulation and an almost linear loss of the tracer [15]. The molecular mechanisms behind this male-specific metabolism of selenium in the reproductive tract have been intensively studied in recent years [19]. Our current picture comprises some detailed knowledge on the importance of certain selenoproteins for selenium transport to the testes, retention within testes, and functional importance of selenoproteins for spermiogenesis and sperm motility (for details please see Chap. 32).

Briefly, liver is the major organ for initial metabolism of dietary selenium, its uptake from the circulation and organification. Selenium becomes fast and efficiently converted into selenoprotein P (SePP), which serves as a transporter being taken up by other organs including brain and testes. Uptake is mediated by receptors of the lipoprotein receptor-related protein (Lrp) family, especially Lrp2 (megalin) in kidney [20, 21] and Lrp8 (ApoER2) in both brain [22] and testes [23]. SePP supply to testes is essentially needed for supporting the biosynthesis of testes selenoproteins and generation of vital and motile sperm [24]. Besides SePP, GPx4 has been identified as a second essential selenoprotein needed for male fertility [25], and the mitochondrial GPx4 isozyme is mainly responsible for normal spermiogenesis [26]. Accordingly, polymorphisms in the human GPx4 gene have been associated with male infertility [27]. In testes, Sertoli cells bind and internalize SePP [23], causing a strong selenium enrichment specifically in late spermatids, which apparently use SePP as a selenium source for GPx4 biosynthesis [28]. During spermiogenesis, GPx4 undergoes a functional metamorphosis from an active enzyme into a structural component needed for stability and motility of spermatids [29].

Accordingly, SePP and ApoER2 are abundantly expressed in male testes, but their expression is marginal or absent in female ovary or uterus. This pronounced sex-specific difference in selenoprotein expression may contribute to the differential selenium retention in males and females. But it is unlikely that sperm and seminal fluid are major factors controlling male-specific selenium metabolism and flux, for the total amount secreted with one ejaculation averages in humans at 100–250 ng selenium (mean volume: 2–5 mL, mean selenium concentration in seminal plasma: 50 μg/L) [30]. In comparison, the blood loss during menstruation is around 35 mL corresponding to an average loss of 1,750–3,500 ng selenium per month, i.e., in a similar range as the selenium loss via sperm in males. In vivo, thus, there must be other and more important pathways causing the sexual dimorphic kinetics of selenium uptake, retention, and selenoprotein expression patterns; unfortunately, the molecular details have not been fully characterized yet. But especially the growing number of sexual dimorphic effects observed in epidemiological studies and selenium supplementation trials highlighting sex-specific disease associations argue that these differences are real and of importance for human health.

33.3 Sex-Specific Regulation of Selenoprotein Expression

Like all other proteins, expression of selenoproteins is regulated at multiple steps, but the strict dependence on the limiting trace element selenium confers some specific oddities to the relative importance of the different regulatory levels [31]. The role of gender-specific circuits and sex steroid hormones controlling transcription of selenoproteins in the different tissues is a very complex and multilayer issue. In experimental animals, castration alters the expression of a number of selenoproteins in a sex-specific way [32]. The effects are not only exerted at the transcription level but involve posttranscriptional mechanisms giving rise to tissue-specific expression patterns in males and females which vary with the selenium status (Fig. 33.2) [33].

The following section illustrates the underlying complexity of trying to elucidate the mechanisms controlling selenoprotein expression in males and females, and highlights that different levels of regulation are involved which converge under physiological conditions ensuring a time-, tissue-, and cell-specific expression pattern of a given selenoprotein. The aforementioned multifunctional selenoenzyme GPx4 is chosen as a very instructive example, as it is ubiquitously expressed and of functional importance for diverse processes including brain development, arachidonic acid metabolism, and fertility. According to its sexual dimorphic importance in reproduction, GPx4 expression in testes depends on gonadotropin stimulation and increases after puberty in rat testes [34]. Surprisingly, testosterone or gonadotropins

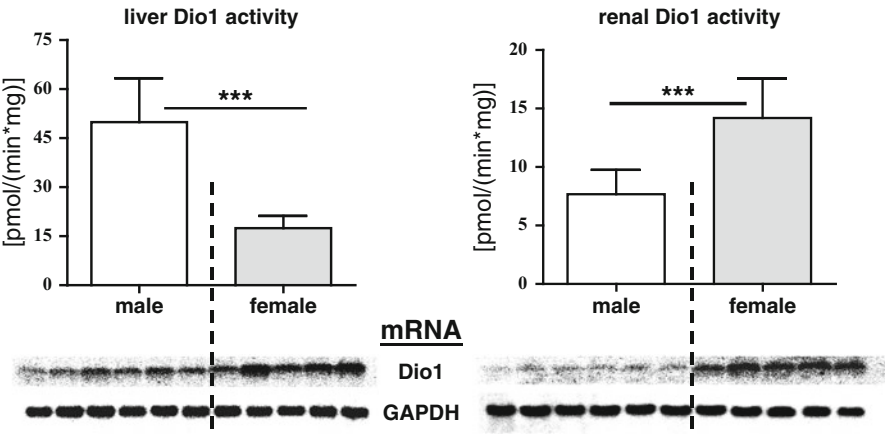

Fig. 33.2 Pre- and posttranscriptional mechanisms control sex-specific selenoprotein expression. Selenoproteins are sex-specifically expressed (*top*, activity; *bottom*, mRNA) in liver and kidney of adult mice (modified from Riese et al. [33]). Enzymatic activity of type 1 iodothyronine deiodinase (Dio1) is higher in male compared to female liver, whereas it is higher in female compared to male kidneys. This sexually dimorphic expression pattern is paralleled by respective differences in Dio1 mRNA concentrations in kidney but not in liver, where the differences in enzyme activity and mRNA levels do not correlate. Apparently, translational efficiency of Dio1 mRNA is higher in male compared to female hepatocytes by unknown molecular mechanisms

do not directly affect transcription of the GPx4 gene. The increased biosynthesis of GPx4 rather correlates to the maturation stage of spermatids, i.e., to a differentiation process which in turn is controlled by local testosterone from Leydig cells [35]. In addition, GPx4 is subject to dynamic alternative splicing and depending on environmental parameters, the cells synthesize different patterns of cytosolic, mitochondrial, and nuclear GPx4 isozymes [36, 37]. On top of this inherent transcriptional complexity, posttranscriptional mechanisms involving sequence-specific RNA-binding proteins recognizing the 5′-untranslated region of GPx4 mRNA control the translation efficiency, e.g., during embryonic brain development [38]. Finally, single nucleotide polymorphisms (SNPs) have been identified in the human GPx4 gene, which affect selenium-dependent GPx4 expression and turnover in a sex-specific way [39]. This gender effect may be of importance for sex-specific effects of selenium supplementation in clinical trials [11, 40].

Collectively, there are specific molecular mechanisms controlling the transcription, alternative splicing, translation, and posttranslational activity of selenoproteins in vivo, all of which may be subject to sex-specific modulation. These regulatory circuits ensure a gene-, cell-, age-, and selenium-status-dependent expression pattern in the tissues. Our molecular insights have mainly been obtained by comparing experimental animals. It has become obvious that the ratio of selenoprotein mRNA and corresponding protein amounts differs between the sexes and between different tissues [33], and that cell-type, age, and selenium status are three additional major regulators of sexual dimorphic selenoprotein expression patterns mainly controlling translational aspects [41]. We will have to take these confounding factors into consideration when clinical effects of selenium supplementation are analyzed and sex-specific differences are discussed.

33.4 Sexual Dimorphic Effects of Selenium in Clinical Studies

33.4.1 Cancer

Selenium belongs to the small number of trace elements and vitamins, which are taken as a nutritional supplement both in clinical studies and as an over-the-counter drug. The enthusiasm for supplemental selenium intake was supported by its alleged function as an antioxidative drug potentially slowing down degenerative processes and protecting genome integrity. Early analyses had indicated an inverse association of cancer prevalence and soil selenium concentrations [42], followed by a large number of respective experimental studies [43].

This general trend has been corroborated in the majority of clinical studies, and finally received tremendous support when the Nutritional Prevention of Cancer (NPC) trial was analyzed [44]. The NPC data indicated that a daily supplementation with 200 μg selenium in form of selenized yeast reduces the incidence of lung, colorectal, and prostate cancers, especially in those participants who entered into the study with relatively low baseline selenium concentrations [45]. Notably, this

conclusion was mainly drawn for males. Females were underrepresented in this important prospective cancer prevention trial and constituted only 25% of the enrolled participants. Nevertheless, the general conclusion was drawn that optimizing selenium intake by supplementation efforts confers chemoprevention and reduces cancer risk in all individuals. The sex-specific lack of information was not appreciated, and consequently the subsequent largest-ever chemoprevention trial testing selenium supplementation in a prospective setting, i.e., SELECT, was again initiated with males only, focusing on prostate cancer [46]. More detailed information on SELECT is found elsewhere in this book (see Chap. 23).

That such a generalization is not necessarily justified is indicated by several respective studies, e.g., the data from the European SU.VI.MAX trial (SUpplementation en VItamines et Mineraux AntioXydants). This randomized double-blind, primary-prevention trial indicated a significantly reduced total cancer incidence in men but not in women 7.5 years after initiating low-dose antioxidant supplementations including 100 μg selenium/day [47]. A detailed analysis of this surprising finding indicated that the baseline antioxidant status was sexually dimorphic, too, but proved insufficient to explain the full differences observed in the supplementation effect between the sexes. This trend of male-specific antioxidant effects is in agreement with two earlier reports from European epidemiological studies; increased cancer risk was associated with lower serum selenium levels in men but not in women in a Dutch [48] and independently in a Finish case–control study, in which the strongest association was observed for stomach and lung cancers [49].

Conversely, a recent meta-analysis indicated that the risk of bladder cancer is inversely associated with selenium concentrations in women but not in men [50]. Sex-specific differences in selenium metabolism and renal secretion are discussed as potential molecular reasons underlying this finding. The sex-specific trend of efficiency is in agreement with a case-control study in the US associating low toenail selenium concentrations with higher bladder cancer risk in women but not in men [51]. A more systematic comparison of sex-specific findings from several studies correlating selenium status and cancer risk has been compiled by Waters et al. [10]. Collectively, the available data indicate that the interactions are in general more pronounced in males (except for bladder cancer), but again, additional studies with both female and male participants spanning a large range of baseline selenium status are needed to get a better idea on the underlying mechanisms, i.e., whether the correction of a deficit ensuring maximal selenoprotein expression or selenocompound-specific, anti-tumor activities underlie the chemopreventive effects in females and males, respectively.

33.4.2 Infectious Diseases and Sepsis

Serum selenium and SePP are negative acute phase reactants and decline in response to inflammatory signals [52]. Plasma selenium concentrations are significantly lower in patients on the intensive care units than in controls [53]. Moreover and

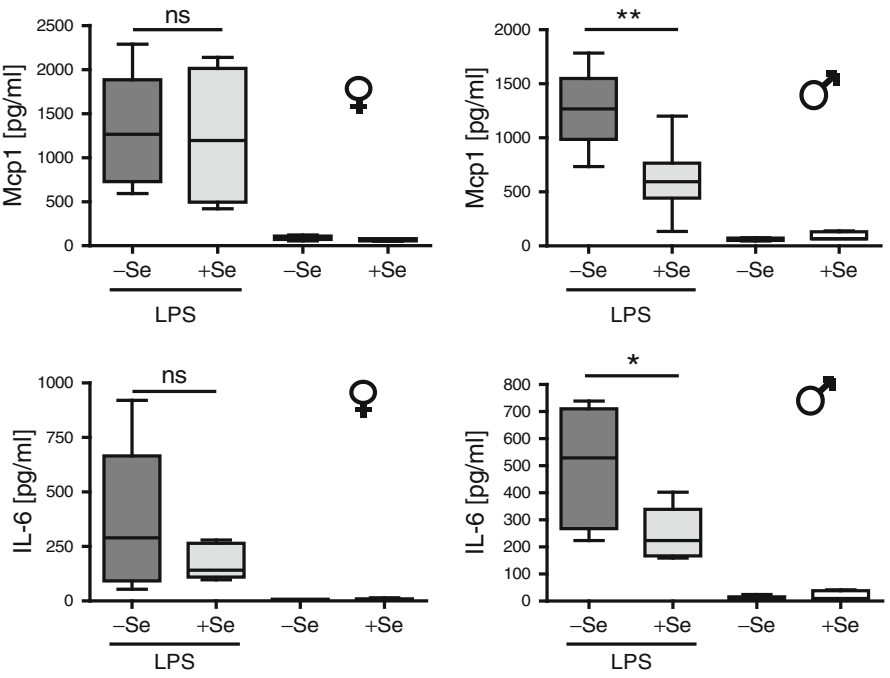

Fig. 33.3 Sexual dimorphic effects of selenite supplementation in a murine model of septic shock (modified from Stoedter et al. [60]). Male and female mice were raised on a selenium-deficient diet and then received regular tab water or water supplemented with selenite for 3 days. At 24 h before being sacrificed, a single injection (i.p.) of endotoxin (LPS) or saline was given. Circulating cytokines (IL-6, interleukin-6; Mcp1, monocyte chemoattractant protein 1) increased sharply upon LPS treatment. Notably, supplemental selenium had a mitigating effect on this acute phase response only in the male but not in the female mice

more importantly, low plasma selenium is associated with reduced survival odds of intensive care patients [54]. This association is valid for both females and males alike, and mortality risk can even be predicted from the minimal selenium levels observed in plasma [55]. Accordingly, clinical trials have been conducted trying to correct this trace element deficiency by a respective supplementation effort. The results are inconsistent, and different selenocompounds, chosen dosages, and application regimen have been discussed as potential reasons underlying this heterogeneity [56, 57].

A recently conducted large placebo-controlled multicentre study, i.e., the Selenium in Intensive Care study, has yielded positive supplementation effects reducing the 28-day mortality rate in patients with severe sepsis [58]. Unfortunately, female participants were again underrepresented, and the positive supplementation effect appeared to be confined to males [59]. This surprising finding was corroborated in a respective mouse study where LPS-induction was used as a model for septic shock. Short-term selenite supplementation efficiently reduced the overshooting immune response (Fig. 33.3) in male but not in female mice [60].

Again, the underlying reason for this sexual dimorphic supplementation effect is unknown, but since it applies to both rodents and humans, it appears to constitute a meaningful phylogenetically-conserved feature.

HIV infection is another strong inflammatory burden causing progressive weight loss and certain mineral and vitamin deficiencies. Part of this problem is given by reduced appetite and nutritional malabsorption, but the cytokine-dependent changes in the intermediary metabolism pose an additional problem to the patients. Serum selenium concentrations decline during HIV disease progression, and low selenium correlates again to poor survival odds [61]. A particular difference has been noted when comparing serum selenium concentrations before and after introduction of a highly-active antiretroviral therapy (HAART); among the most severely diseased individuals, males displayed the lower serum selenium concentrations compared to females before HAART [62]. Interestingly, the selenium status normalized during HAART along with improved weight stabilization, reaching serum selenium concentrations which no longer displayed a sex-specific difference. These findings indicate that the sexually dimorphic selenium status was dependent on the severity of the disease and activity of the immune system, especially in the male HIV patients.

33.4.3 Autoimmune Thyroid Disease

Among the autoimmune diseases, Hashimoto's thyroiditis (HT) is a relatively common destructive disorder of the thyroid gland eventually causing hypothyroidism, goiter, and loss of active thyroid gland tissue. This disease is probably the first one that has been identified as being caused by autoantibodies. It is highly prevalent affecting on average about 1 in 1,000 adults with a skewed sex ratio being about ten times more frequent in adult females than males [63]. Although there is no curative therapy targeting the thyroid destruction process at present, the accompanying hypothyroidism is corrected by a daily supplementation with thyroxin and a personalized dosage to establish euthyroidism and subjective well-being. HT patients are reported to have reduced serum selenium concentrations compared to controls [64]. This finding accords to the aforementioned negative regulation of serum selenium during sepsis and other inflammatory diseases in general.

Accordingly, supplementation trials have been conducted to analyze whether a correction of the selenium deficit improves health and clinical disease parameters [65]. A recent metaanalysis of randomized, placebo-controlled, blinded prospective studies with patients under thyroxin treatment highlights the prospect of selenium supplementation in reducing autoantibody load and improvement of general well-being [66]. This conclusion was based on the pooled analysis of four individual trials comprising in total 123 control and 136 treated HT patients. It is widely accepted as good evidence that selenium supplementation is a beneficial adjunct therapy option in HT. Albeit, in line with disease prevalence, the studies were conducted mostly with women, and only one particular trial enrolled males at all, which

constituted only 9 out of 65 patients [67]. A global statement on the effects of selenium on HT disease can thus not be given for the full population but for women only.

It might well be that the selenium effects will differ between male and female patients. The analysis of the baseline status in participants of the European SU.VI.MAX trial indicated that serum selenium inversely correlates to thyroid volume, risk of goiter, and hypoechogenicity in women [68]. None of these interactions was found in the male participants. We have independently determined the same sex-specific associations in a cohort of Danish adults [69]. Notably, all these sex-specific findings were observed in populations with mild iodine and selenium deficiency, i.e., in regular Europeans; it remains to be seen whether similar correlations are found in other countries with better iodine or selenium supply.

33.4.4 Cardiovascular System

The cardiovascular system is a prime target exposed to oxidative stress with the metabolically highly active myocardium and the widespread network of arteries, veins, and capillaries transporting a colorful cocktail of partly reactive and potentially damaging molecules throughout the body. Key events for development of arthrosclerosis comprise the activity of reactive oxygen species (ROS), especially during oxidation of LDL, triggering the development of proatherogenic foam cells in the vasculature. Selenoproteins of the GPx family are prime candidates for the physiological safe degradation of peroxides as potential precursors of ROS. Accordingly, GPx1 activity has been analyzed in red blood cells of patients with suspected coronary artery disease (CAD) and turned out as a very strong univariate predictor of risk for cardiovascular events [70]. Notably, sex was again an important modifier of the effects and young women expressed the highest GPx1 activities among the probands.

A straightforward test for the functionality, plasticity, and integrity of the cardiovascular system involves the simple measurement of systolic and diastolic blood pressure. In a Belgian cross-sectional and longitudinal analysis, a significant inverse correlation of these two parameters with blood selenium concentrations was found in men but not in women [71, 72]. Notably, the risk of hypertension correlated to baseline selenium status. In extrapolating the data, it appears that increasing the daily intake slightly to improve blood selenium concentrations by a margin of 20 μg selenium/L only might already suffice to lower CAD and myocardial infarction (MI) rates in European men by an impressive 7 and 10%, respectively. This male-specific trend was verified in a Finnish study but was not replicated in a similar French analysis [73].

CAD is the end result of a degenerative process affecting the coronary arteries finally impairing oxygen and nutrient supply to the heart and eventually causing MI. CAD is the leading cause of death of adults in the developed countries. The risk of acute MI is roughly twice as high for men than for women until 60 years of age; thereafter, the difference disappears and equal incidences on a higher level are

observed by the eighth decade of life [74]. The INTERHEART study determined that the median age of the first acute MI is on average 9 years earlier in men than in women [75]. In general, women share the same risk factors as men but their relative contribution to the overall risk differs between the sexes; weight and BMI are of high predictive value in men, while global baseline inflammatory status appears more important in women.

Results on the interaction of selenium status and disease risk differ between the studies, but a protective tendency of higher selenium status can be deduced from the observational studies [76]. In contrast, intervention trials yielded inconsistent results on the efficiency of selenium supplementation to prevent CAD endpoints [73]. A large observational analysis studying patients with stable angina pectoris and acute coronary syndrome, respectively, was conducted in Germany with participants of moderate selenium status [77]. Most patients were between 60 and 70 years of age, and again, only about 25% of the patients were female. Survival rates in stable angina pectoris patients were unrelated to serum selenium status while survival of acute coronary syndrome patients strongly correlated to serum selenium concentrations (hazard ratio of 0.38 (0.16; 0.91), $P=0.03$, for highest vs. lowest tertile of serum selenium) [77]. In this study, the influence of sex was of borderline significance on these associations highlighting the need for a more comprehensive analysis on the interaction of selenium, sex, and CAD.

33.5 Comparison of the Sex-Specific Risk–Benefit Ratio

In general, a low selenium status which is insufficient for full expression of selenoproteins seems to confer an increased risk for developing a number of diseases and impairing the convalescence process. Moreover, a selenium deficiency appears to aggravate during (inflammatory) diseases thereby closing a potentially dangerous feed forward cycle [78]. It is thus widely accepted that selenium supplementation and increased dietary intake offer some health benefits especially in poorly supplied individuals. As for every other medically active substance, an upper limit of intake should not be surpassed to avoid adverse effects. Selenium poisoning (selenosis) is regularly observed both in veterinarian medicine and as sporadic accidents in humans [79].

Besides the acute effects, the long-time intake of supplemental selenium even in the recommended dosages might increase disease risk under certain circumstances. Two independent reports from 2007 highlighted a potentially increased risk of developing type 2 diabetes mellitus (T2DM) upon high selenium intake, i.e., the follow-up analysis of the NPC trial [80] and an epidemiological cross-sectional analysis as part of the Third National Health and Nutrition Examination Survey (NHANES III) [81]. Notably, both studies were mainly conducted in the US and analyzed a population of relatively high baseline selenium concentration.

But most importantly, a detailed analysis of the primary data clearly indicates that the reported increased T2DM risk is confined to males; among the females of

the NPC trial, $n=8$ in the placebo and $n=9$ in the selenium arm developed T2DM, which is statistically insignificant [80]. Similarly, there was no significantly increased T2DM risk with high selenium status in post or premenopausal women of the NHANES III study [81]. While the former notion is somewhat limited due to the relatively low number of females enrolled in the NPC trial, the latter cross-sectional analysis needs to be interpreted in a sex-specific manner. The obvious sexually dimorphic risk of T2DM and selenium supplementation needs to be emphasized more actively, for it is not yet appreciated in the media or public. Similar sexual dimorphic data are available for the interaction of selenium and serum lipids, LDL- or HDL-cholesterol concentrations. At present, a U-shaped interaction curve with a minimal disease risk at an optimal selenium status is emerging, but it remains to be seen whether the optimal selenium status differ between the sexes.

33.6 Concluding Remarks

Even though there is some clear lack of mechanistic insights into the underlying molecular pathways, both animal experiments and clinical data highlight that the health effects of selenium, the associations of selenium intake and status with certain disease risks, and the side-effects from too high a daily selenium supply differ between the sexes. In general, males seem to be more dependent and more responsive to acute changes in the selenium supply, their status responds with faster kinetics and stronger amplitude to inflammatory stimuli, and likewise they are more likely to develop adverse health effects upon surplus intake. The current data do not yet allow for deducing sex-specific intake recommendations, especially with respect to the different health aspects of selenium, but the studies at hand which have compared males and females strongly argue for a more balanced study design in future trials. Wherever possible, we should not conduct studies with one sex only, if the funding allows a more complete approach. But more importantly, we should refrain from generalizations of the findings at hand when one sex only has been analyzed. Males and females differ considerably with respect to selenium metabolism, selenoprotein expression, and medical selenium effects, and these sex-specific differences appear to be conserved across the species and may thus be meaningful for health and disease.

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Chapter 34
Selenium in Alzheimer's Disease

Frederick P. Bellinger and Edwin J. Weeber

Abstract Alzheimer's disease is a devastating and invariably fatal neurodegenerative brain disorder with no cure. Recent studies suggest that selenium may be beneficial in reducing Alzheimer's pathology. Selenoprotein P is associated with Alzheimer's pathology, and deletion of selenoprotein P impairs memory formation and synaptic function. Selenoproteins may be beneficial by reducing oxidative stress and ER stress, regulating intracellular calcium levels, and signaling through specific lipoprotein receptors. The benefits of different forms of selenium supplementation are currently being explored in research and clinical trials.

34.1 Alzheimer's Disease

Alzheimer's disease is a mind-robbing, fatal illness associated with aging. Despite years of study, the etiology of the disease remains poorly understood and current treatment is only mildly effective in maintaining cognitive function. Recent findings suggest that selenium in different forms may be effective in prevention or treatment of this disorder. In this chapter, we will explore the possible roles for selenium and selenoproteins in Alzheimer's disease.

Alois Alzheimer first described in a presentation in 1906 and subsequent article in 1907 the case of Aguste Dieter, a woman who died in her 50s after being institutionalized with severe memory and cognitive impairments [1, 2]. Alzheimer characterized

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a neuropathology of plaques and neurofibrillary tangles found in postmortem brain utilizing newly developed silver staining methods pioneered by Camillo Golgi and Ramón y Cajal. The disorder was later named after Alzheimer by his colleague Emil Kraepelin [3].

Today we recognize Alzheimer's disease as the leading cause of dementia and a leading cause of death in developed countries [4]. The production of amyloid plaques and the presence of intracellular neurofibrillary tangles characterize the disease. Alzheimer's disease can only properly be diagnosed at autopsy by the presence of these features. However, this definition has come under scrutiny in recent years by the discovery of plaques in individuals who had no symptoms of dementia [5]. Whether these are cases where the disease had simply not progressed enough to cause dementia is unclear. However, incidence of dementia does correlate well with the total amount of Alzheimer's and non-Alzheimer's brain lesions [6].

Amyloid plaques are composed primarily of β-amyloid peptide fragments cleaved from amyloid precursor protein (APP), a cell-surface and ER/Golgi membrane protein of the notch intracellular signaling family [7]. β-amyloid is formed by cleavage of APP by beta secretase and the gamma secretase complex [8]. After cleavage, β-amyloid spontaneously aggregates into oligomeric forms with 2–6 peptides and more complex insoluble aggregates and fibrils [9]. Soluble monomers and oligomers are increased in Alzheimer's cerebral spinal fluid (CSF) as the disease progresses, and the buildup of fibrils is believed to facilitate the formation of amyloid plaques. *Diffuse plaques* with spread-out aggregates are thought to progress to *neuritic plaques* of thick insoluble mass, often with a dense core, and surrounded by dystrophic neurites, or remnants of axonal and dendritic processes from dead neurons. Another hallmark of Alzheimer's pathology is the neurofibrillary tangles, formed by aggregates of the protein tau within the large pyramidal neurons [10]. Soluble tau functions in axons to assemble and stabilize microtubules. Hyperphosphorylation of tau reduces solubility and causes dissociation from microtubules and polymerization of tau into paired helical fragments [11]. The breakdown of microtubule structure results in loss of axonal transport and neuronal signaling, and eventually neuronal death [12, 13].

The majority of Alzheimer's cases are defined as "sporadic" or "late-onset," and occur in elderly beyond 65 years of age. Late-onset Alzheimer's disease does not appear to have a single genetic cause, although some genetic risk factors for the disorder have been identified. The best-characterized genetic risk factor is the apoliprotein E (ApoE) gene, which codes for a protein involved in extracellular transport of cholesterol and other fatty acids. This gene in humans has three alleles, termed ApoE2-4, of which ApoE3 is the most common. Having one copy of the ApoE4 allele increases risk of Alzheimer's by fourfold, and having two copies of the ApoE4 allele increases an individual's chances of contracting Alzheimer's disease by almost 20-fold [14]. Various other health factors are also thought to play a role in Alzheimer's, such as general health, diet, exercise, cholesterol levels, incidence of type II diabetes, and history of head injury [15, 16]. Additional environmental factors are hypothesized to increase risk, e.g. exposure to toxins such as heavy metals, but these remain poorly understood.

Although most Alzheimer's is late onset, about 5% of cases are classified as "early-onset," defined as occurring before 65 years of age [17]. Genetic mutations may be responsible for all early-onset cases. Multiple mutations in each of three separate genes – APP, presenilin 1, and presenilin 2 – have been identified as causative of early onset Alzheimer's [18]. These mutations are all autosomal dominant, i.e., a single copy of the mutation will cause the host to have Alzheimer's disease. It should be noted that the case first described by Aldus Alzheimer, in a woman in her 50s, was likely early-onset Alzheimer's [1, 19].

Metals and trace elements have long been suspected as contributing to Alzheimer's disease [20]. Early studies suggested an elevation of aluminum in postmortem Alzheimer's brain, although a causative relation has been difficult to demonstrate [21]. Mercury, a deadly neurotoxin, has been hypothesized to increase risk of Alzheimer's disease [22], and some studies have suggested that mercury used in dental fillings correlates with incidence of the disorder, although these findings are highly controversial [23]. Metals such as zinc, copper, and iron are found associated with amyloid plaques, and promote aggregation of amyloid peptides [20, 24].

Selenium has been proposed for treating or preventing Alzheimer's disease, primarily because of its antioxidant properties. Several recent studies have suggested selenium in various forms, alone or combined with vitamin E, can reduce Alzheimer's associated pathology in cell culture and animals models [25–28]. A study commenced in 2002, the PREADVISE study, to investigate if selenium combined with vitamin E can reduce the risk of Alzheimer's disease. This study is an offshoot of the SELECT trial, which sought to determine if these agents could reduce the risk of prostate cancer. However, early results showed a lack of effectiveness in preventing cancer, and additionally indicated that vitamin E could increase risk of prostate cancer and that selenium could increase risk of type II diabetes, although neither risk was statistically significant. Thus supplements have been discontinued in the SELECT and associated trials, although subjects are still being monitored for possible health benefits or risks.

34.2 Selenium Function in Brain

Selenium is essential for proper brain function. Although the selenium concentration in the brain is not as high as in other organs, selenium is preferentially retained in the brain under conditions of low selenium intake [29]. Studies in areas with low selenium soil content such as some regions of rural China have demonstrated that lower dietary selenium is associated with poorer cognitive function [30, 31]. With the high energy and oxygen utilization and abundance of oxidizable metals, the brain is particularly reliant on antioxidant mechanisms that include several members of the selenoprotein family [32].

Several recent studies have demonstrated that selenium in different forms can reduce Alzheimer's pathology in cell culture and animal models. Seleno-L-methionine protects against oxidative stress and toxicity from β-amyloid in cell culture and in

an Alzheimer's rodent model [26, 28]. Sodium selenite can inhibit amyloid production by decreasing gamma secretase activity [27], and mitigates pathology and cognitive impairment in a streptozotocin-induced rodent model of Alzheimer's disease. Recent studies show that sodium selenate can reduce neurofibrillary tangle formation by acting as an agonist for protein phosphatase 2A, which targets tau phosphorylation [33, 34].

34.3 Selenoprotein P in Brain and Alzheimer's Disease

Selenoprotein P is an unusual selenoprotein abundant in serum. Although most selenoproteins have a single residue of the amino acid selenocysteine, selenoprotein P has 10. This has let to the proposal that selenoprotein P, secreted into serum from liver, supplies selenium to other tissues [29]. Knockout of selenoprotein P leads to decreased selenium, particularly in brain and testes [35–37]. Selenoprotein P has two functional domains, the selenium-rich C-terminal domain that contains 9 selenocyteine residues and the N-terminal domain which contains a single selenocysteine residue in a redox configuration [29]. The N-terminal domain also contains two histidine-rich putative metal binding sites and a heparin-binding domain. Both the N and C-terminal domains are glycosylated. When selenium is limiting, translation of selenoprotein P may terminate at the second UGA codon for selenocysteine, which functions as a stop codon in other proteins. This termination would result in production only of the N-terminal domain. Additionally, selenoprotein P may be cleaved into separate N- and C-terminal domains by the serum protease kallikrein [38].

Recent studies have suggested the importance of selenoprotein P in aging brain and Alzheimer's disease. Selenoprotein P increases in the brain with aging and increases additionally in Alzheimer's brain [39, 40]. We recently reported an association of selenoprotein P with amyloid plaques and neurofibrillary tangles in Alzheimer's brain [41]. Although the function of selenoprotein P in Alzheimer's remains to be elucidated, the antioxidant, metal chelating, and selenium transport properties of selenoprotein P suggest multiple protective roles. This is supported by our findings that knockdown of selenoprotein P increases neurotoxicity caused by amyloid peptides [42].

34.4 Selenoproteins and Signaling Through Lipoprotein Receptors

In addition to a potential role of selenoproteins in oxidative protection, there is mounting evidence to support the role of selenoprotein P as a signaling molecule. The identification of selenoprotein P association to apolipoprotein 2 (ApoER2) in the testis raised the interesting possibility that selenoprotein P may also bind to

ApoER2 in the CNS, the only other tissue where ApoER2 is highly expressed. ApoER2 is a member of the evolutionarily ancient and highly conserved lipoprotein receptor family [43]. Two known ligands of ApoER2 are the extracellular matrix protein Reelin and ApoE [44]. Both ligands associate with the same n-terminal binding domain of ApoER2 and initiates signaling through the intracellular adaptor protein Disabled-1 (Dab-1) followed by internalization of the ApoER2-ligand complex [44]. In this action, ApoER2 can serve as both a signaling receptor and mechanism by which to internalize extracellular proteins. Mice deficient for ApoER2 or genetically altered in their signaling ability show significantly increased sensitivity to dietary reduced selenium intake and alterations during spermatogenesis indicating a causal relationship between selenoprotein P and ApoER2.

There is little known about the role of ApoER2 in the testis; however, ApoER2 is established in the adult CNS to play an important role in synaptic function and memory formation associated specifically with the mammalian hippocampus, a brain region essential for the production of long-lasting memories. Mice deficient for ApoER2 show reduced spatial memory assessed using the hidden platform water maze and impaired hippocampal synaptic function determined by induction of long-term potentiation (LTP) [45, 46], a model for the cellular physiology underlying learning and memory. Interestingly, mice deficient for selenoprotein P have similar deficits in both memory formation and synaptic plasticity [47]. Furthermore, Reelin and ApoE application to the hippocampus can enhance LTP induction. Similarly, addition of recombinant selenoprotein P also enhances LTP induction in an ApoER2-dependent manner (Peters et al., unpublished data). These observations strongly suggest that selenoprotein P plays a necessary role in hippocampal function by signaling through ApoER2 and internalization by ApoER2 could be useful as a neuronal selenium delivery system.

The significant changes in synaptic physiology in selenoprotein P knockout mice [47] and the enhancement of LTP with direct application of the protein raise the question of whether selenoprotein P is more of a signaling molecule than simply a selenium transporter. Several studies illustrate that selenoprotein synthesis and function is still present in brain in the absence of selenoprotein P, suggesting transport is not the primary purpose of this protein. For example, the overall neurological phenotype produced through selenoprotein P deficiency is much less severe than found with a brain-specific knockout of all selenoproteins [48], or with knockout of the selenoproteins glutathione peroxidase (GPx) 4 or thioredoxin reductase (Trxnd) 1 [49, 50]. Conversely, liver-specific selenoprotein deficiency has no affect on neurological function [51]. However, selenoprotein P is likely to be responsible for maintaining brain selenium levels when dietary intake of selenium is low [52], and thus may act as a routing mechanism to target selenium specifically to organs with ApoER2 receptors, i.e., brain and testes.

Regardless, there is a clear correlation between ApoE genotype, lipoprotein receptor-dependent clearing of amyloid, and the progression of Alzheimer's disease. The ability of specific selenoproteins to interact with this system supports the further exploration of selenium and selenoproteins as potential targets for future Alzheimer's disease therapies.

34.5 Selenoproteins and Oxidative Stress in Alzheimer's Disease

Alzheimer's disease is associated with oxidative damage to proteins, lipids, and nucleic acids, and oxidation is particularly strong in areas of amyloid plaques and in cells with neurofibrillary tangles [53]. Findings of increased oxidation lead to the hypothesis that amyloid beta could induce oxidative stress in brain [54]. Metals such as Fe^{2+} and Cu^{2+} are also thought to be sources of oxidization, and promote aggregation of amyloid beta with production of hydrogen peroxide [55, 56]. Because of the reducing properties of selenocysteine, several members of the selenoprotein family are involved in redox and antioxidant pathways, and may play a role in mitigating oxidative stress in Alzheimer's brain. Two classes of enzymes with antioxidant properties are the GPxs and Txnrds. The GPxs use glutathione as cofactor, whereas the Txnrds use thioredoxin. These pathways work in conjunction for the reduction of peroxides, free radicals, and oxidized biomolecules. Amyloid beta toxicity increases GPx activity in cultured neurons [57], and overexpression of GPx1 is protective against amyloid toxicity [58]. Conversely, genetic deletion of GPx1 renders cells more sensitive to toxicity from peroxide and amyloid peptides [59]. AD mice with one allele of GPx4 have increased lipid oxidation and AD pathology [60, 61]. Additionally, a polymorphism in GPx1 has been shown to increase risk of Alzheimer's disease in a South American population [62]. Txnrds may also have a role in AD. Amyloid beta increases oxidation of Txnrd1, and overexpression of Txnrd1 reduces amyloid beta toxicity [63]. Txnrd levels are increased in AD, although a concurrent decrease in thioredoxin may prevent any protective benefit from this increase [64].

Other selenoproteins may also have antioxidant properties. Sepp1 has a redox domain, and can reduce oxidized lipids as well as peroxide, using either glutathione or thioredoxin as substrates [65]. Selenoproteins K, M, and W have all been reported to have direct redox properties as well [66–68]. Thus, the selenoprotein family is an important component of the overall response and mitigation of oxidative stress, and is likely to be important in prevention of neurodegenerative disorders involving high levels of oxidative stress.

34.6 Alzheimer's and Selenoproteins in ER Stress

Proper folding of proteins in the endoplasmic reticulum (ER) is essential for their intended function, and errors in this process require correction. ER-associated protein degradation (ERAD) is a process involving removal of misfolded proteins from within the ER and subsequent breakdown by the proteosome [69]. A buildup of misfolded proteins within the ER causes ER stress, which may lead to an increase in proteins that either fold or remove misfolded proteins (the unfolded protein response, or UPR), or may trigger apoptotic cell death [70].

Recent studies suggest an important role for ER stress in Alzheimer's disease. ER stress is indicated by the presence of binding immunoglobin protein (BiP) and other markers in Alzheimer's brain [71–73]. ER stress can be triggered by the presence of extracellular amyloid beta [74, 75], and can also be triggered by the processing of overexpressed APP [76]. ER stress can increase the phosphorylation of the Tau protein and promote formation of neurofibrillary tangles [74].

Selenoprotein S (SelS) is an ER membrane-spanning protein that is an essential part of the retrotranslocation of misfolded proteins from the ER lumen [32, 77]. SelS, also termed VIMP for VCP-interacting membrane protein [78], forms a complex with the Derlin proteins, which are believed to form a channel to shuttle misfolded proteins across the ER membrane. SelS is connected with a p94 ATPase transporter (also known as VCP) outside the ER. This complex is also associated with an E3 ubiquitin ligase for tagging proteins with ubiquitin, a signaling protein used to target proteins for proteosomal degradation. Thus, proteins are transported out of the ER, tagged with ubiquitin and shuttled off to the proteosome. The important role of SelS in ERAD suggests it may have a preventative role in neurofibrillary tangle formation. SelS present in astrocytes helps prevent cell damage from ischemia or ische-mia [79], but a role for SelS in neurons has not been reported.

SelS was also identified as a glucose-regulated protein in a rodent diabetes model and named Tanis [80]. SelS is upregulated by inflammatory cytokines [81]. A SelS promotor polymorphism decreases SelS expression, causes an upregulation of inflammatory cytokines [81], and is associated with risk of cardiovascular disease and certain types of cancer [32].

Other selenoproteins may have a role in ER stress as well. Selenoprotein 15 (Sep15) is upregulated by the UPR [82]. Selenoprotein K (SelK) is reported to be involved in ER stress in cardiac cells [83]. Several other selenoproteins are also associated with the ER, including SelM, SelN, and SelT, and may have roles in ER stress [77]. Selenoprotein synthesis may be essential to controlling ER stress and for preventing some aspects of AD pathology such as neurofibrillary tangle formation.

34.7 Selenoproteins and Calcium Regulation

Calcium has important roles in neuronal signaling, survival, and cell death, and loss of calcium regulation may be an important part of Alzheimer's pathology [84, 85]. A growing number of selenoproteins have been implicated in regulating calcium flux from ER stress. We have previously shown that selenoprotein M alters ER calcium signaling in neurons and protects from oxidative stress [68]. Selenoprotein N expression alters calcium signaling though the calcium-sensitive ryanodine ER receptors [86]. Selenoprotein T can also alter calcium release from ER stores in neuroendocrine cells in response to the neuropeptide polyadenylate cyclase-activating polypeptide (PACAP). Thus, the selenoprotein family appears to have great importance in ER calcium regulation and homeostasis [87].

34.8 Selenium and Alzheimer's: Clinical Implications

If selenoproteins have a protective role against neurodegeneration in Alzheimer's disease and other disorders, can their expression and activity be increased for treatment or prevention of Alzheimer's disease? As mentioned earlier, the PREADVISE clinical trial was commenced to determine whether selenium and vitamin E can reduce risk of Alzheimer's [88]. As subjects of the parent SELECT trial discontinued taking supplements, the PREADVISE trial has moved into a centralized follow-up phase, and subjects will continue to be monitored for potential health benefits.

However, concerns remain over findings of the SELECT and previous studies that selenium supplementation may increase risk of type II diabetes. Increasing selenoprotein P levels appears to promote insulin resistance. The promotion of type II diabetes, a risk factor for Alzheimer's disease, would counter benefits of selenium supplementation. The use of selenomethionine in these studies has also come under criticism. Although selenomethionine is a more bioactive and biologically relevant form of selenium than sodium selenite, it can be randomly incorporated in place of methionine in other proteins impairing their function [89].

Two recent studies found that sodium selenate, as opposed to selenite, reduces phosphorylation of tau and decreases neurofibrillary tangles in mouse models [33, 34]. The proposed mechanism is through an agonistic interaction of selenate with protein phosphatase 2A (PP2A) resulting in dephosphorylation of tau protein. The studies show that selenate is less toxic to neurons than selenite [33, 34]. Previous studies have shown that selenium from selenate can be incorporated into selenoproteins and that selenate increases selenoprotein expression [90, 91]. The up-regulation of selenoproteins may have further benefits in Alzheimer's in addition to increasing PP2A function. Interestingly, selenate supplementation increases plasma levels of selenoprotein P [90, 92, 93], but increases insulin sensitivity rather than insulin resistance [94, 95]. Thus sodium selenate may be a better form of dietary selenium for the prevention and treatment of Alzheimer's disease.

Additional means of regulating selenoprotein expression may also be beneficial in this disease. The recently discovered selenium compound selenoneine, the main form of selenium in tuna blood, may be an effective source of dietary selenium [96]. Further studies into therapies that may increase effectiveness of selenoproteins should also be explored. For example, pharmaceutical manipulations may be able to increase selenoprotein P uptake through LDL receptors or target selenoprotein synthesis machinery. Investigations into the use of selenium-related therapies for Alzheimer's disease have only just begun, and many avenues remain to be explored.

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Chapter 35
Selenium and Inflammation

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Abstract It is becoming increasingly clear that over-production of reactive oxygen and nitrogen species (RONS) by immune cells, resulting in oxidative stress, plays a prominent role in several disease states, where inflammation forms the underlying basis. Emerging evidence from many studies in humans and animals strongly suggest that the beneficial effects of selenium-supplementation in prevention and/or treatment of some of these diseases occur via the mitigation of inflammatory signaling pathways. Selenium supplementation, over the minimal nutritional requirements, has gained popularity and there is some scientific evidence to support benefits of super-supplementation of Se. However, despite the therapeutic potential of selenium in many inflammatory diseases, very little is known about the mechanism and regulation of inflammation by Se. To explain the health benefits of selenium and define its biochemical role in mitigating oxidative stress-mediated expression of proinflammatory genes and initiate the recovery or resolution phase, it is important to identify those signaling pathways and genes whose expression is regulated strictly by selenium status in macrophages. Given that RONS serves as a double-edged sword in the modulation of inflammatory signaling pathways, it is not surprising to find that selenium-deficiency defects may be related to an "over-worked" system that fails to mitigate oxidative stress. Thus, studies relating to the modulation of signaling of inflammatory gene expression by selenium may open new opportunities to understand the redox-regulation of complex signal transduction pathways.

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35.1 Introduction

Inflammation is a common biological phenomenon that lies at the helm of many pathological states including cancers, atherosclerosis, autoimmune disorders, diabetes, and neurodegenerative diseases. As part of a complex biological response, inflammation provides the first line of defense against harmful stimuli, infections, and pathogenic invasions and, thus, may be considered as a "necessary evil." A variety of specific mediators are released from tissues and migrating cells in response to stress, free radicals, and infections to activate inflammatory pathways, where prostaglandin (PG) E_2, thromboxane (TX)A_2, and leukotriene (LT)A_4 and (LT)C_4 play critical roles. Biological systems have evolved a variety of anti-inflammatory strategies to combat this phenomenon. Alongside, pain management strategies have also advanced with numerous anti-inflammatory agents such as steroids, antibodies to proinflammatory cytokines, and nonsteroidal anti-inflammatory drugs (NSAIDs). Interestingly, many naturally occurring dietary supplements and nutrients have been shown to modulate low-grade inflammation [1]. These molecules or compounds reduce inflammation by specific mechanisms. Selenium is one such well-known antioxidant that has gained significant attention in the recent past due to its diverse role in the etiology of numerous physiological and pathological processes. Selenium has been found to possess anti-inflammatory properties by modulating a number of cellular signaling pathways.

35.2 Selenium and Inflammation: Human trials

Literature is replete with studies that suggest a critical role for selenium in stress-induced inflammatory processes and diseases of viral or bacterial origin. High levels of C-reactive protein (CRP), which is a commonly used biomarker of inflammation, is associated with reduced serum selenium levels [2]. It has been found that increased RONS production induced by low selenium levels has pathological implications as seen in systemic inflammatory response syndrome (SIRS) and patients afflicted by sepsis that is characterized by extensive tissue damage and organ failure [3, 4]. Studies also suggest the ability of selenium supplementation to reduce the rate of secondary infections in patients with burn injuries and trauma that are characterized by low serum selenium levels and glutathione peroxidase (GPx) activity [5]. In a randomized multi center study, selenium administration reduced the mortality in patients with severe sepsis and septic shock [6]. On the contrary, in another randomized placebo controlled study, infusion of high doses of selenium failed to provide any protective outcome [7]. Although the reasons for such discrepancies are not clear at present, these studies suggest the need for further studies to understand the role of Se in mitigating inflammation.

Selenium possesses protective roles in pathologies involving inflammation associated with rheumatoid arthritis, pancreatitis, autoimmune disorders, cancers,

and asthma. Numerous epidemiological studies in different geographical areas support this fact. In a case-control study involving 18,709 men and women who had no arthritis at baseline, the adjusted relative risk between the highest and lowest tertiles of serum selenium was 0·16 (p for trend = 0.02) for rheumatoid-factor-negative arthritis [8]. In another double-blind randomized trial in a small group of patients with rheumatoid arthritis, supplementation of 200 μg selenium (as selenium-yeast) for 3 months significantly reduced pain [9]. Similarly, the protective effect of selenium was evident in pancreatitis, a disorder associated with a high level of oxidative stress and inflammation. Administration of selenium (600 μg/day) along with other antioxidants to patients with chronic and recurrent pancreatitis significantly reduced pain and frequency of attacks [10]. In a small controlled trial in Rostock, Germany, intravenous administration of selenium to patients with acute necrotizing pancreatitis significantly reduced mortality [11].

An inverse relationship was found between dietary selenium intake and asthma in adults in a large population-based case–control study in London [12]. In a small nested case–control study, current wheeze among New Zealand children was more common in those with low concentrations of selenium in serum samples [13]. Hasselmark et al. [14] demonstrated significant clinical improvement in asthma upon supplementation with selenium at 100 μg per day as sodium selenite.

Several interventional studies over the past few years have demonstrated a variable decrease of anti-thyroid-peroxidase (TPO) in patients with autoimmune thyroiditis (AIT) supplemented with selenium [15–17]. In pregnant women with AIT, selenium supplementation was found to alleviate hypothyroidism and impede the manifestation of postpartum thyroiditis by suppressing anti-TPO levels [18]. A recent study from Austria revealed existence of an inverse causal relationship between AIT and serum selenium levels [19]. Furthermore, in celiac disease (CD), an intestinal inflammatory syndrome, malabsorption-induced selenium deficiency was proposed as a major cause of intestinal mucosal damage as well as a predisposition to AIT via the decreased expression of selenoproteins [7]. Selenium has been recommended as a therapeutic measure in CD to block IL-15-dependent epithelial damage and complications as seen in AIT [20, 21] highlighting the role of selenoproteins as critical regulators of RONS-dependent inflammatory signaling pathways that involve interplay of many immune cells.

35.3 Selenium as an Anti-Inflammatory Agent: Mode of Action

The exact mechanism by which selenium serves as a protective agent in mitigating inflammatory insults is not clear. In immune cells, selenium status is known to modulate a variety of pathways that are pivotal in defining the proinflammatory repertoire. Multiple pathways have been proposed for modes of action of selenium pertaining to its anti-inflammatory properties. These pathways are interrelated to each other, but work at different levels of molecular hierarchy as discussed below.

35.4 Selenium Mediated Scavenging of ROS via Selenoproteins

RONS occupy a major role as key modulators central to many pathways of inflammation. RONS, particularly H_2O_2 and $ONOO^-$, have the ability to interact with many cellular molecules, including proteins, to elicit pathways that lead to increased expression of inflammatory mediators. While the effects are, in part, mediated by the ability of selenoproteins to detoxify ROS, including H_2O_2, lipid and phospholipid hydroperoxides, the consequence of such a process to impact gene expression signatures is intriguing. For instance, changes in the cellular redox tone brought about by the reduction of cellular peroxides have an impact on the activation of key enzymes, such as the cyclooxygenases (COX) and lipoxygenases (LOX), which produce lipid mediators in the form of prostaglandins, thromboxanes, prostacyclins, and oxidized fatty acids, respectively [22]. As an example, PGE_2, TXA_2, LTA_4, and LTC_4 are well-known biomarkers of inflammation. Thus, suppression of the production of such mediators by selenium may attest to its role as an anti-inflammatory agent.

The role of selenium in ROS-mediated pathways of inflammation cannot be complete without addressing the effect of selenium on the mitochondria. Given that the mitochondrial electron transport system also serves as an additional source of RONS, mitochondrial redox imbalances can have serious consequences leading to the activation of signal transduction pathways of inflammation and apoptosis. The loss of mitochondrial integrity and activity could be the main cause of tissue oxygenation and development of inflammatory conditions, which are critical events in cell death processes [23]. Thus, protecting mitochondria may prevent tissue damage during inflammation. Subcellular analysis of selenium distribution in human liver samples indicates that this element is preferentially concentrated in mitochondria and nuclei [24]. Recently, it was documented that high selenium diets could affect liver mitochondrial parameters in vivo as a possible mechanism for its chemopreventive effects [25]. Furthermore, high selenium completely prevented the inflammatory and necrotic conditions by directly interacting with mitochondria in an in vivo inflammatory model of colitis. High selenium treatment was also shown to increase the levels of two important mitochondrial transcription factors, nuclear respiratory factor 1 (NRF-1), and mitochondrial transcriptional factor A (mtTFA) upon treatment with inflammatory stimuli [26]. NRF-1 regulates the expression of mitochondrial electron transport chain protein cytochrome C; whereas mtTFA regulates the transcription, maintenance, and replication of the mitochondrial genome. Thus, the protective mechanism of selenium during inflammation may be explained, in part, by its ability to directly target mitochondria leading to upregulation of mitochondrial transcription factors [26]. On the contrary, under selenium deficiency, modulation of the mitochondrial proteins may cause disturbances in the respiratory chain leading to increased free radical production and subsequent activation of inflammatory signaling pathways.

In favor of the anti-inflammatory role of selenium, selenium deficiency is known to impair some of the phagocytic cell functions, while selenium supplementation

completely corrected the defect [27]. For instance, peritoneal macrophages from rats fed with a selenium-deficient diet exhibited increased H_2O_2 production [28]; and granulocytes from selenium-deficient animals were unable to metabolize H_2O_2 leading to the destruction of their superoxide generating system [29]. Many studies demonstrate the importance of selenium in the pathobiology of disease processes. Decreased chemotaxis in selenium-deficient rats [30] and goats [31] were corrected with selenium supplementation [32]. Studies by Pertuz et al. [33] suggest that selenium may function as one of the physiological factors responsible for reducing inflammation, particularly in the joints in rheumatoid arthritis (RA) patients, by downregulating the "respiratory burst" that is critical for neutrophil activation and generation of oxygen-derived free radicals. While the disturbances are explained, in part, by the low GPx level of cells, the role of many uncharacterized selenoproteins need to be elucidated. More importantly, the ability of selenoproteins to impact key signaling pathways to modulate proinflammatory or anti-inflammatory outcomes needs to be addressed to provide a complete understanding of the role of selenium in anti-inflammation.

35.5 Selenium-Mediated Modulation of COX/LOX Pathways of Arachidonic Acid Metabolism

Cellular exposure to stress is reflected by increases in the levels of circulating proinflammatory cytokines, chemokines, and lipid mediators such as PGs and LTs, some of which are already recognized as bonafide biomarkers of inflammation. As discussed earlier, in addition to its role in the detoxification of peroxides during the activation of phagocytic cells, selenium is also involved in the modulation of COX and LOX pathways of PG and LT from arachidonic acid (AA), a common polyenoic fatty acid esterified in the sn-2 position of membrane phospholipids. The initial step in eicosanoid production requires the release of AA from membrane phospholipids through the activity of phospholipase A_2 (PLA_2), which is activated under conditions of oxidative stress. Because of accumulation of peroxides in Se-deficient conditions, selenium status has been indirectly implicated in increased PLA_2 activity through decreased GPx activity [34]. In addition, selenium participates in several steps of both the COX and LOX pathways of the arachidonic acid cascade through the activity of GPx1, which can reduce lipid hydroperoxide intermediates to their corresponding alcohols. GPx1 reduces the COX product PGG_2 to PGH_2 efficiently (Fig. 35.1). In platelets, GPx1 mediates the reduction of 12-lipoxygenase product, 12-hydroperoxyeicosatetraenoic acid (12-HPETE) to 12-hydroxyeicosatetraenoic acid (12-HETE) [35]. Platelets from selenium-deficient rats produce more trihydroxyeicosatetraenoic acid (THETE) and less 12-HETE than platelets from control rats, indicating that THETE is an alternate pathway when the peroxidase-mediated conversion of 12-HPETE is impaired [33]. Increased 12-HETE levels, particularly in keratinocytes, could play an important role in shaping the immune response during bacterial infections [36]. Similarly, the conversion of 5-HPETE to the inactive

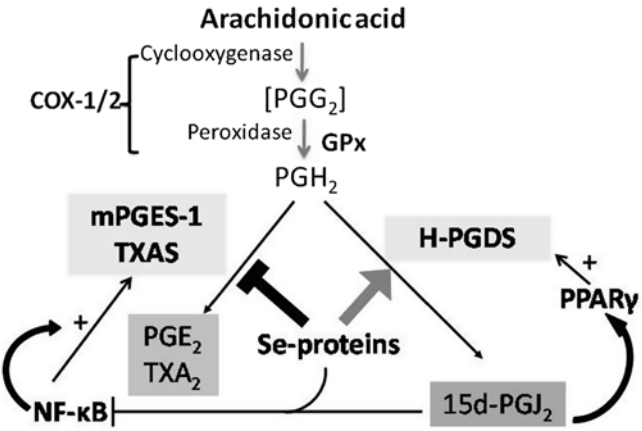

Fig. 35.1 Schematic illustration of the shunting of arachidonic acid metabolism by selenoproteins in macrophages. Macrophages cultured in the presence of bioavailable selenium leads to the enhanced expression of H-PGDS and its product, PGD_2 and 15d-PGJ_2; while pro-inflammatory PGE_2 and TXA_2 that are products of m-PGES-1 and TXAS, respectively, are decreased. Inhibition of NF-κB and activation of PPARγ are two major pathways that are affected by selenoproteins in these cells to modulate pathways of shunting. Increased activation of PPARγ facilitates a positive feedback upregulation of H-PGDS leading to increased levels of CyPGs. COX, cyclooxygenase; mPGES-1, microsomal PGE_2 synthase; H-PGDS, hematopoietic PGD_2 synthase; PPARγ, peroxisome proliferator activated receptor-γ

metabolite, 5-HETE, in leukocytes is also mediated by GPxs [33]. Many of the lipid hydroperoxides display a higher proapoptotic potential in many cell types, apart from activating upstream kinase (MAPK) pathways that increase the expression of many proinflammatory genes, including COX and LOX enzymes. Thus, selenium status serves as a critical determinant of the levels of these reactive intermediates, which appears to be critical in the pathophysiology of many diseases.

Based on these findings, it is speculated that selenium supplementation alters the synthesis of LTs from 5-HETE, while selenium deficiency promotes the conversion of AA to LTA_4, and other products derived from LTs. On the contrary, selenium was shown to promote the synthesis of LTs. Indirect evidence for such a phenomenon comes from studies of pulmonary alveolar macrophages isolated from selenium-deficient rats that produce less LTB_4. Thus, some investigators suggest that GPx activity should be inhibited rather than enhanced in inflammatory diseases [37]. Despite these conflicting data, there is a general consensus that selenium and GPxs play a significant role in PGs and LTs biosynthesis via the ability of these enzymes to modulate the cellular redox tone, particularly to impact such redox-sensitive transcription factors as NF-κB, whose activation has a direct bearing on the production of these lipid hydroperoxides and inflammatory lipid mediators (Fig. 35.1). Therefore, optimal levels of selenoproteins, mainly those with high peroxidase activity, may be clinically beneficial in inflammatory disorders. To determine their relative importance, further in vivo studies are required.

35.6 Selenium-Dependent Modulation of the NF-κB Pathway and Its Role in Macrophage Activation

The NF-κB family of transcription factors, comprising p50, p65 (RelA), p52, RelB, c-Rel, is termed as the "central mediator of immune and inflammatory responses." Diverse stimuli, including cytokines, bacterial and viral products, oxidants, and mitogens, lead to phosphorylation of two regulatory serine residues on IκB, which targets it for polyubiquitination and proteolytic degradation. This leads to nuclear translocation of NF-κB, where it binds to and stimulates the transcription of target genes, including COX-2, iNOS, and several other proinflammatory cytokines and chemokines. Consistent with the notion that decreased selenium deficiency increases intracellular ROS levels, our laboratory has previously demonstrated an increased activation of NF-κB in selenium-deficient RAW 264.7 cells when compared with macrophages supplemented with supraphysiological levels of selenium [38, 39]. More importantly, our studies have further demonstrated that changes in the cellular selenium status serve as a critical regulator of pathways of macrophage activation.

Classical macrophage activation is characterized by the production of several proinflammatory mediators such as IL-1β, IL-6, TNFα, PGE_2, and TXA_2 [40]. PGE_2, TXA_2, PGD_2, and 15d-PGJ_2 are the major eicosanoids derived from arachidonic acid in macrophages. The initial step of PG synthesis involves formation of PGH_2 from arachidonic acid by the action of COX-1 and COX-2, which is subsequently acted upon by specific PG synthases microsomal PGE synthase-1 (mPGES-1), thromboxane synthase (TXAS), and PGD synthase (PGDS), to form PGE_2, TXA_2, and PGD_2, respectively. While the initial phases of inflammation involve increases in levels of proinflammatory mediators like PGE_2, TXA_2, a switch toward the pro-resolving and anti-inflammatory mediators like PGD_2 and 15d-PGJ_2 during the latter stages suggests the involvement of a critical regulator. NF-κB serves as a key transcription factor for mPGES-1 and TXAS, leading to the upregulation of PGE_2 and TXA_2, respectively [41]. Our laboratory has shown that selenium supplementation of macrophages downregulated NF-κB with a corresponding increase in the activation of peroxisome proliferator activated receptor, PPARγ, through the increased production of 15d-PGJ_2 (also called cyclopentenone prostaglandins, CyPGs) [42] (Figs. 35.1 and 35.2). 15d-PGJ_2 serves as an endogenous ligand for PPARγ in addition to acting as an inhibitor of NFκB. On the contrary, Nrf-2 is activated by 15d-PGJ_2 perhaps as a compensatory mechanism to keep the levels of CyPGs under check via detoxification by glutathionylation. Interestingly, recent studies in our laboratory have demonstrated that selenium supplementation of macrophages caused the eicosanoid pathway to be shunted toward PGD_2 and 15d-PGJ_2 rather than PGE_2 and TXA_2 by the differential modulation of NF-κB and PPARγ (Fig. 35.2). Furthermore, preliminary studies have demonstrated the requirement of selenoproteins to effect the switching of eicosanoid pathways. Thus, the role of selenoproteins as key regulators involved in this "switch" toward anti-inflammatory mediators is intriguing and needs to be further elucidated. More importantly, the

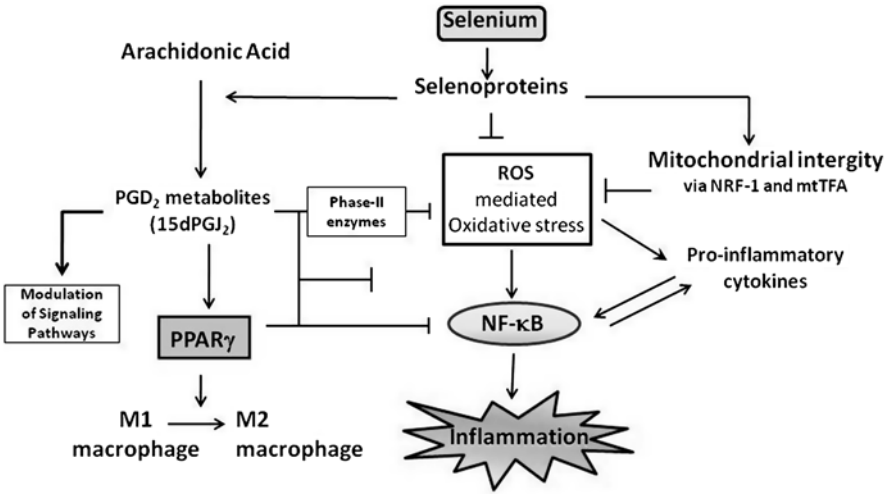

Fig. 35.2 Schematic representation of the implication of selenium-dependent eicosanoid shunting on pathways of anti-inflammation by macrophage phenotype switching. Based on our recent studies, selenoproteins are essential for the upregulation of cellular markers of M2 (anti-inflammatory) macrophages. Selenoproteins effectively mitigate RONS production by protecting the integrity of the mitochondria as well as downregulating the NF-κB pathway. As a result of shunting of arachidonic acid metabolism towards CyPGs, changes in the transcriptional programs within the pro-inflammatory (M1) macrophages facilitates their switching to anti-inflammatory (M2) macrophages to activate proresolution pathways. Such a process is inhibited by treatment of cells with COX or H-PGDS inhibitors or even organoselenium compounds that do not increase selenoproteins in cells

shunting of the arachidonic acid pathway, particular in macrophages, may have many implications; the most notable being a switch from the classically activated "M1" macrophage to the alternatively activated "M2" macrophage phenotype that are endowed with wound-healing and resolving properties [43] (Fig. 35.2).

In addition to producing proinflammatory eicosanoids, M1 macrophages produce proinflammatory cytokines and mediators, such as IL12, IL1β, TNFα, and nitric oxide (NO) [44]. Stimulated by factors like LPS and IFNγ, M1 macrophages lead to tissue damage and cellular immunity [43]. Within the macrophage, the specific enzyme, inducible nitric oxide synthase (iNOS), acts on L-arginine (L-Arg) to produce nitric oxide (NO) [43]. Our laboratory has shown that Se supplementation decreases the presence of iNOS, leading to decreased production of NO [39]. Interestingly, the abundance of the substrate L-Arg does not increase when NO is inhibited, indicating it may be available for the competing enzyme, arginase (Arg-I). Arg-I acts on L-Arg to form urea and L-ornithine, which help in polyamine (collagen) synthesis and enhance the production of anti-inflammatory cytokines and mediators [45].

Alternatively activated macrophages are recognized by their production of anti-inflammatory cytokines, such as IL-10, the expression of distinctive cell surface markers, like mannose receptor, and the secretion of mediators like Ym-1 and

FIZZ-1 [43]. While a function of M2 macrophages is to initiate wound healing through the production of collagen and granuloma formation, M2 macrophages also serve to resolve inflammation. Numerous studies have shown that uncontrolled inflammation can lead to tissue injury and cell death [44]. Based on the preliminary studies, we believe that selenium supplementation positively regulates Arg-I expression to mitigate inflammation and initiate wound-healing (catabasis) responses. Such a switch in macrophage phenotype by selenium could be important in the immune responses to parasites, which remains to be tested.

35.7 Modulation of Inflammatory Pathways by Selenium and Its Effect on HIV Transcription

While micronutrient deficiencies may contribute to HIV/AIDS, selenium deficiency has been singled out as being a major cause for disease progression and mortality in individuals infected with HIV [46, 47]. The significance of selenium against autoimmune disorders is seen in a recent cohort study indicating increased mortality and morbidity among children born to HIV-infected mothers with selenium deficiency [48]. It has also been shown that HIV infection shifts cellular processes toward a prooxidant state leading to increased levels of oxidation products [49] and, hence, accelerated oxidative stress. These changes are concurrent with a simultaneous decrease in plasma selenium levels and depletion of selenoproteins in T cells and erythrocytes [50, 51]. On the contrary, selenium supplementation was found to improve the immunity, diffusion pattern of HIV/AID'S and health of the HIV infected patients [52–54]. Furthermore, the spread of *Mycobacterium tuberculosis* that is commonly associated with HIV-positive patients was reduced with selenium supplementation with a decrease in neuropathy and genital ulcers, accompanied by an increase in $CD3^+$ and $CD4^+$ cells [55].

It is well documented that oxidative stress induces the expression of the transcription factor NF-κB, which is a key molecular event in the initiation of proviral transcription. Increased activation of NF-κB in selenium deficient monocytes or T cells lowers the threshold for increased proviral expression. Thus, the redox modulation of NF-κB by selenium in immune cells could play a regulatory role in the modulation of HIV transcription and replication. Consistent with the observation of Gladyshev et al. [50], our laboratory has recently reported that HIV infection leads to a decrease in the expression of selenoproteins, GPx1, and Txnrd1 in macrophages [56]. Supplementing such infected cells with selenium (in the form of sodium selenite) not only increased the expression of GPx1 and Txnrd1, but also inhibited HIV transcription and replication. These positive effects of selenium on GPx1 and Txnrd1 may be attributed to alleviation of oxidative stress and decreased expression of NF-κB and other pro-inflammatory cytokines that are required to establish a successful HIV infection.

A recent study from our laboratory has shown that selenium, via the increase in Txnrd1 activity, modulated the redox status of a key HIV protein, Tat, a viral protein

expressed early during infection, by reduction of two disulfide bonds to inhibit its transactivation activity, and expression of other viral (structural) genes [56]. In addition, the selenium-dependent production of Δ^{12}-PGJ_2 and 15d-PGJ_2 also impacted the activity of Tat by covalently modifying the thiols that are reduced by Txnrd1. Thus, by a concerted effort, selenium affects the proviral transcription of HIV-1 possibly leading to a reduced rate of disease progression. Such evidences are suggestive of selenium supplementation as a potent adjuvant therapy alongside conventional therapies against HIV/AIDS. Studies are being performed to further characterize the cross talk of HIV with inflammatory signaling pathways, and the role of specific selenoproteins, in downregulating pathways of oxidative stress.

35.8 Selenoprotein S, Its Polymorphism, and Inflammation

Genetic and environmental factors are likely to influence the inflammatory response, but little is known about the genes underlying its regulation. Selenoprotein S (SEPS1) is an endoplasmic reticulum (ER) membrane protein and human homolog of Tanis protein [57], which putatively functions in stress responses of ER that are closely linked to immune and inflammatory signaling pathways [58, 59]. SEPS1 has been found to have a role in inflammatory pathways as an interacting protein of serum amyloid A, which is an acute phase inflammation response protein [58, 60]. SEPS1 participates in the processing and removal of misfolded proteins from the ER to the cytosol [61]. This selenoprotein has a critical role in mediating inflammation through its protection of the ER from unfolded protein stress responses. When the ER is functionally impaired by the build-up of such misfolded proteins, the expression of a number of genes is induced leading to activation of the transcription factor NF-κB [62]. Activated NF-κB induces the expression of SEPS1 in a positive feedback loop. Increased expression of SEPS1 in turn suppresses cytokine production by its ability to remove misfolded proteins from the ER. This system constitutes a SEPS1-dependent regulatory loop in the presence of inflammation. Variations in the SEPS1 gene are known to affect circulating levels of the inflammatory cytokines, IL-1β, IL-6, and tumor necrosis factor-alpha (TNF-α). Interestingly, polymorphism in the 5′ upstream sequence (−105G→A) was associated with impaired expression of SEPS1 and siRNA suppression of SEPS1 resulting in the increased production of inflammatory cytokine production in macrophages [61].

Inflammation in the arterial wall is recognized to be an important component in the development of acute coronary disease syndromes [63]. Given the known association of SEPS1 with inflammation, the effect of genetic polymorphisms in SEPS1 on the risk of cardiovascular disease was investigated in two independent prospective Finnish cohorts. A significant association was found with increased coronary heart disease risk in females carrying the minor allele of rs8025174 in the combined analysis of both cohorts (HR 2.95; 95% CI 1.37–6.39). Another variant, rs7178239, increased the risk for ischemic stroke significantly in females (HR 3.35; 95% CI 1.66–6.76) and in the joint analysis of both sexes in both cohorts (HR 1.75; 95%

CI 1.17–2.64). Suggestive associations of both variants were also seen with the known cardiovascular risk factors of BMI and waist to hip ratio implicating the selenoprotein SEPS1 in cardiovascular disease risk [64]. These studies indicate that genetic variation in selenoproteins, particularly SEPS1, affect cytokine production that could impact cellular stress and inflammation.

35.9 Selenium Containing Drugs and Compounds as Anti-Inflammatory Agents

As a consequence of the growing recognition of the role of selenium in human health, a number of novel pharmaceutical agents are being developed. The beneficial effects of selenium, in the form of selenoproteins and organo-selenium compounds, have been studied for their role as antioxidants, cytokine inducers, enzyme inhibitors, and anticancer agents. Selenium may complement the actions of COX inhibitors and anti-histamines to effectively reduce major inflammatory mediators. Previously it has been described that derivatives of sulfonamide drugs bearing the selenophene with pyridine, pyridazine, and quinoline nuclei, such as selenolo[2,3-Ib]pyridine, selenolo[2,3-c]pydriazine, selenolo[2,3-b]quinoline, respectively, possess anti-inflammatory and analgesic activities [65]. These sulfa drugs with selenium-containing heterocyclic compounds were demonstrated to increase their biological activities in the form of anti-inflammatory, analgesic, fungicidal, and bactericidal agents [66]. Such novel findings of selenium-based drugs have opened new vistas to explore the enhanced spectrum of biological activity of sulfonamides (sulfadiazine, sulfadimidine, and sulfacetamide) with organo-selenium derivatives.

Along these lines, 1,4-phenylenebis(methylene)selenocyanate (*p*-XSC), a selenium-derivative of benzylthiocyanate was shown to have significant chemopreventive properties in a few rodent cancer models [67]. Interestingly, *p*-XSC effectively inhibited COX-2 expression via the inactivation of NF-κB [68] and displayed enhanced chemopreventive activity in rodents when compared with its sulfur counterpart, 1,4-phenylenebis(methylene)thiocyanate (*p*-XTC). Along the same lines, recent studies by Desai et al. [69] demonstrated that substitution of sulfur in PBIT (S,S′-(1,4-phenylenebis[1,2-ethanediyl])bisisothiourea), a well known iNOS inhibitor, with Se [Se′-(1,4-phenylenebis[1,2-ethanediyl])bisisoselenourea (PBISe)] increased the proapoptotic ability of the isosteric analog toward many cancer cell lines by inhibiting the PI3-kinase and Akt pathway. Recently, we demonstrated a similar strategy with celecoxib, a well-known nonsteroidal anti-inflammatory drug that selectively inhibits COX-2 activity. Interestingly, clinical trials are in progress using a combination of celecoxib and selenium yeast for the prevention of colon cancer [70]. Thus, the concept of synthesis of selenium-derivatives of celecoxib with anti-inflammatory and chemopreventive properties could, thus, represent an effective method to treat inflammatory processes, a hallmark of tumorigenesis. Therefore, to enhance the anti-inflammatory properties at extremely low doses and protect against potential side effects of these drugs, selenium-containing

derivatives were synthesized. One of the selenium-derivatives of celecoxib, namely, 4-(3-selenocyanatomethyl-5-*p*-tolyl-1-yl)-benzenesulfonamide (selenocoxib-2) significantly inhibited bacterial endotoxin LPS-induced activation of NF-κB leading to the down-regulation of expression of pro-inflammatory genes, *COX-2*, *iNOS*, and *TNFα* more effectively than the parent celecoxib at least in a murine macrophage model [71]. Surprisingly, these studies also revealed that selenocoxib-2 effectively suppressed NF-κB activation without increasing the selenoprotein pool, which suggests that such placement of selenium within the celecoxib molecule is critical to target key inflammatory signaling axes in immune cells to mitigate inflammation [[71]]. The ability of selenium in these pharmacophores to interact with Cys thiols in proteins to (redox) modulate their activity could be one of the mechanisms of action, which needs to be further investigated. Nonetheless, these interesting findings open possibilities for a new generation of inhibitors with significant and broader anti-inflammatory potential.

35.10 Concluding Remarks

Increasing evidence shows that selenoproteins and possibly selenium metabolites play a pivotal role in down regulating cellular signaling pathways critical in the expression of proinflammatory mediators. It is now clear that selenium status of immune cells, particularly macrophages, leads to the decreased activation of NF-κB through a variety of mechanisms, including the production of novel anti-inflammatory PG metabolites. The implications of such an increased CyPGs production is vast and can explain many anti-inflammatory properties of selenium. However, defining the mechanism(s) by which selenoproteins increase CyPGs to dampen pathways of proinflammatory signaling while increasing pathways of anti-inflammation still remain to be investigated.

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Chapter 36
Selenium Metabolism in Prokaryotes

Michael Rother

Abstract Biologically active selenium occurs as a modification in tRNA, non-covalently attached cofactor, or as the amino acid selenocysteine, exerting functions key to the metabolism of the organism harboring it. In prokaryotes, selenocysteine is found in the catalytic site of numerous redox-active enzymes. It was designated as the 21st genetically encoded amino acid because it is cotranslationally inserted into growing polypeptides and universally encoded by the stop-codon UGA on the mRNA. The pathway of selenocysteine biosynthesis and incorporation is well understood in Bacteria, but considerable gaps of knowledge still exist in the respective system of the Archaea. This chapter aims to summarize details on prokaryal selenium biology with a focus on emphasizing the differences of the bacterial and the archaeal pathways of selenoprotein synthesis.

36.1 Introduction

Selenium has long been considered to be toxic element and only in the 1950s were its beneficial qualities recognized [1, 2]. It is common knowledge today that selenium is an essential trace element for many organisms from bacteria to humans due to the essential nature of selenium-containing cellular macromolecules.

The element occurs in four oxidation states, selenate Se(VI), selenite Se(IV), elemental selenium Se(0), and selenide Se(−II), the latter including also organoselenium compounds (Fig. 36.1). Selenium amounts to 0.05–0.14 ppm in the earth's crust and is typically associated with metal sulfides [3]. Except for regions unusually

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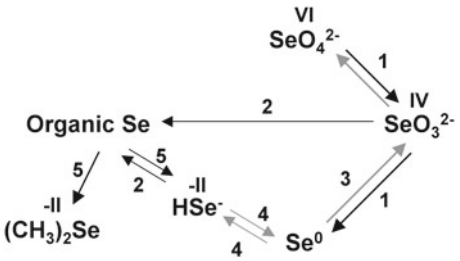

Fig. 36.1 Scheme of the selenium cycle. 1: selenium respiration; 2: biosynthesis; 3: predominantly abiotic reactions; 4: predominantly microbial activities; 5: degradation/detoxification; the processes indicated by *black* arrows bear greater relevance in the context discussed here than those indicated by *gray* arrows; adapted from Stolz et al. [5], see text for details

high or low in selenium, environmental concentrations of selenate range from 20 nM to less than 100 pM [4]. Some microbes exploit selenium as electron acceptor by anaerobically respiring oxyanions of this "precious" element, contributing significantly to the geochemical cycle of selenium [5] (Fig. 36.1). Methyl-selenides and methyl-selenoxides are common degradation and detoxification products. Dimethylselenoniopropionate (DMSeP) is the selenium-containing analog of the osmolyte dimethylsulfonionpropionate found in plants and algae. DMSeP can be degraded to acrylate and dimethylselenide by bacterial lyases [6]. Unusually high tolerance towards selenium in plants (>75 μM) is also conferred via methylated selenium species; a class of *S*-adenosylmethionine- and *S*-methylmethionine methyltransferases methylates selenocysteine [7] yielding Se-methylselenocysteine, which cannot be incorporated into proteins unspecifically anymore and, thus, be accumulated to more than 1 mg Se/g [8].

Higher organisms usually meet their needs for reduced selenium species via their complex diet; unicellular microbes usually take up oxidized forms of this element from the environment. It is interesting to note that, in spite of selenium's rather narrow biologically beneficial "window" (deficiency below ca. 0.5 ppm and toxicity above ca. 5 ppm in mammals [9]) and the low concentrations of bioavailable selenium species, it is still unclear if selenium is transported into the cell via a dedicated transporter and what selenium species is transported. Even for *Escherichia coli*, one of the best-studied organisms, a specific, active selenium-specific transport mechanism across the cytoplasmic membrane remains uncharacterized. Above a critical concentration, however, selenium utilizes the sulfur pathways and is metabolized via the respective assimilation pathways (Fig. 36.2). Selenate enters the cell via sulfate transporters and is reduced by the assimilatory reduction system. The biosynthetic machineries synthesizing cysteine, and from that eventually methionine, do not discriminate much between selenium and sulfur, which leads to the formation of selenocysteine and selenomethionine. Selenocysteine can serve as substrate for cysteyl-tRNA synthetase, which forms selenocysteyl-$tRNA^{cys}$ leading to unspecific selenocysteine incorporation during protein translation at cysteine positions [10]. This nonspecific incorporation is greatly reduced when either the pathway for

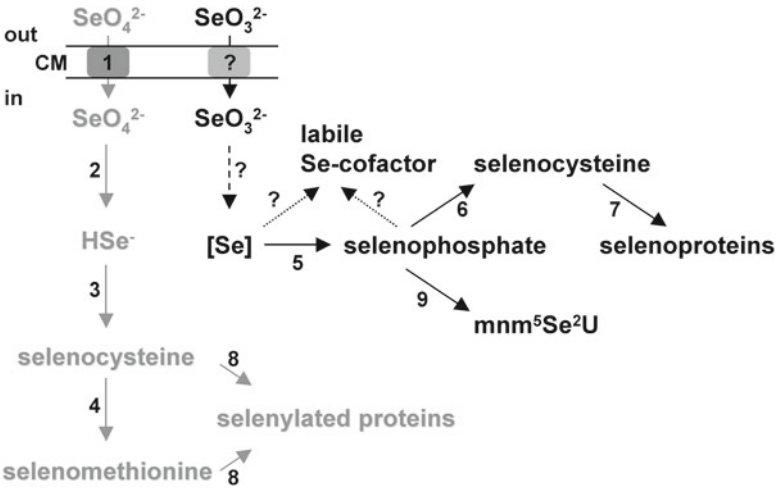

Fig. 36.2 Scheme for nonspecific (*gray*) and specific (*black*) metabolism and incorporation of selenium into macromolecules. 1: sulfate transporter; 2: sulfate assimilation pathway; 3: cysteine biosynthesis pathway; 4: methionine biosynthesis pathway; 5: selenophosphate synthetase; 6: selenocysteine biosynthesis pathway; 7: selenocysteine translation pathway; 8: canonical translation pathway; 9: 2-selenouridine synthase; *CM* cytoplasmic membrane; mnm^{5}Se^{2}U: 5-[(methylamino) methyl]-2-selenouridine; *[Se]*: reduced selenium species used by selenophosphate synthetase; the *dashed* arrow represents unknown selenium assimilation pathway(s); the *dotted* represent hypothetical reactions; adapted from Böck et al. [101]; see text for details

cysteine biosynthesis is repressed (e.g., in the presence of cysteine in the medium) or disrupted by mutation [11]. Selenomethionine is incorporated almost indiscriminately into protein in place of methionine.

The specific incorporation of selenium into macromolecules is already effective at very low external selenium concentrations. For example, cotranslational selenocysteine insertion in *E. coli* is already saturated at 0.1 μM selenite in the medium [12]. While the two other forms of biologically active selenium, as modification of RNA bases and as non-covalent cofactor of enzymes such as xanthine dehydrogenase, will be briefly considered, the major part of this chapter is dedicated to the mechanism of selenocysteine synthesis and its specific incorporation into proteins in the domains Bacteria and Archaea.

36.2 Biological Forms of Selenium Other than Selenocysteine

One form of biologically active selenium occurs as a base modification (5-[(methylamino)methyl]-2-selenouridine) in certain glutamate, proline, or lysine accepting tRNAs [13, 14] derived by specific substitution of selenium for sulfur in 2-thiouridine, which is catalyzed by 2-selenouridine synthase [15]. The precise molecular role of

this modification in the respective tRNAs is still not completely understood, but efficiency of the aminoacylation reaction in vitro depends on the selenium to be present [16]. Since this modification is located at the "wobble" position of the anticodon of a glutamate isoacceptor in *Clostridium sticklandii* [17], both capacity and fidelity of translational decoding in vivo should strongly depend on the modified base. Seleno(mono)phosphate, the product of the selenophosphate synthetase (SPS) reaction (see below), is the selenium donor in the substitution reaction [18, 19]. Interestingly, there is an apparent inverse correlation between the number of tRNA species in the total tRNA population of a particular organism that is modified with selenium and the extent to which the organism can tolerate oxygen during growth [20].

Less is known about the other form of biologically active selenium, cofactors with noncovalently bound selenium, found in anaerobic bacteria. Members of the purinolytic clostridia ferment purines to CO_2, ammonia, acetate, and formate. The compounds are converted to the common intermediate xanthine via pathways that involve xanthine dehydrogenase and purine hydroxylase, depending on the substrate [21]. The initial observation that the level of nicotinic acid hydroxylase activity in *Clostridium barkeri* was greatly enhanced by supplementation of the growth medium with selenium [22] was followed by characterization of the enzyme demonstrating the noncovalent nature of the cofactor [23]. Both xanthine and nicotinate dehydrogenases are molybdo-flavoenzymes of the molybdenum hydroxylase class [24] and electron paramagnetic resonance studies showed that selenium is coordinated with molybdenum [25, 26]. Since the molybdenum atom in molybdopterin is coordinated by a dithiol inserted during cofactor biogenesis [24], it is thought that selenium can be inserted instead of sulfur. Analysis of more than 500 prokaryal genomes led to the idea that insertion of selenium into these enzymes potentially involves a protein homologous to SPS [27, 28].

36.3 Specific Incorporation of Selenocysteine by *E. coli*

The third form of biologically active selenium is that of the amino acid selenocysteine. It was discovered as a unique amino acid in 1976 [29] and was simultaneously shown to be cotranslationally inserted into growing polypeptides at the position of an in-frame UGA (opal) nonsense codon on the mRNA in mammals [30] and *E. coli* [31]. The mechanism of selenocysteine biosynthesis and incorporation in *E. coli* (Fig. 36.3**a**) was the first to be elucidated in elegant studies conducted in the laboratory of August Böck, not least because large numbers of mutants could be generated and screened. Based on the fact that *fdhF* of the organism coding for formate hydrogen lyase-linked formate dehydrogenase contained an in-frame TGA stop-codon suppressed with selenocysteine, the genetic strategy consisted of the isolation of strongly acidifying strains, since a defect in selenium metabolism should prevent the synthesis of all formate dehydrogenase isoenzymes and result in massive excretion of formic acid. Four genes were identified which caused pleiotropic *fdh* mutations affecting selenium metabolism, today known as *selA*, *selB*, *selC,* and

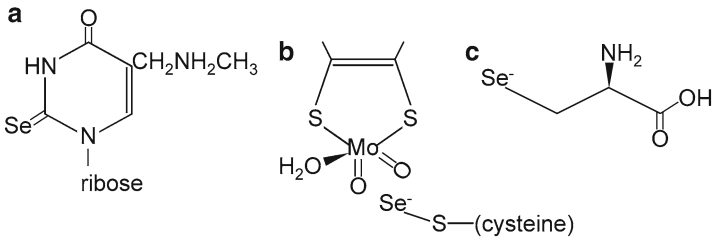

Fig. 36.3 The biological forms of selenium. Structures of 5-[(methylamino)methyl]-2-selenouridine (**a**), of the one proposed ([105]) for the active site of selenium-dependent purine hydroxylases (**b**), and of selenocysteine (**c**)

selD [32]. *selC* encodes the selenocysteine-specific tRNA ($tRNA^{sec}$, $tRNA_{UCA}$) [33]. Its anticodon UCA is complementary to the selenocysteine codon, and besides being the largest tRNA in *E. coli*, a number of normally invariant sequence positions deviate from the consensus [34]. It has an unusually long extra arm and a D stem closed to a six base-pair helix minimizing the D loop to four nucleotides, which restricts the types of tertiary interactions within the molecule [35]. The tRNA also has a unique modification pattern [36], but the most conspicuous difference to canonical elongator tRNAs, however, is the eight-base-pair aminoacyl-acceptor stem; all other tRNA species have a seven-base-pair stem.

$tRNA^{sec}$ is first "mis-"aminoacylated with L-serine by canonical seryl-tRNA synthetase (Fig. 36.4) [33]. The conversion of seryl-$tRNA^{sec}$ into selenocysteyl-$tRNA^{sec}$ is catalyzed by selenocysteine synthase (the *selA* gene product). Selenocysteine synthase of *E. coli* binds pyridoxal 5′-phosphate (PLP) as cofactor, the carbonyl of which forms an aldimine linkage with the α-amino group of serine, and 2,3-elimination of a water molecule generates enzyme-bound dehydroalanyl-$tRNA^{sec}$ [37]. Nucleophilic addition of selenide to the aminoacrylyl double bond forms selenocysteyl-$tRNA^{sec}$ [37]. The activated selenium donor for this reaction is seleno(mono)phosphate synthesized by SPS, the *selD* gene product [18]. The protein is an AMP-releasing ATPase [18, 38]. Interestingly, both of the anhydride bonds of ATP are cleaved by SPS, liberating the β-phosphate and channeling the γ-phosphate to the selenium donor [38]. The exact nature of the reduced selenium species used for this reaction is still unknown, but could be furnished by thioredoxin reductase [39]. Principally, selenocysteine synthase does not require the activated selenium species since selenophosphate can be substituted with high concentrations of selenide in vitro. Instead, the phosphate probably serves as a specificity "handle" to discriminate selenide from sulfide in vivo [40]. This notion is supported by the finding that thiophosphate serves as substrate in the selenocysteine synthase reaction in vitro leading to cysteyl-$tRNA^{sec}$, albeit at a much lower catalytic efficiency [40]. As SPS from *E. coli* is highly specific for selenium [40], the phosphate moiety of selenophosphate probably provides the selenocysteine synthase reaction with the necessary discrimination specificity against sulfur. Interestingly, mammalian SPS2

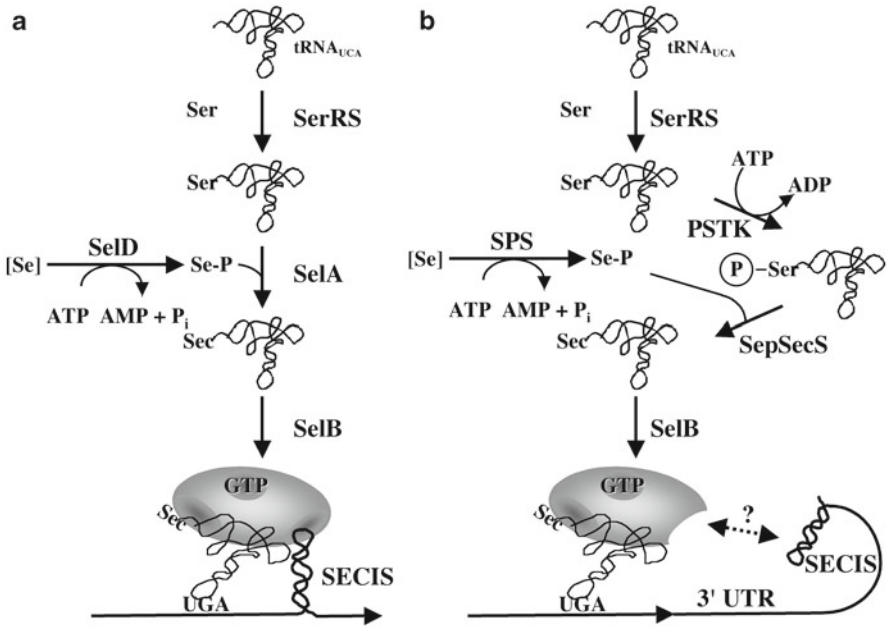

Fig. 36.4 Schematic of selenocysteine biosynthesis and incorporation in *E. coli* (**a**) and in *M. maripaludis* (**b**). *3′UTR* 3′-untranslated region; *PSTK* seryl-tRNA^{sec} kinase; *[Se]*: reduced Se-species; *SelA* selenocysteine synthase; *SelB* selenocysteine-specific elongation factor; *SelD,SPS*: selenophosphate synthetase; SepSecS: *O*-phosphoseryl-tRNA^{sec}:selenocysteine synthase; *Ser* serine; *SerRS* seryl-tRNA synthetase; *Se-P* seleno(mono)phosphate; the *dashed* arrow indicates an unknown recoding mechanism; adapted from Thanbichler and Böck [106]; see text for details

(a selenoprotein itself) displays a much higher ambiguity between selenium and sulfur, maybe to be able to "intentionally" generate cysteyl-tRNA^{sec} in times of selenium starvation [41] (and see Chap. 2 of this book).

It appears that once the tRNA is charged with serine, it is immediately bound to selenocysteine synthase and stays in the activated form until selenophosphate is available. The cellular stoichiometry of tRNA^{sec} molecules (ca. 250 [42]) and selenocysteine synthase (ca. 150, 5 seryl-tRNA^{sec} binding sites per decamer [43]) argues for selenocysteine synthase functioning as a sink for "capturing" seryl-tRNA^{sec}, which is less stable than other aminoacyl-tRNAs [44].

Because of its unique structural features, tRNA^{sec} is not recognized by the canonical elongation factor EF-Tu [45]. Instead, a selenocysteine-specific translation elongation factor, SelB (the *selB* gene product), is employed [46]. SelB from *E. coli* shares significant homology to EF-Tu in its N-terminal portion; it binds selenocysteyl-tRNA^{sec} and GTP stoichiometrically and discriminates not only tRNA^{sec} from the other elongator tRNAs [47], but also with high stringency selenocysteyl-tRNA^{sec} from uncharged tRNA^{sec}, seryl-tRNA^{sec}, or alanyl-tRNA^{sec} [46, 48]. This amino acid specificity explains why in *E. coli* only selenocysteine is incorporated at

the dedicated UGA codons, even in the presence of the seryl-tRNA^{sec} precursor. However, the apparent ambiguity in UGA suppression with cysteine recently reported suggests that cysteyl-tRNA^{sec} forms a sufficiently stable complex with SelB in vivo to be incorporated into a "seleno"-protein [49].

A unique property of bacterial SelB crucial for its function is that it interacts with another RNA molecule, the selenoprotein mRNA itself. The interacting segment, designated SECIS element (the term was originally coined for the eukaryal mRNA element directing UGA decoding with selenocysteine [50]), is a stem-loop structure of approximately 40 nucleotides that follows the UGA at the immediate 3′-side. The binding to the *E. coli* SECIS is mediated by the 17 kDa C-terminal domain (IV) of SelB, which is not present in EF-Tu [51–53]. The complex interaction of the tip-portion of the SECIS with the L-shaped domain IV of SelB consisting of four winged helix domains, derived from several X-ray crystallography and NMR studies, was recently summarized [54]. Formation of the quaternary complex between SelB, selenocysteyl-tRNA^{sec}, the SECIS, and GTP is cooperative; the SECIS is bound with tenfold higher affinity in the presence of selenocysteyl-tRNA^{sec} [55]. Furthermore, binding of the SECIS not only tethers the tertiary GTP •SelB•tRNA complex to the site of translation, but also serves to activate the molecule: GTP hydrolysis activity is stimulated at the ribosome when the SECIS is bound [56]. These findings indicate that SECIS binding induces a conformational switch in the complex, which renders SelB compatible for productive interaction with the ribosome, followed by GTP hydrolysis, and subsequent release of selenocysteyl-tRNA^{sec} in the proximity of the A site. This causes the affinity of SelB to the mRNA to decrease resulting in dissociation of the SelB-SECIS complex. Since SelB has, unlike EF-Tu, a ca. tenfold higher affinity for GTP than for GDP, no nucleotide exchange factor (EF-Ts) is needed, but guanine nucleotide exchange probably occurs chemically [46, 57].

36.4 Selenoprotein Synthesis in Other Bacteria

It is generally assumed that all bacteria follow the *E. coli* paradigm both during selenocysteine synthesis and selenocysteine translation. Whether this assumption is really true cannot be answered satisfactorily as no other (non-entero) bacteria have been thoroughly investigated in this respect. Furthermore, the lack of facile genetic systems often limits such efforts to biochemical analyses or to complementation analyses in *E. coli*. Still, surprising details emerged when members of other bacterial groups were analyzed. For example, *Haemophilus influenzae*, a γ-proteobacterium, like many Gram-positive bacteria, archaea, and eukaryotes, synthesizes a selenocysteine-containing selenophosphate synthetase [58], which poses the question how selenocysteine synthesis can be initiated employing an enzyme which itself is a selenoprotein. Apparently, this problem is solved by initially operating selenophosphate synthetase that is either devoid of selenocysteine, or by forming selenocysteyl-tRNA^{sec} in a selenophosphate-independent fashion [58].

Another unexpected finding was that the spirochete *Treponema denticola* synthesizes glycine reductase, a selenoprotein thought to occur only in Gram-positive bacteria [59]. Furthermore, when selenium-dependent gene expression was analyzed in *Treponema primitia*, an acetogenic inhabitant of termite guts, it was found that the level of selenoprotein mRNA upstream of the deduced SECIS element was unaffected by the selenium supply while its abundance downstream of the SECIS was greatly reduced when the selenium concentration was low [60]. This observation suggests that the SECIS either modulates transcription elongation or transcript stability in a selenium-responsive fashion.

The machinery decoding UGA as selenocysteine in Gram-positive bacteria appears to deviate somewhat from the *E. coli* paradigm, which is illustrated by the fact that putative SECIS elements in Gram-positive bacteria are not well defined and selenoprotein genes cannot be functionally expressed in *E. coli* [61]. In fact, it is difficult to derive a plausible consensus for a Gram-positive SECIS, even in one organism expressing several selenoprotein genes [62]. On the other hand, the observation that the $tRNA^{sec}$/SelB pair of *Eubacterium acidaminophilum* effected UGA read-through in *E. coli* of selenoprotein mRNAs from *Desulfomicrobium baculatum*, *Campylobacter jejuni*, and *T. denticola*, respectively, suggests that the SelB/SECIS interaction of Gram-positive bacteria is rather promiscuous [63]. The generally higher number of selenoproteins present (thirteen in *E. acidaminophilum* vs. three in *E. coli*) [64, 65] might be the reason for this less stringent recognition.

As some bacteria, which strictly depend on selenoproteins for growth, are also of considerable clinical relevance, the selenium metabolism in these organisms represents a potentially useful target for antimicrobial compounds. It is, therefore, surprising that research in this area is rather scarce, probably due to the elaborate methodology required to cultivate these pathogens. Still, susceptibility of *Clostridium difficile* and *T. denticola* to growth inhibition by auranofin, which scavenges selenide in the cells, shows that such an approach is feasible [66, 67].

36.5 Selenoprotein Synthesis in Archaea

36.5.1 Archaeal Selenoproteins

The second prokaryal domain encompasses the Archaea, only recognized as a distinct phylogenetic group some 30 years ago. According to 16S rRNA phylogenetic analysis, the Archaea have a common evolutionary origin with eukaryotes [68, 69]. This fact has led to debates about the meaningfulness to classify Bacteria and Archaea as "prokaryotes" in order to delineate them from eukaryotes [70, 71]. The only Archaea for which selenoproteins are known are methanogenic archaea obligatorily depending on the hydrogenotrophic pathway of methanogenesis for growth. In this pathway, CO_2 is sequentially reduced to methane in seven steps via coenzyme-bound intermediates using H_2 as the electron donor [72]. If formate is the substrate, it

is first reduced to CO_2 via formate dehydrogenase. Biochemical, genetic, and genomic analyses have so far confirmed eight selenoproteins in methanogens: one subunit of formate dehydrogenase [73], one of formyl-methanofuran dehydrogenase [74], one of heterodisulfide reductase [75], one of F_{420}-reducing hydrogenase [76], two of F_{420}-nonreducing hydrogenase [77], selenophosphate synthetase [78, 79], and HesB-like selenoprotein [79, 80]. Strikingly, of the eight known archaeal selenoproteins, six are directly or indirectly involved in the organism's primary metabolism, methanogenesis [81].

It is important to note that by far not all hydrogenotrophic methanogens employ selenocysteine. In fact, members of the same genus, even strains of the same species, can have different selenium requirements. The reason for this unevenly distributed essentiality of selenoproteins among methanogens is that these organisms employ, to various degrees, isoforms where the selenocysteine residue is replaced by cysteine [74, 77, 82, 83]. In *M. voltae*, for example, growth with $H_2 + CO_2$ depends on the selenium supply [84], whereas in *M. maripaludis* strain JJ, no such effect is observed because the selenoproteins can be efficiently complemented with the corresponding cysteine-isoforms. Growth on formate, however, is impaired when selenium is limiting or when the path for selenoprotein synthesis is disrupted because formate dehydrogenase in this organism is strictly selenium-dependent [82]. Strikingly, a very close relative, *M. maripaludis* strain S2, cannot do without its selenoproteins under any growth condition, probably because, for at least one of them, no complementing cysteine-isoform exists or is sufficiently active [79].

36.5.2 Selenocysteine Synthesis in Archaea

Archaeal $tRNA^{sec}$ possesses all the structural features which differentiate this tRNA from canonical elongator tRNAs. Altogether, it is structurally more similar to the eukaryal than to the bacterial counterparts [34]. $tRNA^{sec}$ from *Methanocaldococcus jannaschii* could be charged with L-serine by the *E. coli* seryl-tRNA synthetase and converted to selenocysteyl-tRNA by the *E. coli* selenocysteine synthase, which led to the assumption that the archaeal pathway for selenocysteine synthesis would be identical to the bacterial one [85]. However, no homolog of SelA could be found encoded in any archaeal genome. Instead, the occurrence of a kinase-encoding gene restricted to archaea and eukaryotes with selenoproteins was noted [86]. Subsequent biochemical analysis showed that the protein transfers a phosphate group to seryl-$tRNA^{sec}$ generating *O*-phosphoseryl-$tRNA^{sec}$, which led its designation as *O*-phosphoseryl-$tRNA^{sec}$ kinase (PSTK) [86, 87]. Still, the significance of this reaction in vivo was unclear, as *O*-phosphoseryl-tRNA had been known in eukaryotes for decades [88]. The answer came when the coding sequence of soluble liver antigen/liver pancreas (SLA/LP), the antigen for autoantibodies leading to autoimmune hepatitis, which was known to interact with eukaryal $tRNA^{sec}$ [89], was determined [90, 91]. It was proposed to be the eukaryal selenocysteine synthase due to its predicted PLP-dependence and its predicted overall similarity to SelA [92].

The presence of an archaeal SLA/LP homolog was shown by cross-reactivity of SLA/LP-specific antisera with *M. jannaschii* crude extract [93] and the role in selenocysteine synthesis was demonstrated by heterologous genetic analysis [94]. Biochemical analysis of the SLA/LP homolog from *M. maripaludis* demonstrated that the protein catalyzes the selenophosphate-dependent conversion of *O*-phosphoseryl-tRNA^{sec} to selenocysteyl-tRNA^{sec}, which led to its designation *O*-phosphoseryl-tRNA^{sec}:selenocysteine synthase (SepSecS) [94]. An elaborate biochemical study, conducted simultaneously, showed that the pathway in mammals is identical (yet there, SepSecS was designated SecS [95]). Thus, the function of bacterial selenocysteine synthase in converting seryl-tRNA^{sec} to selenocysteyl-tRNA^{sec} is expanded and separated to PSTK and SepSecS in Archaea and Eukarya. As this pathway can be traced back to the split between the archaeal and eukaryal sister lineages [94], the important question arises as to what selective advantage could employing an "extra" enzyme (PSTK) have to be so stably retained in evolution. Our idea that this pathway could render activation of selenium in the SPS reaction unnecessary turned out to be wrong [79]. Currently, there are three plausible explanations: (i) as *O*-phosphoseryl- would provide a better leaving group (phosphate) than seryl- (water) for replacement with selenium, the overall kinetic efficiency may be improved in case of PSTK/SepSecS as compared to SelA. (ii) Since seryl-tRNA^{sec} is more sensitive to deacylation than other aminoacyl-tRNAs [44] and SelA functioning as a sink for seryl-tRNA^{sec} (see above) is absent, phosphorylation by PSTK could stabilize the aminoacyl-tRNA [86], thus serving the purpose of storing precursors of selenocysteyl-tRNA^{sec} [96]. (iii) As cysteyl-tRNA^{sec} is formed in vitro with thiophosphate, more efficiently from *O*-phosphoseryl-tRNA^{sec} [41] than from seryl-tRNA^{sec} [40], targeted suppression of UGA with cysteine could be achieved, i.e., an "emergency route" for cysteine insertion being created. Indeed, severely selenium starved rats incorporate cysteine at the selenocysteine-position of thioredoxin reductase, probably in order to salvage at least some enzymatic activity [97]. However, for selenoprotein-containing archaea like *M. maripaludis* or *M. voltae*, it seems not plausible to employ a system which allows replacement of selenocysteine with cysteine because these organisms synthesize whole sets of separately encoded isoenzymes containing cysteine at the position of the selenocysteine, which substitute for the selenoproteins under selenium-deprived conditions [82, 98]. Thus, it appears that the consequential question regarding amino acid ambiguity for UGA decoding in methanogens [49], as well as for the role of *O*-phosphoseryl-tRNA^{sec} in these organisms, remains without a physiologically meaningful explanation for the time being.

36.5.3 Selenocysteine Translation in Archaea

Like in Bacteria and Eukarya, selenocysteine insertion in Archaea is directed by UGA [75, 80]. However, Archaea do not contain conserved secondary structures within the coding region of the selenoprotein mRNAs, which could serve as SECIS.

Instead, such structures can be found in the nontranslated regions (UTRs) of the respective deduced selenoprotein mRNAs [75]. In seven of the eight selenoprotein genes of *M. maripaludis,* the putative SECIS elements are located downstream of the translational stop-codon in the 3′-UTR; however, one appears to be located in the 5′-UTR [99], but its function as SECIS still needs to be demonstrated experimentally. Such proof for the nature of one of the structures in the 3′-UTR was provided in vivo by showing that a selenoprotein gene from *M. jannaschii* could only be heterologously expressed in *M. maripaludis* in the presence of the SECIS element [100]; analysis of point mutants showed that not the SECIS sequence, but rather its structure, is important for selenocysteine insertion [100]. The structures consist of a helical part with only few unpaired bases containing an upper G-C rich segment, which is followed by an internal loop characteristic for the structure. The loop contains four purines with a conserved G-A-A sequence opposed by an A on the other side and continues into a helix of two or three G-C pairs followed by a nonconserved apical loop region [101]. Interestingly, *M. voltae* contains a gene for a cysteine-isoform of one of its selenoproteins, which is succeeded by a mutated SECIS-like structure. This element, which has obviously lost its function, illustrates the evolution of a selenoprotein gene into a cysteine encoding isogene by making the SECIS susceptible to mutation after the selenocysteine codon had changed into a cysteine codon [102].

As SelB is a key component of the bacterial selenoprotein synthesis machinery (see above), it was assumed that the situation would be the same in Archaea. Indeed, inspection of the genome sequence of *M. jannaschii* [103] revealed a plausible archaeal SelB homolog (aSelB) and purified aSelB binds guanosine nucleotides and aminoacyl-tRNA^{sec} as expected [85]. Demonstrating that a *M. maripaludis* strain lacking SelB could not produce selenoproteins anymore proved its in vivo role [82]. The C-terminal extension of aSelB is rather short and completely unrelated to that of bacterial SelB, where it is responsible for SECIS binding (see above). For aSelB from *M. jannaschii,* no binding of the archaeal SECIS element in vitro could be demonstrated [85], but the crystal structure of aSelB from *M. maripaludis* gave rise to speculations that it may bind the SECIS element [101, 104]. However, all our subsequent efforts to demonstrate direct aSelB-SECIS interaction in the *M. maripaludis* system were so far unsuccessful (C Sattler, S Goetz, M Rother, unpublished data). These observations and the fact that the situation is similar in the eukaryal system (see Chaps. 2 and 3 of this book) suggest that in Archaea communication between the SECIS element in the UTR effecting recoding and the site of that recoding (the ribosome at the UGA) is established by one (or more) as of yet unknown factor(s). Identifying the full complement of *trans*-acting factors required for selenocysteine insertion is a prerequisite for a full appreciation of the evolutionary events, which have led to the three principally similar but still unique strategies for selenocysteine utilization found in the three domains of life.

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Chapter 37
Selenoproteins in Parasites

Gustavo Salinas, Mariana Bonilla, Lucía Otero, Alexey V. Lobanov, and Vadim N. Gladyshev

Abstract Parasites are organisms that live, at least part of their lifecycle, inside another organism (the host), which they exploit for their own benefit. Parasite infections are highly prevalent in vast tropical regions and cause a wide variety of human-neglected diseases. Significant advances have been made in the identification and characterization of selenoproteins of both major groups of human parasites: protozoan (unicellular) and helminth (worm) parasites. Some selenoprotein mRNAs had a highly efficient noncanonical form of eukaryotic SECIS element. A major finding has been the identification of the selenoprotein thioredoxin glutathione reductase (TGR) as the single redox wire for electron transfer to both thioredoxin and glutathione pathways in flatworm parasites (phylum Platyhelminthes). Further studies validated TGR as a novel drug target and identified drug leads that show great promise for treatment of flatworm infections by disrupting parasite redox homeostasis. Interestingly, lineage-specific selenoprotein families are present in medically important protozoan parasites, but are absent in their hosts.

37.1 Introduction

The control of parasitic infections, a major cause of disability and mortality in many developing countries, remains as one of the most important challenges for medicine in the twenty-first century [1]. Yet, the tools to control these infections are very

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limited: there is no single vaccine available for a human parasite, and the pharmacological arsenal for many of them consists of just a single drug, for which there is justified concern of drug resistance emergence [2, 3]. The redox homeostasis of parasites is precarious due to the oxidative stress imposed by the host, and in the case of flatworm parasites, it is absolutely dependent on selenium. This has led to successful targeting of thioredoxin glutathione reductase (TGR) in these parasites. The possibility of targeting plasmodia selenoproteins is also interesting given the unique selenoproteome found in these organisms, but further studies are needed to functionally characterize their selenoproteins.

37.2 Parasites: Diverse Organisms with Precarious Redox Homeostasis

Despite the broad definition of parasites, in medicine (and in this chapter), the term is restricted to protozoan and helminth parasites. Both protozoa and helminths are polyphyletic groups and also include free-living organisms [4]. Protozoan parasites are protists, which multiply quickly within the host, and are, in most cases, intracellular in habitat. In contrast, helminths are metazoan with complex organization, which undergo complex metamorphoses and migrations within the host. Protozoan parasites include diverse phyla, among them Apicomplexa (characterized by the presence of a distinctly polarized cell apex containing unique organelles) and Kinetoplastida (characterized by the presence of a kinetoplast, a granule containing many copies of the mitochondrial genome, within the single mitochondrion associated with the base of the cell's flagella) are the ones that cause the most devastating human diseases. Apicomplexa includes *Plasmodium* spp., the causative agents of malaria. Kinetoplastida includes the Trypanosomatid species, which cause sleeping sickness, Chagas disease, and Leishmaniasis. There are two major phyla of helminths. The nematodes (also known as roundworms) include the major intestinal worms (or soil-transmitted nematodes) and the tissue-dwelling filarial worms that cause lymphatic filariasis and onchocerciasis. The platyhelminths (also known as flatworms) include the flukes (also known as trematodes), such as the schistosomes (causative agents of schistosomiasis) and the tapeworms (also known as cestodes), such as *Taenia solium*, that cause cysticercosis.

A biological problem faced by all parasites, due to their lifestyle, is the oxidative stress to which they are exposed. The host mounts an inflammatory response that includes a repertoire of powerful oxidants that can inactivate enzymes and initiate the process of lipid peroxidation, leading to radical chain reactions that further damage membranes, nucleic acids, and proteins [5]. This response may lead to parasite death if it is not controlled by the parasite. Although parasites cope with the oxidative stress imposed by the host's immune response by a series of cellular chemicals and antioxidant enzymes that neutralize or detoxify oxidant species, redox metabolism of parasites is precarious and particularly susceptible to destabilization. Indeed, thiol- and selenol-based redox metabolism is currently a key pharmacological target for both protozoan and helminth parasites [6, 7].

37.3 Linked Thioredoxin-Glutathione System in Flatworms: A Simplified Pathway Entirely Dependent on the Selenoprotein Thioredoxin Glutathione Reductase

In most living organisms, glutathione (GSH) and thioredoxin (Trx) systems are two parallel supporting enzymatic systems that control cellular redox homeostasis, providing reducing equivalents to essential enzymes and participating in a variety of cellular processes [8, 9] (Fig. 37.1, see also Chaps. 12 and 13). The tripeptide GSH constitutes the major nonprotein thiol-based redox buffer of the cell. GSH acts as a general antioxidant molecule within the cell and provides electrons to glutathione peroxidases (GPxs, see below) and also recycles glutaredoxins (Grxs) to their reduced state. In addition, GSH serves a detoxifying role: hydrophobic electrophiles can be ligated to GSH, in reactions catalyzed by glutathione S-transferases, rendering them less reactive and easily excretable. Grxs are small thiol-disulfide oxidoreductases that belong to the Trx superfamily, have a similar redox active site, and transfer electrons to their substrates and substrate reductases such as ribonucleotide reductase, an essential catalyst for the synthesis of deoxynucleotides [10]. Grxs also catalyze deglutathionylation of protein-GSH mixed disulfides that can be formed under oxidative stress. There are also emerging functions for Grxs, such as their participation in Fe/S biogenesis and mobilization in mitochondria [11] and signal transduction through deglutathionylation. Trx, in turn, is a powerful protein disulfide reductase [12]. The conserved redox active site of Trx provides reducing equivalents to enzymes containing disulfides as part of their catalytic cycle. Targets of Trxs include ribonucleotide reductase and antioxidant enzymes such as peroxiredoxins (Prxs) and repair enzymes such as methionine-S-sulfoxide and methionine-R-sulfoxide

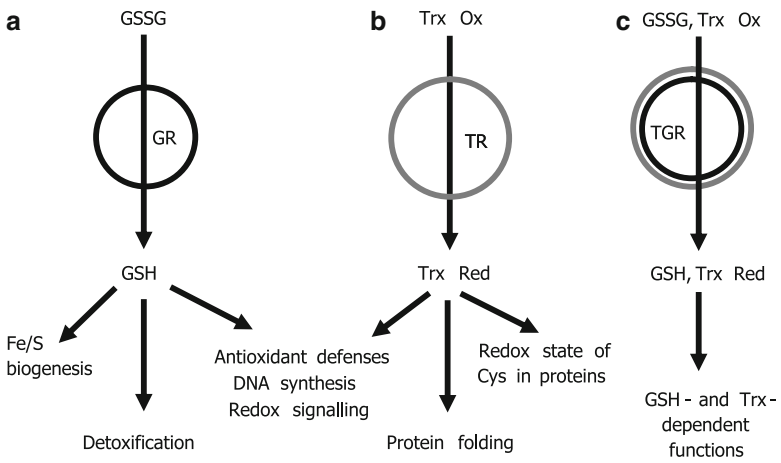

Fig. 37.1 TGR function includes combined functions of GR and TR. GR (**a**), TR (**b**), and TGR (**c**) activities are illustrated. Trx and GSH systems have both overlapping and distinct functions, and all of them can be supported by TGR alone. Flatworm parasites possess a single Sec-containing TGR; in contrast, their mammalian hosts possess GR, TRs, and TGR

reductases (Msrs, see below). Trxs are also directly involved in blocking oxidative stress as generic protein disulfide reductases and as backup reductases for GSSG and hydrogen peroxide. In addition, Trxs exert redox control of regulatory proteins involved in signal transduction and gene transcription [13].

GSH and Trx are usually reduced by pyridine-nucleotide thiol-disulfide oxidoreductases GSH reductase (GR) and Trx reductase (TR), respectively, at the expense of NADPH oxidation. Biochemical studies with parasitic flatworms have established that these organisms lack "conventional" GR and TR, in contrast to their hosts. Instead, they rely exclusively on a linked Trx-GSH system in which the selenoenzyme TGR is the single pyridine-nucleotide thiol-disulfide oxidoreductase that provides reducing equivalents to both pathways [14–17] (Fig. 37.1). TGR achieves its versatility by a fusion of conventional TR domains to an N-terminal Grx domain. TGR, like GR and TR, is a homodimer, with monomers oriented in a head-to-tail manner [18]. Biochemical and X-ray crystallographic data indicate that electrons in TGR flow from NADPH to FAD, to the CX_4C redox center, then to the C-terminal GCUG redox center of the second subunit, and finally to the CX_2C redox center of the Grx domain of the first subunit. The fully reduced enzyme can reduce either oxidized Trx using the C-terminal active site GCUG or GSSG through the CX_2C redox center of the Grx domain [19, 20]. By combining crystallographic data with computer modeling, it has been recently proposed that selenocysteine (Sec) is located on a flexible C-terminal arm that exhibits conformational changes after the transfer of reducing equivalents from NADPH to the redox active sites of the enzyme [19]. Furthermore, it has been proposed that the external Trx domain and the internal Grx domain do not compete for the same binding site; rather the C-terminal arm shuttles electrons to either of the two alternative positions. In addition to the GR and TR activities, it has been recently demonstrated that TGR also possesses glutathione-independent deglutathionylase activity [21]. Unlike conventional deglutathionylation by Grxs, the glutathionylated enzyme intermediate is resolved by Sec and not by GSH. This intramolecular electron pathway of TGR would assure deglutathionylation of target proteins under a broad range of conditions (e.g., at a low GSH concentration or under oxidative stress).

In silico, molecular and cellular studies have provided additional insights into the unique redox array of parasitic flatworms. In addition to the biochemical data from different parasitic flatworms, definitive support for the existence of a single multifunctional enzyme in these organisms arises from the analysis of trematode (*Schistosoma* spp.) and cestode (*Echinococcus multilocularis*) genomes, which did not identify genes encoding conventional GR and TR, but a single TGR gene [17, 22]. Data from transcriptomic surveys from *Fasciola hepatica* (another trematode) and *T. solium* (another cestode) are also in agreement with this unique biochemical scenario present in parasitic flatworms. It is interesting to note that the genome of *Schmidtea mediterranea* (class Turbellaria), a free-living flatworm, encodes TGR and conventional GR and TR, suggesting that these genes were lost in the parasitic lineage that includes the Trematoda and Cestoda classes [17]. Finally, the existence of TGR variants derived from a single gene [15] and the occurrence of functional TGR-dependent linked systems in both cytosol and mitochondria have been demonstrated in parasitic flatworms [20].

37.4 High-Throughput Screening of TGR Inhibitors Led to the Identification of New Drug Hits for Flatworm Infections

The existence of a single TGR in parasitic flatworm contrasts the situation of their mammalian hosts, where in addition to GR, two TR isozymes, encoded by different genes, function in the cytosol and mitochondria, and TGR is also present, being expressed mainly in testis [23]. The dissimilar arrangements of redox pathways as compared to their hosts, the lack of backup systems, and the fact that parasitic organisms are subjected to the oxidative challenge imposed by the host's immune system provide a strong rationale to disrupt flatworm homeostasis by targeting platyhelminth TGRs. In this context, it is important to mention that these organisms lack catalase and rely exclusively on GPx and Prx for hydrogen peroxide removal, and both of these enzymes fully depend on TGR. Recent studies strongly support this concept: inhibition of TGR expression by RNAi caused death of *Schistosoma mansoni*. Furthermore, auranofin, a potent inhibitor of Sec-containing TGR and TRs, kills *S. mansoni* worms, *Taenia crassiceps* metacestode, and larval worms of *Echinococcus granulosus* [20, 24, 25]. Moreover, administration of auranofin to *S. mansoni*-infected mice caused a partial cure in experimental infection [24]. TGR also fulfills all other requirements as a drug target: it is expressed constitutively, there is a low cost and simple biochemical assay to test its activity, and it is a "druggable enzyme": the Sec residue contains a highly reactive side chain, susceptible for targeting by electrophiles.

Validation of TGR as a drug target prompted a quantitative high-throughput screen for TGR inhibitors using a chemical library from the NIH consisting of ~70,000 compounds. As a result, several compounds showed IC50 values in the micromolar to nanomolar range. Most active series include oxadiazoles 2-oxides and phosphinic amides [7] (Fig. 37.2).

Subsequent studies demonstrated that one of them, 4-phenyl-1,2,5-oxadiazole-3-carbonitrile-2-oxide, was effective against all *S. mansoni* developmental stages and showed low toxicity against mammalian cell lines. Furthermore, when administered to mice, the compound was well tolerated and the lethal effect on parasites was above 90% [26]. The anti-schistosome activity was associated with nitric oxide donation through the action of the target enzyme, and it was suggested that inhibition of TGR occurs through the modification of catalytically active Cys/Sec residue(s). Preliminary results indicate that oxadiazol N-oxides are also effective against *F. hepatica* (another trematode) and the cestode *E. granulosus* (our unpublished observations), providing additional evidence that TGR is a drug target for platyhelminth parasites of both classes.

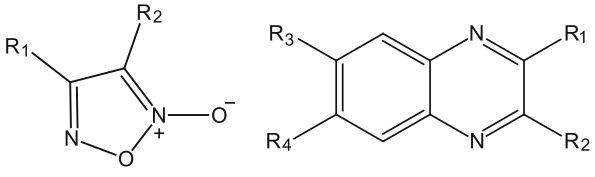

Fig. 37.2 Structure of the TGR inhibitors identified. *Left*: generic oxadiazol 2-oxide, *right*: generic phosphinic amide [26]

37.5 The TGR Redox Network in Flatworm Parasites Includes Additional Selenoenzymes

As already mentioned, TGR is the only redox wire in flatworm parasites, and its Sec residue is its "redox hub." Additional selenoenzymes are part of the TGR-dependent redox network. The first one to be characterized in parasites was a *S. mansoni* GPx [27]. This enzyme has biochemical properties similar to those of mammalian phospholipid hydroperoxide GPx (GPx4); its activity being highest with phosphatidyl choline hydroperoxide [28]. GPx and superoxide dismutase, another antioxidant enzyme, colocalize in the tegument and gut epithelium of adult worms, which are the exposed interfaces of the parasite towards the host [29]. Its expression is developmentally regulated, with the highest levels present in the adult worm [29], the stage most resistant to oxidative stress and immune elimination [30]. In addition, GPx expression is upregulated by hydrogen peroxide and xanthine/xanthine oxidase-generated ROS [31]. A second GPx (GPx2) has been identified in Schistosoma genomes. GPx2 also encodes a Sec residue at the active site and possesses an N-terminal signal peptide, which targets this isoform to the extracellular compartment, suggesting that this secreted variant would be important for extracellular hydroperoxide removal, helping to protect the parasite. Only one gene encoding a GPx is present in *Echinococcus* genome. This gene encodes a Sec-containing enzyme and is represented by a highly expressed transcript.

Another selenoprotein encoded by flatworm parasites genomes is selenoprotein W (SelW). The members of this class of selenoproteins have been shown to be glutathione-dependent antioxidant proteins in vivo, but their precise function is not known. Although functional studies have not been reported for flatworm SelW, the transcriptomic surveys revealed that this gene is highly expressed. In mammals, other selenoenzymes, such as methionine sulfoxide reductases (Msrs, see Chap. 38), function as antioxidant repair enzymes. Methionine is an amino acid particularly prone to oxidation, affecting protein function. Oxidation produces a diastereomeric mixture of S and R forms of methionine sulfoxide (Met-SO), which can be reduced back to methionine by two stereospecific Msrs: MsrA and MsrB, respectively. In mammals, there is one MsrA gene, and three MsrB genes, one of them encoding a Sec-containing enzyme. In contrast to their mammalian hosts, no Sec-containing Msrs are present in Schistosoma and Echinococcus genomes. Even though the entire selenoproteome of flatworm parasites has not been reported, two other selenoproteins, SelT and Sep15, have been identified in these organisms in addition to TGR, GPx, and selW (G Salinas and VN Gladyshev, unpublished).

37.6 Selenoproteins of Nematode Parasites

No experimental studies have yet been performed with selenoproteins from parasitic nematodes. The only information available is based on data mining of selenoprotein genes using SECISearch and by screening for homologs of known

selenoproteins in the nematode ESTs [32]. These analyses identified selenoprotein homologs of selK, selT, selW, Sep15, selenophosphate synthetase, and GPx. Two interesting points were noted from these analyses. First, various nematodes encode different selenoproteins, and the distribution of selenoprotein families within this phylum is a mosaic. Second, it was found that all detected nematode selenoprotein genes contained an unusual form of SECIS element, with G rather than a canonical A at the conserved position preceding the quartet of non-Watson-Crick base pairs [32].

37.7 Selenoproteomes of Protozoan Parasites Revealed the Presence of Selenoenzymes Absent in Their Hosts

The selenoproteomes of the main protozoan parasites have been determined in recent years. Yet, the field remains largely unexplored with regard to the function of identified selenoproteins. Within the phylum Apicomplexa that includes *Plasmodium falciparum*, the causative agent of malaria, the Sec-incorporation trait is present in the six plasmodial genomes and in *Toxoplasma gondii* and absent in *Cryptosporidium* (these two latter organisms are opportunistic human parasites) [33]. The Sec-incorporation trait is also absent in *Babesia* and *Theileria* spp, apicomplexa species that only infect agricultural animals, but have profound indirect effects on human welfare. Therefore, some apicomplexa lineages have lost the capacity to incorporate Sec. Particularly interesting is the fact that the genome-wide searches for SECIS elements in the six *Plasmodium* genomes revealed the evolution of several lineage-specific selenoproteins. The four selenoprotein genes encoded by Plasmodium spp. have no homologs outside Apicomplexa [33]. Although the function of these selenoproteins remains unexplored, the absence of these selenoproteins from the host may be relevant, in particular if they are implicated in redox homeostasis. This can make these selenoproteins attractive targets for anti-malaria drug development. The search for selenoprotein genes in *T. gondii* identified five selenoprotein genes, four homologs of mammalian selenoproteins (SelW, SelK, SelS, and SelT) and a previously undescribed selenoprotein, SelQ [34]. In addition, this study identified a functional, noncanonical form of eukaryotic SECIS element: the SECIS quartet of SelT had a GGGA sequence instead of AUGA. The U in the AUGA sequence was considered invariant and present in all previously known eukaryotic SECIS elements. This SECIS, detected only in two aplicomplexan parasite selenoproteins (*Toxoplasma* and *Neospora caninum*), supports Sec insertion in mammals. Because a high level of expression was obtained in mammalian cell lines with this form of SECIS element, its sequence was used to develop a tool for efficient expression of recombinant selenoproteins in mammalian cells: pSelExpress1, a vector that contains an SBP2 gene, and the most efficient tested SECIS element: an AUGA mutant of the GGGA-type Toxoplasma SelT structure.

The kinetoplastids (members of the phylum Euglenozoa) represent the other major group of protozoa that includes parasites responsible for serious diseases in

humans (Chagas disease, sleeping sickness, and leishmaniasis). The analysis of Trypanosoma and Leishmania genomes revealed the presence of the Sec-decoding trait and three selenoproteins [35]. The selenoproteins include distant homologs of mammalian SelK and SelT, and a novel multidomain selenoprotein, designated SelTryp. SelTryp has neither Sec- nor cysteine-containing homologs in the human host and appears to be a Kinetoplastida-specific protein. In all three selenoproteins, Sec is predicted within redox motifs. Subsequent studies indicated that Sec incorporation is dispensable in vitro. One of these studies was instrumental in providing experimental evidence for Sec-tRNA$^{[Ser]Sec}$ formation in vivo [36]. *Trypanosoma brucei* null mutants of either *O*-phosphoseryl-transfer RNA$^{[Ser]Sec}$ kinase (PSTK) or Sec synthase (SecS, also known as SepSecS) abolished selenoprotein synthesis, demonstrating the essentiality of both enzymes for Sec-tRNA$^{[Ser]Sec}$ formation and the requirement of Sep-tRNA(Sec) as an intermediate. At the same time, this study revealed that the selenoproteins are not required for viability under laboratory conditions: growth of the two knockout strains was not impaired. Further studies in cell culture showed normal growth of procyclic and bloodstream *T. brucei* SecS null mutant and that *T. brucei* sensitivity to auranofin, a compound known to target selenoproteins, is not due to selenoprotein targeting, since the same sensitivity as in wild-type *T. brucei* was observed in the SecS knockout cell line. These authors also showed that the absence of selenoproteins did not increase sensitivity to H_2O_2-induced oxidative stress [37]. No studies have been carried out with this mutant strain in experimental infections in mice. The existence of lineage-specific selenoproteins in different protozoan parasites suggests that unicellular protists contain undiscovered and orphan-function eukaryotic selenoproteins.

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Chapter 38
Selenium and Methionine Sulfoxide Reduction

Hwa-Young Kim and Vadim N. Gladyshev

Abstract Methionine sulfoxide reductases (Msrs) are repair enzymes that catalyze the reduction of methionine sulfoxide in both free and protein-based forms back to methionine in a stereospecific manner. Methionine sulfoxide reduction is an important pathway that occurs in most organisms, protects cells against oxidative stress and regulates protein function. This pathway is also implicated in delaying the aging process and progression of neurodegenerative diseases. Selenoprotein Msr forms are found in bacteria, unicellular eukaryotes, and animals, and contain selenocysteine, an essential catalytic residue. Selenoprotein MsrB1 is the main Msr in the liver of mammals and its expression is easily regulated by dietary selenium. This chapter discusses the physiological roles of Msrs with the focus on mammalian MsrA and MsrBs and recent studies involving selenoprotein Msrs.

38.1 Introduction

Reactive oxygen species (ROS) are generated by designated enzymes such as NADPH oxidase and xanthine oxidase and may also be produced as by-products of a respiration process in aerobic organisms. Accumulated ROS may damage macromolecules, such as proteins, lipids, and nucleic acids, as well as smaller biomolecules. This oxidative damage has been implicated in the incidence of a variety of diseases and in accelerated aging. Virtually all amino acid residues in proteins can

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be targeted by ROS, especially at their side chains [1], and these modifications may be reversible or irreversible. Among the 20 common amino acid residues, the two sulfur-containing amino acids, methionine and cysteine, are the most susceptible to oxidation.

Methionine can be oxidized to a mixture of two diastereomers, methionine-*S*-sulfoxide and methionine-*R*-sulfoxide, because of the pro-chiral nature of its sulfur atom [2]. Oxidation of methionine may lead to a significant alteration of protein structure and function. In addition, methionine sulfoxide may be further targeted by ROS to generate methionine sulfone and propagate oxidative damage [3]. However, organisms have evolved an enzymatic reduction system to reverse methionine sulfoxide back to methionine [4–6]. Cyclic oxidation/reduction of methionine residues was suggested to serve as an important defense mechanism against oxidative stress [7]. Methionine sulfoxide reductases (Msrs) are protein repair enzymes responsible for methionine sulfoxide reduction; they are viewed as important antioxidants.

Selenium, an essential trace element in humans and other mammals, is co-translationally incorporated into proteins in the form of the 21st amino acid, selenocysteine (Sec) [8]. The Sec-containing proteins, selenoproteins, are found in all three domains of life. Twenty five selenoprotein genes have been identified in human and 24 in rodent genomes [9]. Of the selenoproteins with known functions, most are oxidoreductases, such as thioredoxin reductase [10], glutathione peroxidase [11], and formate dehydrogenase [12], in which Sec occupies the active sites. Selenoproteins typically exhibit 100–1,000 fold higher enzyme activities than their Cys mutants or natural Cys-containing forms [13, 14]. A key reason for Sec utilization in biological systems is thought to involve the high catalytic activity of Sec-containing enzymes.

About 10 years ago, application of bioinformatics tools for identification of selenoprotein genes by searching for SECIS (Sec insertion sequence) elements identified selenoprotein R (SelR) [15]; this protein was also independently identified by another group and named selenoprotein X (SelX) [16]. Comparative genomic analyses were then used to link the function of SelR to the pathway of methionine sulfoxide reduction, and further biochemical studies revealed that SelR had a specific methionine-*R*-sulfoxide reduction activity and contained a zinc atom [17]. This protein was later renamed as MsrB1.

38.2 Methionine Sulfoxide Reductases: Three Distinct Families

Msrs reduce free and protein-based methionine sulfoxides back to methionine. For the reduction of methionine sulfoxide residues in proteins, two stereospecific enzyme families have evolved. MsrA can only reduce the *S*-stereoisomer of methionine sulfoxide in both free and protein-based forms. The other family, MsrB, is specific for the *R*-form of methionine sulfoxide (Fig. 38.1). However, MsrB efficiently acts only on the protein-based methionine sulfoxide, whereas it has a very low activity with the free form of methionine sulfoxide.

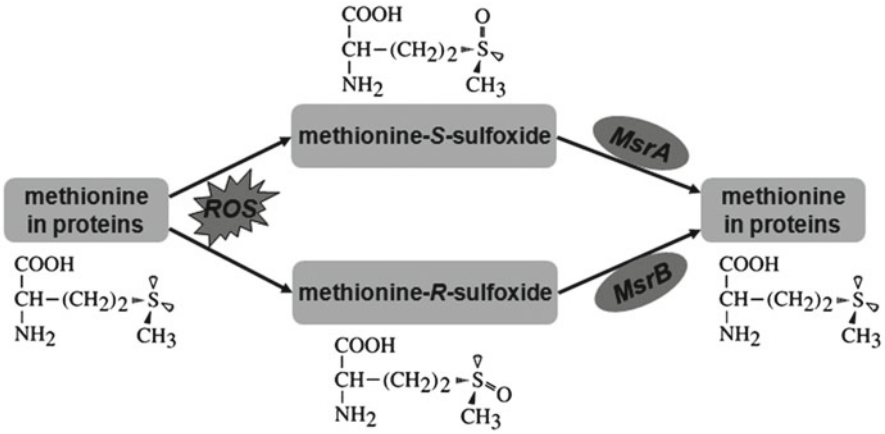

Fig. 38.1 A pathway of methionine sulfoxide reduction in proteins. Methionine residues can be readily oxidized by ROS to a mixture of diastereomers of methionine sulfoxide. MsrA stereospecifically reduces the *S*-form of methionine sulfoxide, while MsrB is specific for the *R*-form. Reproduced with permission from Kim and Gladyshev (see details in [4])

Msrs are found in most organisms from bacteria to humans, even in anaerobic organisms. However, some hyperthermophiles and intracellular parasites do not have MsrA, MsrB, or both proteins [17, 18]. MsrA and MsrB typically exist as separate enzymes. However, in some bacteria such as *Streptococcus pneumonia*, *Neisseria gonorrhoeae*, and *Helicobacter pylori*, MsrA and MsrB are directly fused to form a single polypeptide (MsrAB). The number of MsrA and MsrB genes in different organisms is variable. Single MsrA and MsrB genes were found in organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, and many animals (e.g., *Caenorhabditis elegans* and *Drosophila melanogaster*) [17, 18]. On the other hand, plant genomes, such as *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*, contain multiple MsrA and MsrB genes. Also, multiple MsrA and/or MsrB genes may be present in bacteria.

MsrA was discovered three decades ago as an enzyme that could restore the biological activity of a ribosomal protein L12 [19]. The *Escherichia coli* and bovine MsrA genes were cloned [20, 21] in the 1990s and the corresponding proteins were found to stereospecifically reduce methionine-*S*-sulfoxide [22]. On the other hand, MsrB was identified much later in the early 2000s [23]. It was first found that an *E. coli* YeaA (MsrB ortholog), together with MsrA, could fully reduce methionine sulfoxides in proteins [23]. Further studies found that mammalian and *Drosophila* MsrBs are zinc-containing proteins specific for methionine-*R*-sulfoxide [17, 24].

Recently, a new type of Msr from *E. coli*, designated as fRMsr, was discovered [25]. This enzyme catalyzes the reduction of the free form of methionine-*R*-sulfoxide, but is not active with the protein-based form. Interestingly, fRMsr contains a GAF-domain, which is present in cyclic GMP phosphodiesterases, but does not bind cGMP [26]. fRMsr only occurs in unicellular organisms including *S. cerevisiae*, and is absent in multicellular organisms [27].

38.3 Mammalian Msrs

Human and mouse genomes contain a single MsrA gene (Fig. 38.2). The most abundant MsrA form generated from this gene has a typical N-terminal mitochondrial targeting sequence, but interestingly, rat and mouse MsrA proteins were found to localize to both cytosol and mitochondria [28, 29]. SDS-PAGE analysis showed that the cytosolic MsrA form migrated faster than the mitochondrial form. It was found that structural and functional elements in mouse MsrA play a role in cellular distribution of the protein [29]. Apparently, unfolded precursor or misfolded MsrA forms are targeted to mitochondria, whereas robust folding of MsrA retains a significant portion of MsrA in the cytosol. In addition, a recent study revealed that the cytosolic MsrA was myristoylated and generated by alternative translational initiation at Met21 [30]. This study suggested that the dual sites of protein initiation control the cellular location of MsrA. An additional MsrA form, which is generated by alternative first exon splicing, was also identified [31]. This MsrA isoform is enzymatically active and resides in the cytosol and nucleus, but has low abundance.

In contrast to a single MsrA gene, there are three MsrB genes in mammals [14]. As discussed above, MsrB1 was the first selenoprotein identified computationally [15, 16]. This selenoenzyme contains Sec as the catalytic residue and localizes to cytosol and nucleus. In two other MsrBs, catalytic Cys are present in place of Sec. The second mammalian MsrB was first reported as CBS-1 with high similarity to bacterial PilB [32] and was later renamed as MsrB2. This enzyme contains an

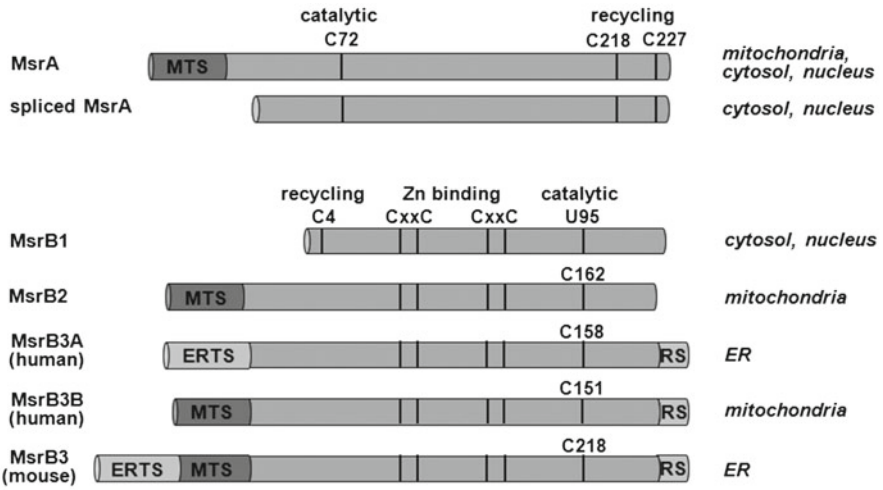

Fig. 38.2 Mammalian Msrs: one MsrA and three MsrB genes. *MTS* mitochondrial targeting signal; *ERTS* ER targeting signal; *RS* ER retention signal consisting of a tetrapeptide; and *U* Sec. Reproduced with permission from Kim and Gladyshev (see details in [4])

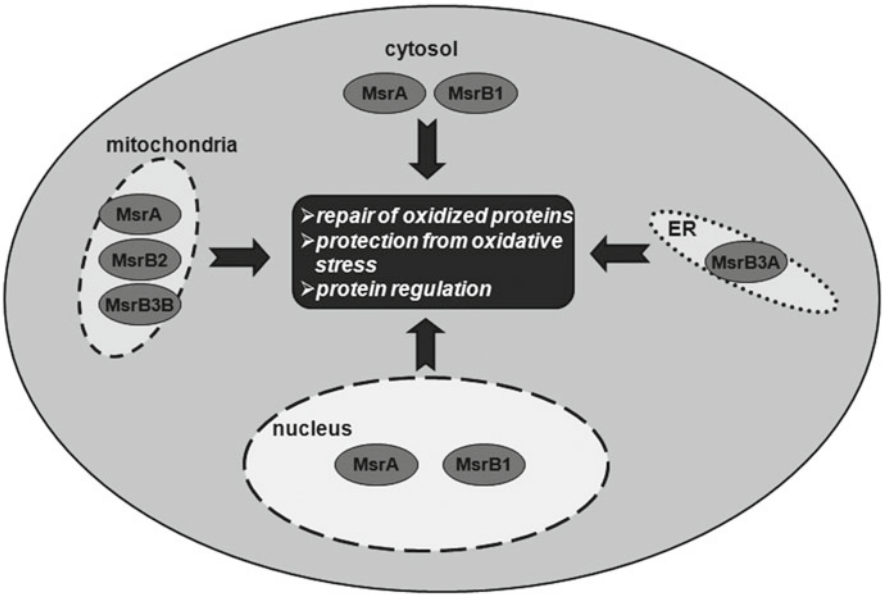

Fig. 38.3 Methionine sulfoxide reduction system in mammals. Different cellular compartmentalization of MsrA and MsrBs maintains the methionine sulfoxide reduction system in mammals for repairing oxidatively damaged proteins, protecting cells from oxidative stress, and regulating protein function. Reproduced with permission from Kim and Gladyshev (see details in [4])

N-terminal mitochondrial signal peptide that targets the protein to mitochondria [14]. The third MsrB, MsrB3, gives rise to two forms, MsrB3A and MsrB3B, in humans by alternative first exon splicing [14]. MsrB3A contains an N-terminal endoplasmic reticulum (ER) signal peptide and a C-terminal ER retention signal (KAEL tetrapeptide), and is targeted to the ER. The other alternatively spliced form, MsrB3B, contains a mitochondrial signal peptide at the N-terminus and is targeted to mitochondria. However, no evidence for alternative splicing of MsrB3 was found in mouse [33]. Instead, mouse MsrB3 contains consecutive ER and mitochondrial targeting signals at the N-terminus. The function of the mitochondrial signal appears to be masked by the ER signal peptide and consequently this protein is targeted to the ER.

The findings of multiple cellular locations of both MsrA and MsrBs suggest that the methionine sulfoxide reduction systems are maintained in different compartments in mammalian cells for repair of oxidatively damaged proteins, regulation of protein function, and protection against oxidative stress (Fig. 38.3). Further studies are needed to address the physiological roles of each protein form with regard to its location and to identify its substrates.

38.4 Physiological Roles of Msrs

Reversible interconversion between methionine and methionine sulfoxide residues has been implicated in various biological and pathological processes, including oxidative stress, cellular signaling, aging, and neurodegenerative diseases [4, 5]. First, MsrA and MsrB repair oxidatively damaged proteins. Second, Msrs can regulate protein function by reducing specific methionine sulfoxide residues involved in activation or inactivation of proteins. Finally, Msrs function as antioxidant enzymes. Some surface-exposed methionine residues can be oxidized without any impact on protein function. Thus, it was proposed that such methionine residues, in combination with Msrs, function as antioxidants by scavenging ROS [7]. This cyclic methionine oxidation/reduction is thought to be an important antioxidant mechanism.

In experiments involving gene overexpression or deletion, MsrA was found to protect cells against oxidative stress in microorganisms such as *S. cerevisiae* and *N. gonorrhoeae* [34, 35]. MsrA was also found to play an important role in viability of mammalian cells including lens and retinal pigmented cells by conferring resistance to oxidative stress [36, 37]. In addition, MsrA was suggested to play a photoprotective role in skin cells against ultraviolet irradiation [38]. The antioxidant role of MsrBs has also been characterized. The siRNA-mediated silencing of each of MsrB genes in human lens cells led to the increased oxidative stress-induced cell death [39]. On the other hand, overexpression of MsrB2 protected leukemia and retinal pigmented cells against oxidative stress-induced cell death [40], while heterologous expression of MsrB2 or MsrB3 in yeast protected cells against oxidative stress [41]. Recently, fRMsr was also found to function as an antioxidant in yeast [27].

38.5 Target Proteins for Msrs

Oxidation of methionine residues can affect biological activities of proteins whereas reduction of methionine sulfoxides back to methionines can restore these activities. Previously identified proteins that serve as substrates for MsrA include ribosomal protein L12, α-1-proteinase inhibitor, calmodulin, Fft (a prokaryotic signal recognition particle component), HIV-2 protease, and *shaker* potassium channel, whose functions are impaired by oxidation of methionines and restored by MsrA [4]. Recently, it was shown that methionine oxidation can activate calcium/calmodulin-dependent protein kinase II (CaMKII) in the absence of calcium and MsrA can reverse the activation caused by oxidation [42]. In addition, MsrB1 can recover the TRPM6 magnesium channel activity by reducing an oxidized methionine residue [43].

It is expected that there are numerous additional Msr targets in cells. However, methodological difficulties precluded identification of these proteins on a large scale. Recently, antibodies against methionine sulfoxides have been developed [44, 45]. Although the currently available anti-methionine sulfoxide antibodies seem to have a rather narrow specificity, further development of these reagents may lead to the identification of additional Msr target proteins.

38.6 Methionine Sulfoxide Reduction, Aging and Neurodegenerative Disorders

Oxidatively damaged proteins produced by ROS may accumulate with age and the accumulation of damaged proteins is considered as one of the major causes of aging. It may be easily inferred that Msrs are directly implicated in the regulation of the aging process. Total Msr activity was reduced in aged rat kidney and liver [46], and decreased expression levels of MsrA and MsrB2 were observed in senescent WI-38 fibroblasts [47]. Indeed, overexpression of bovine MsrA in *Drosophila* increased lifespan by 70% [48]. Recently, it was found that overexpression of *Drosophila* MsrA also extended fruit fly lifespan [49]. However, MsrA knockout in mice did not alter lifespan, although it led to an increased sensitivity to paraquat-induced oxidative stress [50]. Overexpression of MsrB in yeast extended lifespan under caloric restriction conditions, but not in a regular medium, whereas MsrA overexpression increased yeast lifespan under either condition [51]. A recent study showed that overexpression of either *Drosophila* MsrB or mouse MsrB2 had no effects on the lifespan of fruit flies, suggesting that MsrA and MsrB, the two proteins with opposing stereoselectivity for methionine sulfoxide reduction, have different effects on aging in fruit flies [52]. To understand the precise role of MsrB in the aging process in mammals, additional studies are needed involving knockout and transgenic models. In addition, studies with the combined MsrA and MsrB deficiency and overexpression in animals should be informative for understanding the precise role of methionine sulfoxide reduction in aging.

In addition to the roles of Msrs in aging, a growing evidence implicated Msrs in regulation of neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases. For example, decreased MsrA activity was observed in the brains of Alzheimer's disease patients [53]. Deposition of β-amyloid caused the neurotoxicity of Alzheimer's disease, and oxidation of Met35 was crucial for aggregation and neurotoxicity of this protein [54]. MsrA knockout mice showed an elevated neurodegeneration in hippocampus characterized by elevated levels of β-amyloid deposition and damaged astrocytes [55]. Similarly, aggregation of α-synuclein was found to be critical for pathogenesis of the Parkinson's disease and oxidation of methionine residues in this protein altered its fibrillation [56]. In addition, inhibition of α-synuclein degradation and elevated accumulation of fibrillated proteins were observed in MsrA null yeast strain [57]. Finally, MsrA knockout mice exhibited abnormal dopamine levels suggesting impaired dopamine regulation in MsrA-deficient mice [58].

38.7 Msrs as Selenoproteins

Of the selenoproteins characterized thus far, the majority are oxidoreductases in which Sec is used for catalytic function. Selenoprotein forms of MsrA and MsrB have been identified from bacteria to humans, but their distribution is different, suggesting independent origin of MsrA and MsrB selenoproteins [4, 5]. The

selenoprotein forms of MsrA have been found in bacteria, algae, and invertebrate animals, but not in vertebrates. In contrast, the selenoprotein forms of MsrB have only been described in animals including humans and some invertebrates. Interestingly, there is no evidence for the occurrence of selenoprotein forms of fRMsr.

To elucidate the role of Sec in catalysis, mammalian MsrB1 was engineered, in which a bacterial SECIS element was introduced immediately downstream of the UGA Sec codon by site-directed mutagenesis, and expressed in *E. coli* [14]. This recombinant selenoprotein had an 800-fold higher activity than the corresponding Cys form, indicating that Sec was essential for catalysis. A native selenoprotein MsrB1 form was also expressed in mammalian cells [59] and the purified selenoenzyme had a 100-fold higher activity than the Cys mutant form. Additionally, an engineered selenoprotein MsrB from *Clostridium* sp. (also known as *Alkaliphilus oremlandii*) OhILAs was found to have a 100-fold higher activity than the wild-type Cys-containing MsrB [60]. Higher catalytic activities were also observed in naturally occurring selenoprotein MsrAs from *Clostridium* and *Chlamydomonas* [61, 62]. These selenoenzyme MsrAs exhibited at least tenfold higher activities than their Cys mutant forms. Taken together, these studies demonstrated catalytic advantages provided by Sec in Msrs and likely other thiol-dependent oxidoreductases.

Multiple sequence alignments revealed different sets of active site features in selenoprotein and non-selenoprotein MsrBs [59]. There are three highly conserved residues (His77, Val/Ile81, and Asn97; numbering is based on the mouse MsrB1 sequence) in Cys-containing proteins. However, these three residues are absent in selenoprotein forms. Instead, the corresponding residues in selenoprotein MsrBs are Gly77, Glu81, and Phe97, respectively. It was found that the three conserved residues in Cys-containing MsrBs are critical for the enzyme activity in MrsB2 and MsrB3, but introducing these residues into selenoprotein MsrB1 was detrimental for the activity of this enzyme [59]. These data suggested that Sec- and Cys-containing MsrBs evolved distinct sets of active site features that maximize their catalytic efficiencies.

38.8 Regulation of Selenoprotein MsrB1

Mammalian MsrA and MsrBs are ubiquitous proteins but with distinct expression patterns [63, 64]. MsrA is highly expressed in detoxification organs, such as liver and kidney [63]. Selenoprotein MsrB1 is also highly expressed in these organs, and in fact, it is the main Msr in the liver of mice [64]. In the case of MsrB2 and MsrB3, their elevated expression levels were observed in heart and skeletal muscle [65, 66].

The expression of MsrB1 is highly regulated by dietary selenium in mice [64, 67]. Selenium deficiency led to a large reduction in MsrB1 expression and MsrB activity in mouse liver and other organs [64]. In addition, MsrB activity was decreased with age in mice and this decreased MsrB activity could be explained by the decrease in MsrB1 levels [64]. It was also reported that expression of selenoprotein MsrB1 may be regulated at the transcriptional level by the Sp1 transcription factor and epigenetic modifications (e.g., methylation) [68].

Recently, a knockout mouse deficient in selenoprotein MsrB1 was developed [69]. Its characterization revealed a significant contribution of MsrB1 to the redox regulation in liver and kidney, but MsrB1 was found to be a nonessential selenoprotein. Increased levels of lipid peroxidation, protein oxidation, and oxidized glutathione were observed in the MsrB1 knockout mice, and the levels of free and protein thiols were reduced. Additionally, it was found that a previously undescribed 5 kDa selenoprotein band specifically disappeared in the MsrB1 knockout mice. This 5 kDa selenoprotein was identified as the C-terminal portion of MsrB1 containing the Sec residue using ^{75}Se labeling and mass spectrometry analyses. Computational analysis of MsrB1 sequences also revealed a difference in gene structure between rodents and most other mammals, including humans [70]. The mouse MsrB1 gene consisted of five exons, whereas the corresponding human gene had four exons. An extra intron (intron 4) in mouse MsrB1 existed in the 3′-untranslational region immediately following the stop codon. Interestingly, further analyses showed that this sequence can be either intronized or non-intronized, thus yielding two spliced forms of MsrB1 in mice, which, however, give rise to identical open reading frames of the protein [70].

38.9 Catalytic Mechanisms of Msrs

The catalytic mechanisms of all Msrs are characterized by a common sulfenic acid chemistry and generally consist of three steps [4, 5]. A catalytic Cys (CysA) attacks the sulfur of methionine sulfoxide and forms a sulfenic acid intermediate, with concomitant release of the product, methionine. The catalytic Cys sulfenic acid then forms an intramolecular disulfide bond by interacting with a resolving Cys (CysB). Finally, the disulfide bond (CysA–CysB) is reduced by reductants, and consequently the enzyme becomes active again. Thioredoxin and glutaredoxin are generally considered the *in vivo* reductants for Msrs, whereas dithiothreitol is often used *in vitro*.

38.9.1 MsrA

Crystal structures of MsrAs from *E. coli*, *Bos taurus*, *Mycobacterium tuberculosis*, *Populus trichocarpa*, and *S. pneumoniae* have been described [4, 71]. The central feature of MsrA structure is an α/β plaits motif, which includes a conserved active site GCFWG sequence. MsrA proteins can be divided into three groups with regard to the involvement of the resolving Cys in the catalytic reaction. Group I MsrAs, such as the enzymes from *E. coli* and *B. taurus*, have two recycling Cys (CysB and an additional CysC) in the C-terminal region involved in the catalytic mechanism. CysC attacks CysB of the CysA–CysB disulfide bond leading to a new CysB–CysC disulfide, which is then reduced by reducing agents. Group II MsrA proteins, such as the enzyme from *M. tuberculosis*, include a single resolving Cys, in which only

CysB is involved in the recycling of the sulfenic acid intermediate. Notably, some MsrAs lack any resolving Cys residues and are classified into group III. For example, all selenoprotein MsrAs identified thus far do not have candidate resolving Cys [61]. In the case of group III MsrAs, the sulfenic acid (or selenenic acid) intermediate is likely directly reduced by reductants.

38.9.2 MsrB

Crystal structures of Cys-containing MsrBs from *N. gonorrhoeae*, *S. pneumoniae*, *Bacillus subtilis*, and *Xanthomonas campestris* have been reported [4, 71]. Interestingly, MsrB fold is completely different from that of MsrA, but the comparison of MsrB and MsrA structures revealed a mirror-like relationship of their active sites. Although MsrB and MsrA structures differ, their catalytic mechanisms are very similar [4, 5]. Approximately 60% of MsrBs contain a conserved resolving Cys in the middle of their sequences, whereas the remaining 40%, including all three mammalian MsrBs, do not have this conserved Cys. It was found that mammalian MsrB2 and MsrB3 do not require resolving Cys and thus the sulfenic acid intermediate could be directly reduced by thioredoxin [72]. Interestingly, in contrast to MsrB2 and MsrB3, selenoprotein MsrB1 uses an alternative Cys (Cys4) in the N-terminal region as the resolving residue [59] (Fig. 38.2). Cys4 is conserved in selenoprotein MsrB forms and required for regeneration of the selenoenzyme by thioredoxin. It was also reported that *X. campestris* MsrB reduces the sulfenic acid intermediate with an alternative resolving Cys residue located in the N-terminal region [73]. Many MsrBs, including all three mammalian enzymes, contain a zinc atom coordinated by two CxxC (X, any amino acid) motifs (see Fig. 38.2). Mutation of any zinc-coordinating Cys to Ser in *Drosophila* MsrB resulted in the complete loss of the metal and catalytic activity [24]. It was suggested that zinc is not directly involved in the catalytic function, and that this metal plays a structural function in the metalloprotein MsrBs [24].

The first structure of the mammalian selenoprotein MsrB1 determined by high resolution NMR spectroscopy has recently been reported [74]. The MsrB1 structure is characterized by an overall β-fold protein consisting of two antiparallel β-sheets, and by a highly flexible N-terminal region. This NMR study confirmed a catalytic mechanism involving catalytic Sec95 and resolving Cys4 residues and a structural role of zinc in the MsrB1 molecule, as previously suggested. The study also suggested that hydrophobic interactions between the substrate and the active site aromatic residues play an important role in the catalytic function of MsrB1.

38.9.3 fRMsr

Two crystal structures of fRMsrs from *E. coli* and *S. cerevisiae* have been reported [25, 26]. Initial studies were consistent with Cys101 functioning as a catalytic

residue, Cys125 as a primary resolving Cys, and Cys91 as a secondary resolving residue (numbering is based on the *S. cerevisiae* fRMsr) [25, 27], but several unusual features were reported such as the essentiality of every Cys for catalysis. Recently, *in vivo* growth complementation experiments, using *S. cerevisiae* cells lacking all three Msrs, showed that Cys125 is the catalytic residue in fRMsr [75]. Furthermore, structural and biochemical analyses of fRMsrs from *Staphylococcus aureus* and *Neisseria meningitidis* suggested a catalytic mechanism of fRMsr in which Cys125 functions as the catalytic residue and Cys91 as the resolving Cys that forms a disulfide bond with Cys125 [76, 77]. The structural studies also revealed that conformational changes occur in the active site during catalysis, particularly in the loop containing the catalytic Cys125, and provided insights into the enzyme–substrate interaction.

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Chapter 39
Inactivation of Glutathione Peroxidase 1 and Peroxiredoxin 2 by Peroxides in Red Blood Cells

Chun-Seok Cho and Sue Goo Rhee

Abstract Glutathione peroxidase 1 (GPx1), peroxiredoxin II (Prx II), and catalase are the principal enzymes responsible for peroxide elimination in red blood cells (RBCs). GPx1, which contains a selenocysteine (Sec) residue at its active site, is irreversibly inactivated by its own substrate as the result of the oxidation of selenium atom followed by the conversion of oxidized Sec to dehydroalanine (DHA). Prx II is inactivated when its catalytic cysteine (Cys) is hyperoxidized to cysteine sulfinic acid during catalysis. The hyperoxidation can be reversed by sulfiredoxin. The activity of sulfiredoxin in RBCs is sufficient to counteract the Prx II hyperoxidation that occurs during elimination of H_2O_2 molecules resulting from hemoglobin (Hb) autoxidation. We developed a blot method for detection of DHA-containing proteins, with the use of which we observed that the amount of DHA-containing GPx1 increases with aging of RBCs as well as in RBCs exposed to H_2O_2 generated either externally by glucose oxidase or internally as a result of aniline-induced Hb autoxidation. Given that the conversion of Sec to DHA is irreversible and that protein turnover mechanism is lacking in RBCs, the content of DHA–GPx1 in each RBC likely reflects total oxidative stress experienced by the cell during its lifetime. Therefore, DHA–GPx1 in RBCs might be a suitable surrogate marker for evaluation of oxidative stress in the body.

39.1 Introduction

When O_2 binds to the iron of deoxyhemoglobin, which is in the Fe^{2+} state in the red blood cells (RBCs), an electron is delocalized between the iron and the O_2 and the Fe-O bonding becomes intermediate in character between that in Fe^{2+} bonded to

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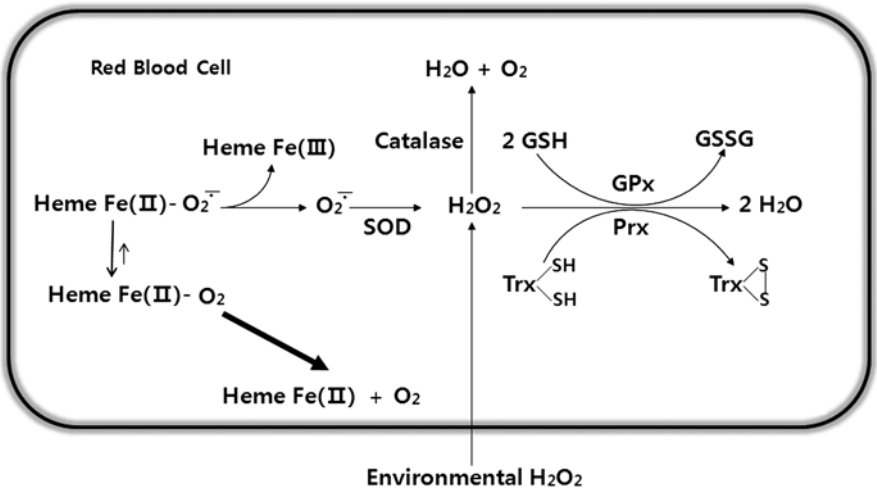

Fig. 39.1 Production of ROS by oxygenated Hb and their elimination of by antioxidants in RBCs. *SOD* superoxide dismutase; *GPx* glutathione peroxidase; and *Prx* peroxiredoxin

$O_2$and that in Fe^{3+} bonded to superoxide anion ($O_2^{\bullet}$) [1, 2]. Occasionally, a molecule of oxyhemoglobin releases $O_2^{\bullet}$ instead of O_2 (Fig. 39.1). The Fe^{3+}-containing product, methemoglobin cannot bind O_2 and should be converted to ferrous hemoglobin (Hb) by methemoglobin reductase to permit continuous O_2 transport. It has been reported that about ~4% of total Hb in human RBCs undergo the autoxidation-reactivation each day [3, 4]. Given that the concentration of oxygenated Hb is 5 mM and that RBCs make up 40% of the blood volume, even a small rate of autoxidation not only produces substantial levels of oxidative stress within RBCs but also causes damage to other components of the circulation. Oxygen transport by RBCs is thus a substantial contributor to oxidative stress. To cope with reactive oxygen species (ROS) produced during O_2 transport, RBCs are equipped with various antioxidant enzymes. The superoxide anion is dismutated (disproportionated) to H_2O_2 by Cu, Zn superoxide dismutase (CuZnSOD), which is abundant in RBCs (Fig. 39.1). No MnSOD is present, correlating with the absence of mitochondria in RBCs. Enzymes responsible for the elimination of H_2O_2 in RBCs are catalase, glutathione peroxidase 1 (GPx1), and peroxiredoxins (Prxs) [5–8].

The catalase reaction is essentially a dismutation; one H_2O_2 is reduced to H_2O and the other is oxidized to O_2. GPxs, many of which contain a selenocysteine (Sec) at the active site, catalyze the reduction of H_2O_2 and lipid peroxides by utilizing reduced glutathione (GSH) [9]. There are four distinct GPx enzymes in mammalian cells, and GPx1, the most abundant mammalian form, is the only type present in RBCs. Mammalian cells express six different Prx enzymes, with Prx I, II, and VI being found in human RBCs [8, 10]. Thus, human RBCs contain five peroxide-eliminating enzymes (catalase, GPx1, Prx I, Prx II, Prx VI). All Prx enzymes contain a conserved Cys residue, which is the site of oxidation by peroxides. Reduction of the oxidized Cys is mediated by thioredoxin (Trx).

The relative importance of peroxide-eliminating enzymes has been discussed for many years. The amounts of peroxide-eliminating enzymes decrease according to the rank order Prx II > GPx1 > catalase Prx I > Prx VI. When measured in lysates of RBCs from 17 healthy African-American subjects, the amounts of Prx II, GPx1, and catalase were 26.8 ± 7.7, 6.0 ± 2.0, and 4.4 ± 0.4 μg/mg of soluble protein, respectively [11]. Prx II is the most abundant antioxidant enzyme and the third most abundant protein in RBC cytosol after Hb and carbonic anhydrase. Prx I and Prx VI, with their amounts being only 1–2% that of Prx II, might be required for erythroid differentiation but may not be an important antioxidant in the differentiated RBCs [11]. The role of GPx1, catalase, and Prx II in RBCs has been studied using their respective knockout mice. RBCs of GPx1-deficient mice did not exhibit any signs of oxidative injury [6]. A difference between wild-type and GPx1-deficient RBCs was apparent only when catalase was depleted or when RBCs were exposed to high exogenous H_2O_2. In support of this notion, sporadic cases of GPx1 deficiency have been noted in humans without any clinical symptoms [12]. Similar to GPx1, neither catalase knockout mice nor humans with congenital acatalasemia exhibited signs of oxidant sensitivity [5, 13]. In contrast, Prx II knockout mice developed severe hemolytic anemia and splenomegaly as the result of increased destruction of abnormal RBCs [14]. Furthermore, RBCs from Prx II knockout mice contained Heinz bodies, which are composed of oxidatively damaged Hb. It is not clear, however, why Prx II knockout mice show severe phenotype, while deficiency of catalase or GPx1 caused no obvious defect associated with RBC function. It might indicate that Prx II is more important than the other two enzymes in eliminating peroxides. Alternatively, the protective effect of Prx II may not be attributable to its peroxidase function but to its chaperone function [15] or to its capacity to interact with stomatin, a monovalent cation transport [16].

39.2 Irreversible Inactivation of GPx1 Through Oxidative Conversion of Sec to Dehydroalanine

Because the Sec selenol has a considerably lower pK_a than cysteine thiol (5.2 of –SeH vs. ~8.5 of –SH), Sec exists in a fully ionized selenolate (–Se^-) state at physiological pH. The ionized selenolate or thiolate (–S^-) reacts faster with peroxides than their protonated forms. During catalysis, the selenolate of active site Sec (GPx–Se^-) reacts with H_2O_2 to yield selenenic acid (GPx–SeOH), which, in the presence of GSH, is rapidly converted to a glutathionylated intermediate (GPx–Se–S–G). This intermediate then reacts with another GSH molecule to produce GPx–Se^- plus oxidized glutathione (GSSG). GPx is susceptible to inactivation by its own substrates. Exposure of purified GPx1 to various hydroperoxides gradually results in its irreversible inactivation, whereas similar treatment has no effect on catalase [17, 18]. GPx1 also undergoes irreversible inactivation in the presence of nitric oxide as a result of the formation of a selenenyl sulfide (Se–S) linkage between Sec and Cys^{91}, which leads to its irreversible inactivation [19]. Selenium atom is often

removed from selenoprotein P and GPx1 during purification. It was suggested, therefore, that the selenium loss is likely through oxidation of selenolate to selenoxide followed by β-elimination of selenenic acid [20].

Among the antioxidant enzymes in RBCs, the activity of GPx1 was shown to be strongly influenced by lifestyle and environmental factors such as use of dietary supplements and smoking habit and proposed as a strong predictor of cardiovascular risk, which is associated with oxidative stress [21–23]. We therefore investigated if GPx1 is inactivated during 120-day life span of RBCs [8]. Given that the density of RBCs increases with RBC aging, human RBCs from healthy adult donors could be fractionated by centrifugation on a discontinuous density gradient of Percoll to obtain cells of four different mean ages [24]. The age-dependent separation was verified by decreasing activity of glucose-6-phosphate dehydrogenase (G6PDH), a marker of RBC aging [25]. The activity of GPx1 also decreased with aging (Fig. 1 in ref. [8]). To test whether the loss of GPx1 activity during aging was accompanied by loss of selenol, we subjected RBC lysates to alkylation at pH 6.5 with a biotinylated ethylenediamine iodoacetamide, *N*-(biotinoyl)-*N*-9′-(iodoacetyl)ethylenediamine (BIAM). GPx1 was then immunoprecipitated and subjected to blot analysis with HRP-conjugated streptavidin. Although human GPx1 contains five Cys residues in addition to the active site Sec, selenol is selectively alkylated at pH 6.5 because it exists in the ionized form ($–Se^-$), whereas thiols are in the protonated form (–SH) at this pH. The band intensity for BIAM-labeled GPx1 decreased with aging, suggesting that a substantial proportion of GPx1 molecules in aged RBCs do not contain selenol (Fig. 1 in ref. [8]). Experiments with a mutant GPx1, in which Sec 49 was changed to Cys, provided strong evidence that the selenol is the site of alkylation by BIAM [8]. Wild-type GPx1 was intensively labeled with BIAM, whereas no labeling was apparent with the mutant. In addition, H_2O_2 treatment decreased the labeling intensity of wild-type GPx1. These results indicate that Sec 49 is the only site of modification by BIAM and that oxidation of the selenol prevents BIAM labeling.

To elucidate the mechanism of inactivation, GPx1 purified from human RBCs was incubated with 1 mM H_2O_2 for 1 h at 37°C. Such treatment resulted in a ~40% loss of peroxidase activity and mass spectral analysis of tryptic peptides derived from inactivated GPx1 indicated that Sec at the active site was converted to dehydroalanine (DHA) [8]. The conversion is believed to be achieved via the oxidation of Sec by H_2O_2 followed by the loss of selenium oxide (Fig. 39.2). This conversion reaction is similar to the reaction in which the synthesis of DHA-containing peptides is achieved by incorporating phenylselenocysteine into growing peptide chains via standard peptide synthesis procedures, followed by oxidative β-elimination of phenylselenol to yield a DHA at the desired position [26]. In the conversion of GPx Sec to DHA, the catalytic intermediate GPx1–SeOH itself can be the source of DHA. However, further oxidation to $–SeO_2H$ will provide better opportunity for efficient β-elimination because $–SeO_2H$ is a better leaving group than –SeOH (Fig. 39.2). Treatment of GPx with H_2O_2 has been shown to generate GPx–SeO_2H [27].

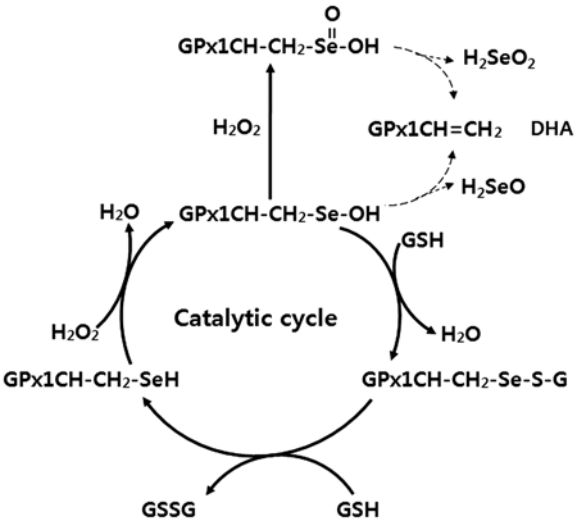

Fig. 39.2 Catalytic cycle of GPx1 and the conversion of oxidized selenocysteine to dehydroalanine (DHA) by the β-elimination of the oxidized selenium moiety

For estimation of the amount of DHA–GPx1 in cell homogenates, we developed a blot-based method that depends on specific addition of biotin-conjugated cysteamine to the DHA residue followed by detection of biotinylated protein based on its interaction with streptavidin (Fig. 39.3). This method is based on the fact that DHA readily reacts with nucleophiles such as thiols via Michael-type addition [28]. Cys residues of many proteins are present in the form of Cys–SO_2H in normal tissues [29] and some of them are converted to DHA [30]. However, DHA is not frequently found in positions corresponding to Cys residues because the strength of the C–S bond (272 kJ/mol) greater than that of the C–Se bond (234 kJ/mol) [20]. Because of the multiple sources of DHA, direct blot analysis of crude RBC extracts yielded many positive bands. It was thus necessary to immunoprecipitate GPx1 before alkylation and labeling with biotin-conjugated cysteamine in order to measure DHA specifically in GPx1.

39.3 Reversible Inactivation of 2-Cys Prx Through Hyperoxidation of Catalytic Cysteine to Cysteine Sulfinic Acid

All Prx enzymes, which form an obligatory homodimer, contain a conserved Cys residue (called the peroxidatic Cys, C_p) at the NH_2-terminal region, which is the primary site of oxidation by H_2O_2. Mammalian tissues express six distinct gene products of Prx (Prx I–VI), which can be divided into three subgroups, namely 2-Cys, atypical 2-Cys and 1-Cys subgroups. The 2-Cys members, which include Prx I–IV, contain an additional conserved Cys (often called the resolving Cys, C_R)

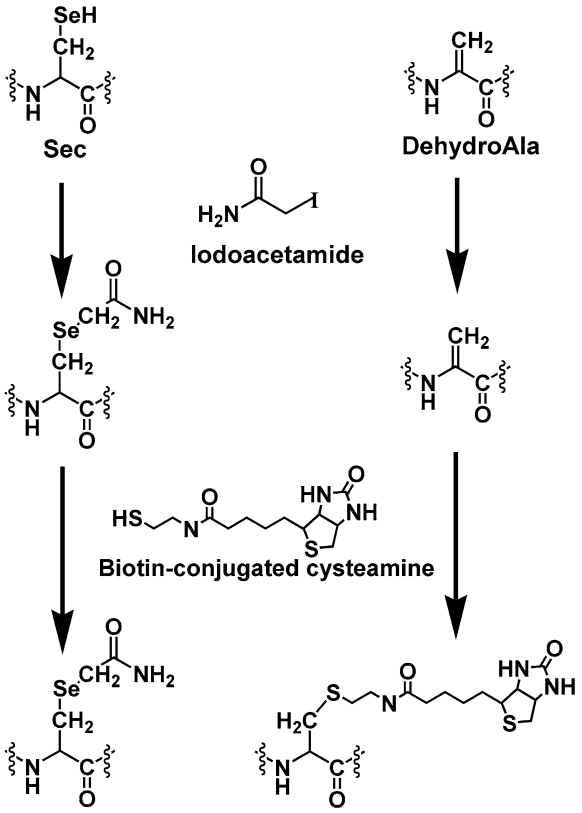

Fig. 39.3 Chemical reactions underlying the biotinylation of DHA-containing GPx1. After alkylation of free thiol and selenol groups by iodoacetamide, DHA residues are biotinylated with biotin-conjugated cysteamine

at the COOH-terminal region, whereas Prx V and Prx VI, the members of the atypical 2-Cys and 1-Cys subgroups, respectively, do not contain this second conserved Cys. During catalysis, the conserved, peroxide-sensitive C_P–SH is oxidized to C_P–SOH by the peroxide substrate (Fig. 39.4). The C_P–SOH of 2-Cys Prx, which corresponds to Cys^{51} in mammalian Prx II, then reacts with the COOH-terminal conserved C_R–SH (Cys^{172} of Prx II) of the other subunit to form an intermolecular disulfide. The disulfide is subsequently specifically reduced by Trx (Fig. 39.4, catalytic cycle). The reducing equivalents for the peroxidase activity of 2-Cys Prxs are thus ultimately derived from NADPH via Trx and Trx reductase. Despite the fact that Cys is much less sensitive to oxidation by peroxides than is Sec, the bimolecular rate constant for C_P of Prx was estimated to be 1.3×10^7 $M^{-1} s^{-1}$ [31], which is similar to that for GPx1 [31]. This unusually high rate constant is attributable to the fact that the tertiary structure of Prx provides a specific environment for C_P such that its pK_a value is lowered to between 5 and 6.

The sulfenic intermediate (C_P–SOH) generated during catalysis occasionally undergoes further oxidation to sulfinic acid (C_P–SO_2H), leading to inactivation of peroxidase function (Fig. 39.4, inactivation/reactivation cycle) [32]. This hyperoxidation occurs only when Prx is engaged in the catalytic cycle. Reactivation of 2-Cys

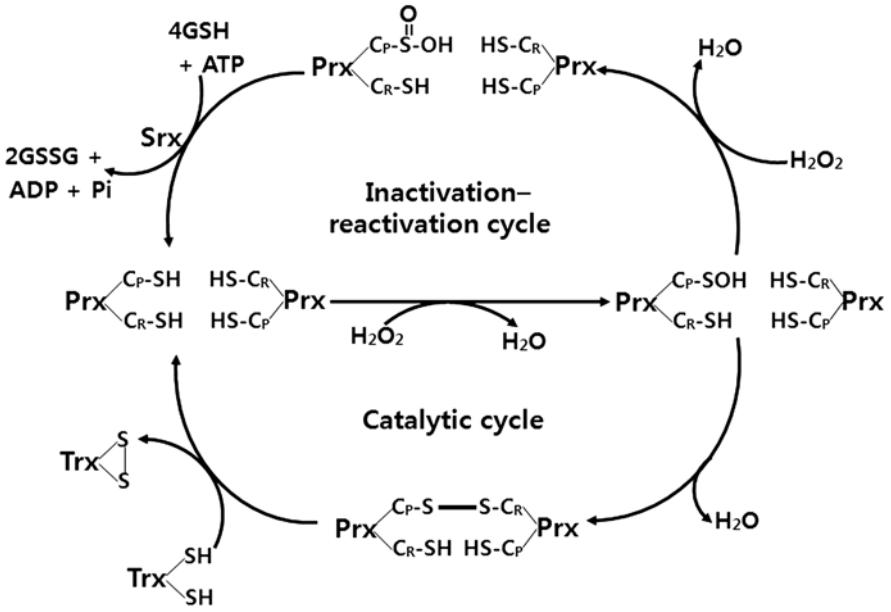

Fig. 39.4 Catalytic and inactivation/reactivation cycles of Prx

Prx enzymes is achieved by reduction of the C_P–SO_2H moiety in a reaction that requires ATP hydrolysis and is catalyzed by sulfiredoxin (Srx), with reducing equivalents being provided by physiological thiols such as GSH and Trx (Fig. 39.4, inactivation/reactivation cycle) [33, 34]. Hyperoxidation to sulfinic acid is not restricted to Prx enzymes. Critical Cys residues of many other proteins including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) also undergo this modification. In contrast, reduction by Srx is highly selective. Among the Prx isoforms, only the sulfinic forms of the 2-Cys Prx subgroup (Prx I to Prx IV), not those of Prx V or Prx VI, are reduced by Srx [35]. Moreover, Srx does not act on the sulfinic form of GAPDH. This specificity is due to the fact that Srx physically associates with the 2-Cys Prxs but not with other sulfinic proteins [35].

Proteins that contain hyperoxidized Cys residues (Cys–SO_2H or Cys–SO_3H) were initially detected as the more acidic satellite spots of the spots corresponding to the reduced form of the protein on two-dimensional polyacrylamide gels [32]. Given that an acidic shift on two-dimensional gels is also caused by protein phosphorylation, as is the case with Prx I [36, 37], mass spectral analysis of the acidic forms of proteins was necessary to ascertain the presence of hyperoxidized Cys residues. To develop an alternative approach to the complex procedure involving isotopic labeling of cells, two-dimensional electrophoresis, and mass spectrometry for the detection of proteins containing hyperoxidized Cys residues, we prepared rabbit antibodies to a sulfonylated peptide based on the active site sequence (DFTPVCTTEL) common to mammalian Prx I to IV [38]. With the use of immunoblot analysis with these antibodies, we could demonstrate reversibility of the hyperoxidation of Prx I to IV [35].

39.4 Inactivation of GPx1 and Prx II in RBCs

With the use of the blot method that detects the specific addition of biotin-conjugated cysteamine to the DHA residue, we investigated whether the conversion of Sec to DHA in GPx1 occurs with aging of RBCs. Indeed, the blot intensity of the band recognized by HRP-conjugated streptavidin increased gradually with aging of RBCs (Fig. 3 in ref. [8]), indicating that the Sec residue of GPx1 is converted to DHA in a time-dependent manner during exposure to the mild oxidative stress resulting from heme autoxidation. We also investigated whether the inactivated sulfinic forms of Prx enzymes accumulate in aged RBCs by the use of immunoblot analysis with antibodies that specifically recognize a sequence surrounding the C_P–SO_2H. Because the active site sequence is the same for 2-Cys Prxs (Prx I–IV) and because the sizes of Prx I and Prx II are identical, the sulfinic forms of Prx I and Prx II cannot be differentiated by immunoblot analysis. Sulfinic Prx VI, however, can be distinguished because its active site sequence (DFTPVCTTEL) differs from that for 2-Cys Prxs and because specific antibodies that recognize the sulfinic form are available. Immunoblot analysis with the antibodies to sulfinic Prxs revealed that neither the sulfinic forms of Prx I or Prx II nor that of Prx VI accumulates in RBCs during the aging process (Fig. 4 in ref. [8]). In contrast, the amount of the sulfinic form of GAPDH increased markedly during aging. Given that Prx II is known to be mainly responsible for dealing with the basal level of H_2O_2 flux originating from Hb autoxidation [4, 7] and that Prx II is abundant in RBCs, the conversion of even a small fraction of Prx II molecules to the sulfinic form would be expected to be readily detected by the blot analysis. The absence of sulfinic Prx II indicates that RBCs contain Srx at a sufficient concentration to counteract the hyperoxidation. Srx is expressed in RBCs and its abundance remained unchanged during aging.

H_2O_2 passes through the plasma membrane of RBCs, and their antioxidant enzymes eliminate ROS that originate from the external environment and thereby protect other cells from oxidative injury induced by phagocytic cells or toxins [1]. To examine the effects of extracellular H_2O_2 on RBCs, we added various amounts of glucose oxidase (GO) to these cells (50% hematocrit) suspended in DMEM containing a high concentration of glucose. GO catalyzes the oxidation of glucose with concomitant production of H_2O_2. Incubation of RBCs with GO at 37°C for 3 h resulted in concentration-dependent decreases in the activity and selenol content (Fig. 7b in ref. [8]) of GPx1 as well as an increase in the DHA content of GPx1 (Fig. 5 in ref. [8]). Although the sulfinic forms of Prx II and Prx VI were not detected in aged RBCs, their accumulation was apparent in cells incubated in the presence of GO at 0.1 mU/mL and increased further at higher concentrations of GO (Fig. 5 in ref. [8]). The amount of Srx in RBCs was not affected by the presence of GO. The sulfinic form of GAPDH was detected in RBCs even in the absence of GO, but its abundance increased in the presence of GO (Fig. 5 in ref. [8]). The amount of H_2O_2 produced by GO under our experimental conditions was estimated. In the absence of RBCs, GO at 1 mU/mL generated H_2O_2 at a rate of ~4.5 μM/min [8]. However, accumulation of H_2O_2 was not detected when the same amount of GO was added to

the suspension of RBCs; indeed, no accumulation of H_2O_2 was detected even at a GO concentration of 50 mU/mL, which could produce H_2O_2 at a rate of ~225 μM/min in the absence of RBCs [8]. These observations indicate that human blood is able to metabolize H_2O_2 efficiently by catalase in RBCs. Nevertheless, the entry of H_2O_2 into RBCs induces oxidative damage to many proteins including G6PDH and GAPDH. In addition, loss of the Sec residue of GPx1 is accelerated even at a low rate of H_2O_2 entry (0.45 μM/min, as generated by GO at 0.1 mU/mL). Furthermore, the hyperoxidation of Prx II and Prx VI, which was not observed during normal aging of RBCs, becomes apparent at this low rate of H_2O_2 entry.

If we assume that autoxidation occurs at a rate of 4% of total Hb a day in the 50% hematocrit suspension and that all superoxide anions produced from the autoxidation are dismutated to H_2O_2, the rate of H_2O_2 production in the suspension would be ~0.12 μM/min. When RBCs at a 50% hematocrit were incubated for 3 h with GO at 0.1 mU/mL, which generates H_2O_2 at a rate of ~0.45 μM/min, ~10% of Prx II was found to be hyperoxidized [8]. This result suggests that the additional flux of H_2O_2 at a rate of ~0.45 μM/min increases the hyperoxidation of Prx II to a level that exceeds the capacity of Srx in RBCs.

A variety of drugs including sulfonamides and industrial chemicals such as aniline induce hemolytic anemia anemia [39, 40]. These arylamine compounds are metabolized in the liver, and the resulting N-hydroxyarylamines react with oxyHb to produce superoxide anion. To examine the effects of such extra oxidative stress produced internally by environmental chemicals, we incubated a 50% hematocrit of RBCs with 20 mM aniline for various times. The activity and selenol content of GPx1 decreased with time whereas the DHA content of GPx1 increased on exposure of RBCs to aniline (Fig. 6 in ref. [8]). ROS produced by aniline also induced hyperoxidation of Prx II, Prx VI, and GAPDH (Fig. 6 in ref. [8]).

39.5 Concluding Remarks

Catalase is resistant to inactivation by its own substrate, whereas GPx1 and Prx II are inactivated as the result of oxidative modification of Sec and Cys residues, respectively, at the active site. GPx1 inactivation is irreversible and does not require continuous turnover, whereas Prx II inactivation is reversible and progresses only when the enzyme goes through the catalytic cycle continuously [32]. We found that the inactive DHA–GPx1 accumulates in RBCs with age even under the basal condition of H_2O_2 flux originating from Hb autoxidation. The inactive, sulfinic form of Prx II, however, does not accumulate under this condition. The amount of sulfinic Prx II increases transiently when the flux of H_2O_2 increases temporarily above the basal level, but it is removed slowly by the action of Srx. When exposed to such an increased H_2O_2 flux for long periods, however, the inactivated forms of both Prx II and GPx1 accumulate, as seen in RBCs exposed to GO or to aniline.

RBCs protect other tissues against oxidative damage by taking up and metabolizing peroxides [1]. Given that the rate of DHA–GPx1 accumulation in RBCs depends

on peroxide flux and that protein turnover mechanism is lacking in RBCs, the content of DHA–GPx1 in each RBC likely reflects total oxidative stress experienced by the cell during its lifetime. In addition to genetic polymorphisms, exposure to chemicals such as aniline and sulfonamides, pathological conditions such as diabetes and local inflammation, and an insufficient intake of antioxidants are all expected to affect the rate of GPx1 inactivation. In this regard, among the several antioxidant enzymes in RBCs, the activity of GPx1 was shown to be most influenced by lifestyle and environmental factors such as use of dietary supplements and smoking habit [21]. GPx1 activity in RBCs has also been proposed as a strong predictor of cardiovascular risk, which is associated with oxidative stress [22, 23]. Our present data together with these previous observations suggest that DHA–GPx1 in RBCs might be a suitable surrogate marker for evaluation of oxidative stress in the body.

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Chapter 40
Functional Aspects of the Genomics of Selenoproteins and Selenocysteine Incorporation Machinery

Catherine Méplan and John Hesketh

Abstract Functional genetic variants have been identified in selenoprotein and related genes. These include mutations in the selenoprotein N and SECIS-binding protein 2 genes as well as single nucleotide polymorphisms in genes encoding selenoproteins P and S, selenoprotein 15 and glutathione peroxidases 1, 3, and 4. Disease-association studies suggest that genotype for these SNPs may affect risk for several disorders. There is evidence for epigenetic regulation of selenoprotein expression and regulation of epigenetic mechanisms by Se supply. DNA microarray studies have identified both selenoproteins and downstream pathways that are sensitive to Se intake. Genomics (transcriptomics, proteomics, data mining and genetics) is providing useful approaches for exploring the roles of selenoproteins in cell function and human health with an integrated perspective.

40.1 Introduction

In the last 15 years the sequencing of the human genome, the development of other genome projects, the identification and cataloguing of genetic variations by the International Hapmap project and the development of high-throughput technologies has led to the emergence of the science of genomics. This encompasses both functional analysis of gene sequences and knowledge of gene expression patterns. Key aspects of genomics are the measurement of multiple parameters whether they are mRNA levels, protein concentrations, sites of DNA methylation or genetic variants and the integration of this information from a pathway or network perspective rather than in terms of a single enzyme or gene. Genomic approaches have been applied to

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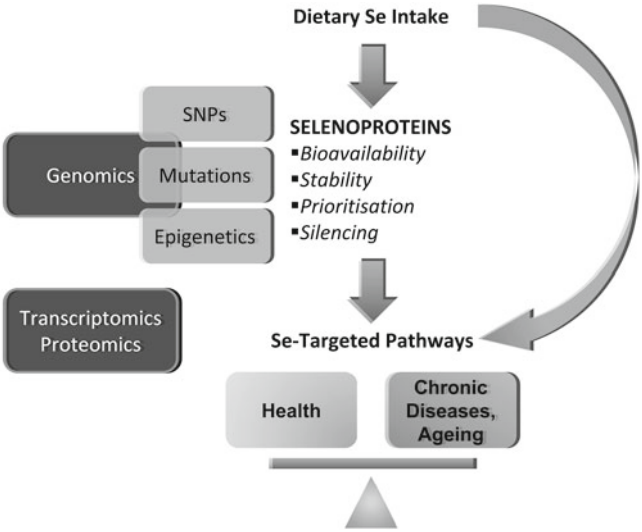

Fig. 40.1 Scheme illustrating the various genomic approaches used to study selenoproteins and associated metabolism

nutrition to assess the reciprocal influences of both genetics on nutrient metabolism and nutrition on gene expression. With regard to selenium (Se) metabolism, as illustrated in Fig. 40.1, genomics has led to the identification of genetic variants that influence selenoprotein metabolism and the description of how alterations in Se metabolism lead to changes in patterns of gene expression. Genomic approaches provide an opportunity to look at Se biology from an integrated perspective including all selenoproteins and downstream targets in other related pathways and to understand the mechanisms by which Se could prevent disease.

40.2 Genetics of Selenoprotein Metabolism

In theory selenoprotein metabolism can be influenced by genetic variants in both the selenoprotein genes and in genes that code for components of the selenocysteine incorporation machinery. There are a huge number of stable genetic variants found within the human population but only a very small proportion of these are expected to alter protein expression or function. Therefore, a major challenge in the nutrigenomics of Se is to identify mutations or stable allelic variations at single nucleotides (single nucleotide polymorphism; SNP) which affect metabolism and selenoprotein function. Importantly, since the SECIS structure within the 3′-untranslated region (3′ UTR) is essential for Se incorporation, it is necessary to consider the impact of genetic variants in gene regions that correspond to the 3′ UTR.

40.2.1 Mutations and Genetic Disease

Several genetic diseases have been linked to mutations that affect selenoprotein synthesis and function. First, mutations in the selenoprotein N (*SEPN*) gene cause a congenital muscular dystrophy [1, 2]. These mutations are located in the gene region that corresponds to the SECIS region of the 3′ UTR where they lower the binding affinity of the SECIS-binding protein 2 (SBP2) to the SECIS region and in the selenocysteine redefinition element (SRE) located adjacent to the selenocysteine-encoding UGA codon. In both cases, these mutations impair selenocysteine incorporation efficiency and result in low levels of SelN mRNA and protein [1, 2]. Second, mutations in *SBP2* cause a disorder characterised by an impaired thyroid function. In this case, the mutation causes a missense that leads to defective SECIS-driven selenocysteine incorporation [3, 4]. For both these mutations there is a clear mechanistic link between the mutation, impaired effectiveness of the SECIS, lower synthesis of a specific selenoprotein and disease outcome. Importantly, and not unexpectedly, Se supplementation does not result in clinical improvement or increase in selenoprotein activity [4]. Third, a recently identified rare complex disorder was observed in carriers of heterozygous defects in the *SBP2* gene. The corresponding phenotype includes a failure of spermatogenesis, muscular dystrophy, an increased sensitivity to UV radiation, increased levels of reactive oxygen species, and reduced expression of all selenoproteins [3].

40.2.2 Functional SNPs

In recent years, mechanistic approaches, human supplementation and gene association studies have highlighted the potential functionality of several variants in selenoprotein genes (Fig. 40.2). Selenoprotein P (SePP), the major selenoprotein found in blood, plays a pivotal role in Se metabolism as it transports and delivers Se to tissues [5]. Thus, variants in the *SEPP1* gene have the potential to affect delivery of Se to tissues such as the brain, prostate, testis and colon, hence affecting Se bioavailability in these organs. So far, three genetic variants in *SEPP1* have been shown to be functionally significant. A complex (TC) repeat sequence variant in the promoter region affects the promoter activity in reporter gene construct in HepG2 cells [6]. However, it is not known if this variant influences Se metabolism in vivo. On the contrary, two polymorphisms identified in the SelGen study, a G/A variant within the 3′ UTR (rs7579) and G/A variant that causes an Ala to Thr change at codon 234 (rs3877899), were found to affect Se metabolism in vivo [7]. Both variants influence the pattern of plasma isoforms of SePP [8] and the levels of various lymphocyte, erythrocyte and plasma selenoproteins in response to Se supplementation [7, 8]. These data illustrate how SNPs in the *SEPP1* gene can potentially affect Se availability for synthesis of other selenoproteins, possibly by modulating SePP capacity to transport and deliver Se.

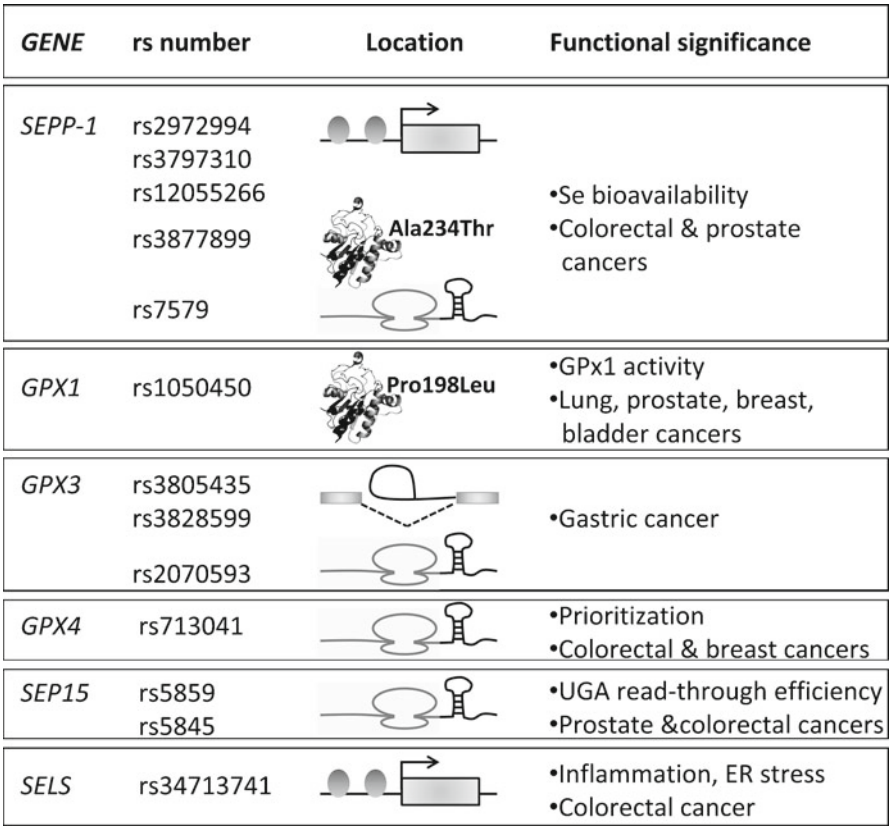

Fig. 40.2 Functional SNPs in selenoprotein genes. Those SNPs for which there is evidence of functional significance are presented. The SNPs are grouped according to the corresponding gene, identified by rs number and basis of functional significance noted. Location of the SNP within the gene is illustrated by the following icons: promoter (), coding sequence (), 3′ untranslated region () and intron ()

Additionally, several SNPs identified in the *GPX1*, *GPX3* and *GPX4* genes have been shown to be functionally significant. A coding SNP (rs1050450) in the *GPX1* gene causes an amino acid change at codon 198 (Pro to Leu) that lowers enzyme activity [9]. In vivo, the association between GPx1 activity and Se concentration differed between groups of different genotype suggesting that this SNP modifies the response of GPx1 activity to Se [10]. In the *GPX4* gene, a SNP (rs713041) results in a C/T base change in the 3′ UTR of the corresponding mRNA, close to the SECIS element. The T and C variants differ in their capacity to induce the transactivation of a reporter gene and in their affinity to bind proteins in RNA-binding assays, the C variants being the strongest in promoting reporter gene activity and binding to proteins from cultured cell extracts with a stronger affinity [11, 12]. Furthermore, in vivo evidence from the SelGen study, in which healthy volunteers were prospectively genotyped for rs713041, revealed that individuals exhibited different responses of lymphocyte GPx4, GPx1 and plasma GPx3 protein in response to selenium

supplementation according to genotypes for rs713041 [12]. Linked variants are found in the promoter region of the GPX3 gene and there is evidence from gene reporter experiments that these variants affect promoter activity [13].

Reporter gene studies have also been used to investigate functionality of variants in the 3′ UTR of the *SEP15* gene. Two linked variants, a C/T substitution at position 811 (rs5845) and a G/A at position 1125 (rs5859), are present in the region of the *SEP15* gene that corresponds to the 3′ UTR and both reporter gene experiments and gene association studies suggest that they are functional [14]. In addition, a G/A allelic variant at position −105 in the *SELS* promoter has been found to modulate the response to endoplasmic reticulum stress and influence markers of inflammation such as TNF-α and interleukin 1β [15].

40.2.3 Disease-Association Studies of SNPs in Selenoprotein Genes

Genome-wide association studies (GWAs) of cancer cohorts have failed to identify any selenoprotein genes as susceptibility loci. However, the limitation of such studies is that they do not take either Se intake/status or interactions between different genetic variants into account. In contrast, a number of association studies, particularly those that include measures of SNP-SNP or SNP-Se status interactions in the analysis, have indicated an association of selenoprotein gene variants with disease risk. A limitation of disease-association approaches is that outcomes depend on the study population and different populations may vary in either genotype frequency for SNPs of interest or exposure to specific environmental/dietary factors; and therefore, it can be difficult to replicate findings between populations. Nonetheless candidate gene studies can highlight the importance of investigating the role of the corresponding protein in relation to a tissue function.

Several studies suggest that carriage of at least one allele coding for the Leu variant of the Pro198Leu variant in *GPX1* increases susceptibility to lung, breast and bladder cancer, possibly when combined with the influence of either a SNP in the gene encoding the antioxidant defence protein manganese superoxide dismutase (*SOD2*) [16] or environmental factors such as alcohol consumption and smoking [16–21]. Additionally, the relationship between prostate cancer risk and serum Se has been reported to be modified by the Pro198Leu variant in *GPX1* [22]. More recently, genotype for SNPs in *SEPP1* (rs7579 and rs3877899) were shown to alter the risk for prostate cancer [22, 23] and colorectal cancer [24]. The risk of prostate cancer was shown to be modulated by a combination of low Se status with a genetic interaction between rs4880 in *SOD2* and rs3877899 in *SEPP1* [23]. So far, association studies investigating a possible link between rs713041 in *GPX4* and colorectal cancer risk have been inconclusive with the T allele being reported to lower risk, increase risk and have no influence as a single variant on risk [11, 12, 25]; a report of association of this SNP with increased risk of breast cancer [26] remains to be confirmed. Additionally, data from the Physicians Health Study carried out on a US population revealed that prostate cancer risk and survival were modified by a combination of genetic variation in *SEP15* gene and low Se status [27].

Two recent studies have shown that the T variant of rs34713741 in the *SELS* gene is associated with increased risk of colorectal cancer risk [24, 25]. Since this association has been replicated in two separate populations of different ethnicity (Caucasians and Korean), the data provide some confirmation of the association of this variant with disease risk. In addition, a second SNP in *SELS,* the variant at position −105 in the *SELS* promoter, has been reported to influence gastric cancer risk in a Japanese population [28].

These studies highlight a potential role of GPx1, SeP15 and SePP in prostate function, SePP, GPx4, SelS and SeP15 in colorectal function, and GPx4, and GPx1 in breast function. Furthermore, it is emerging that other physiological factors known to affect Se or selenoprotein metabolism influence the impact of selenoprotein variants. For example, the influence of rs3877899 and rs7579 in *SEPP1* on plasma Se are modulated by body mass index [7] while the effect of rs713041 in *GPX4* on lymphocyte GPx4 levels following withdrawal of Se supplementation was observed in females but not males [12]. Similarly, the association of a SNP in *SELS* with colorectal cancer risk differs in males and females [24, 25]. Additionally, risk for colorectal cancer was not only increased by genotypes for SNPs in the *SEPP1*, *GPX4* and selenoprotein S (*SELS*) genes but this risk was further modulated by SNP–SNP interactions with polymorphisms in other selenoprotein genes and these interactions overlapped with the biological interactions between the corresponding proteins [24]. The biological mechanisms underlying the interactions observed in these and other studies could reflect either the known selenoprotein hierarchy and selenocysteine incorporation mechanism or complementary roles of various selenoproteins in functions such as antioxidant protection or redox control.

40.3 Epigenetics of Selenoprotein Metabolism

In addition to the rare mutations and SNPs that have been described in selenoprotein genes and found to be associated with complex disorders, recent observations suggest that there is epigenetic control of selenoprotein expression. Epigenetic mechanisms include regulation of gene expression by heritable, but potentially reversible, changes in DNA methylation and chromatin structure [29]. DNA methylation is commonly altered in cancer and is thought to contribute to the carcinogenesis process. Affected genes are either silenced by hypermethylation, which is the case for tumour suppressor genes, or activated by hypomethylation, as for oncogenes for example [30–32].

Downregulation of GPx3 expression in prostate cancer has been linked to changes in the epigenetic pattern in the promoter of GPx3 [33, 34]. Hypermethylation of the 5′ regions of the GPX3 gene and subsequent loss of GPx3 protein expression have been described in primary prostate cancer samples [35], in primary esophageal squamous cell carcinoma tumour tissues [36] and in Barrett's tumorigenesis [37]. This hypermethylation was correlated with reduced GPx3 protein expression in the tumour tissue [35, 36]. The consequences of such silencing of GPx3 may sensitise cells to ROS damage and genome instability and suggest a role of new role for GPx3

as tumour suppressor; such a role may be particularly relevant to prostate cancer tissue in which expression of GPx3 has been shown to be widely downregulated [33, 35]. In addition, methylation of the methionine sulfoxide reductase B1 (*MSRRB1*) promoter and subsequent silencing of the gene has been reported in some breast cancer cell lines [38]. As MsrB1 has been shown to play a major role in the repair of oxidised proteins [39], its silencing would be expected to result in an increase in damaged proteins. Interestingly, downregulation of the *MSRB1/SEPX1* gene expression by age and calorie restriction has recently been found to occur in mouse liver [40]; since calorie restriction is known to induce changes in DNA methylation, the changes observed may well reflect epigenetic modifications of the promoter in the *MSRB1/SEPX1* gene. Overall, these results suggest both that several selenoprotein genes may be regulated by epigenetic mechanisms and that the silencing of particular selenoprotein genes is necessary for the carcinogenesis process and contributes to the accumulation of cell damage in cancer cells.

In addition, there is evidence that Se plays a role in epigenetic regulation. It is well-known that nutrients such as folate act on the one-carbon transfer metabolism, in which methyl groups are made available for subsequent DNA methylation. Se has been shown to affect the activities of key enzymes in one-carbon metabolism, in particular DNA methyltransferase [41]. In the rat colon, effects of folate-deficiency, such as an increase in colonic aberrant crypts and plasma homocysteine concentration, were largely compensated by feeding a Se-deficient diet [41]. In humans, the North Carolina Colon Cancer Study showed an approximately equal risk of colon cancer for individuals with high Se and low folate compared with individuals with low Se and low folate or with low selenium and high folate, whereas combination of high Se and high folate levels were associated with a decrease risk of colon cancer, suggesting an interaction between the two nutrients to regulate DNA methylation mechanisms [42].

The recent development of novel technologies to study gene-specific DNA methylation will certainly help to uncover the contribution of DNA methylation to the regulation of selenoprotein gene expression. On the other hand, the combined study of the epigenome and transcriptome will reveal the importance played by Se on the one-carbon metabolism. In both cases, studying the link between epigenetic mechanisms and Se should provide additional clues to further understand the anti-carcinogenic properties of Se and selenoproteins.

40.4 Transcriptomic, Bioinformatic and Proteomic Analyses of Se Metabolism

Whole genome gene arrays provide an opportunity to assess responses of multiple genes linked in pathways or networks to experimental manipulation. In the context of Se metabolism and responses to Se supply this approach presents four advantages. First, it allows examination of the overall pattern of selenoprotein gene expression. Although a limitation of this approach is that the quantification of the mRNA level may not mirror directly fluctuations in selenoprotein concentration as

a major effect of Se on selenoprotein expression is expected to occur during their translation, there is however evidence that some selenoprotein mRNA levels reflect protein levels [43]. Second, this methodology can analyse how genes other that selenoprotein genes may be affected by Se intake (Fig. 40.1). Third, it integrates the effects on selenoproteins within the context of non-selenoprotein genes and pathways that respond as a consequence of changes in selenoprotein activity or Se status – so-called downstream targets. Fourth, it allows performing microarray data mining to gain new knowledge in Se molecular targets and their relevance to disease. To date, transcriptomic studies have addressed how the pattern of gene expression changes in response to Se supply in the mouse colon [44], human lymphocytes [45] and transformed cell lines cells in culture [46–48].

Microarray analysis of colonic tissue from mice fed a diet marginally low in Se showed that expression of GPx1, SelH, SelW and SelM was particularly sensitive to Se supply. This confirmed the low position of GPx1 and SelW in the selenoprotein hierarchy and indicated that SelH and SelM may also be sensitive biomarkers of Se function in the colon. Pathway analysis highlighted the protein translation apparatus, NFκB and mTOR signalling pathways and both Wnt and Nrf2 pathways as being sensitive to Se supply [44, 49]. Microarray analysis of RNA from human lymphocytes collected from healthy volunteers before and after a modest Se supplementation (100 μg/day) for 6 weeks showed that protein biosynthetic pathways were the most sensitive to Se supply [45]. The finding that protein biosynthetic pathways are sensitive to Se supply in both the mouse colon and human lymphocyte indicates that this may be a widespread response to Se in many tissues. However, in terms of selenoprotein expression microarray experiments showed different responses of mouse colonic tissue and human lymphocytes with SeP15 and SelK responding to Se in lymphocytes but not in the colon.

Transcriptomic analysis using targeted gene arrays to analyse breast, colonic and prostate cell lines after treatment with high concentrations of various forms of selenium (selenite, selenomethionine or methylseleninic acid) has shown consistent changes in the pattern of expression of apoptosis and cell cycle genes (e.g. [48]), compatible with the observed effects of such treatment on apoptosis. A bioinformatic analysis of the genes showing both altered expression in prostate cancer and opposite changes in prostate cells, after addition of Se, highlighted a number of genes in cell proliferation/cell cycle regulation pathways [47].

Additionally, carrying out a data mining analysis of published datasets from gene expression profiling of clinical prostate specimens, Zhang and collaborators identified 42 genes consistently dysregulated in prostate cancer and which expression can be reversed by Se in LNCaP and PC3 cells [50]. The authors also found that Se could counteract the effect of androgen on the expression of a subset of androgen-regulated genes.

Overall, transcriptomic analysis of animal and human studies are providing important new insights into both potentially novel selenoprotein targets (SelH, K, M and W) which deserve greater investigation and biochemical pathways which represent downstream targets that respond to Se supply (protein biosynthetic pathways, NFκB, Wnt, cell cycle control and Nrf2). Understanding the role of these

selenoproteins and pathways should contribute to our knowledge of the links between oxidative stress, inflammation, cell cycle control and carcinogenesis. In contrast, proteomic studies of the cell responses to Se are very limited. Recently, however, using iTRAQ proteomic techniques and pathway analysis in a mouse prostate cancer model, methylseleninic acid and Se-methylselenocysteine were found to affect androgen-receptor signalling, protein folding and endoplasmic reticulum stress responses or phase II detoxification and cell protection functions, respectively [51] supporting observations from transcriptomics approach.

40.5 Conclusions and Future Perspectives

Genomics is proving to be a useful tool to expand our knowledge of selenoproteins, Se biology and Se in health. Both mutations and SNPs have been shown to influence Se metabolism and these variants both affect disease and provide new insights into selenoprotein function. New functional variants will no doubt be discovered and a major challenge will be to assess the impact of SNPs in Se-related genes on Se metabolism and disease susceptibility. Potentially, selenoprotein expression can be influenced by genetic variants in not only selenoprotein genes (promoter, coding region or 3′ UTR) but also genes encoding components of the Se incorporation machinery (e.g. SBP2, EFSec), or in tRNA-Sec and its modification, or Se transport. In the future, it will be important to consider how SNPs in the whole selenoprotein metabolic pathway or *selenome* affect overall selenoprotein function and to assess the impact of both interactions between SNPs in multiple genes within the pathway and their additive effects. Consequences of one SNP may be magnified or counterbalanced by a variants in other genes; it is this net "pathway effect" that is likely to determine the overall physiological interaction between genetics and Se intake. To address these issues, large genetic epidemiology studies will be needed. These will also need to include Se status as a covariate, to take into account multiple variants and SNP−SNP interactions across the selenoprotein pathway and be replicated in second populations before strong arguments about these SNPs and disease risk can be made. These genetic variants will need to be considered when designing Se supplementation or intervention trials since the potential complexity of these interactions influencing disease risk may explain the variation in findings from different populations.

Gene microarrays have provided new insights into effects of both the selenoproteins sensitive to Se intake and biochemical pathways which represent downstream molecular targets that respond to Se supply/changes. This provides an initial overall picture of the biochemical mechanisms which underpin the changes in function. Further use of genomic techniques, siRNA, and proteomics will help improve this picture of the *selenome* and define roles of specific selenoprotein and identify key pathways. The further challenge will be to incorporate system biology approaches to produce an integrated picture of how cell function responds to Se intake and perturbations in selenoprotein function.

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Chapter 41
Selenium: Dietary Sources and Human Requirements

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Abstract Selenium is an essential trace mineral. Typically, in humans, grains and animal products are the primary exposure source. Inadequate information exists about the benefits provided by dietary selenium supplements. Current recommended dietary amounts for healthy adults in the US are 55 μg/day for men and women. In the general US population, higher amounts can be obtained through consumption of typical foods. Regardless, controversies prevail about adequate amounts for health and the individual needs depending on genetic and environmental variables. Information regarding specific target populations potentially sensitive to selenium status continues to be an area of much needed research.

41.1 Introduction

Selenium is one of approximately 60 essential nutrients that are required for human health. Our understanding of the significance and complex metabolic role of this micronutrient in human nutrition has grown rapidly during the past decades [1–3]. Conclusions about human requirements for this trace element have come from

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animal models and from observations in geographical areas where nutritional selenium deficiencies or excesses occur naturally. Appropriate selenium nutriture, as described in other chapters of this book, is necessary for healthy life due to its antioxidative properties and its incorporation into selenium-containing proteins, i.e., selenoproteins. It is recognized that the safe range of selenium intake is relatively small and excess selenium intake can readily result in toxicity [4]. This chapter briefly describes the sources and the normal human requirements of this dietary nutrient. Information regarding potentially sensitive groups within the general population, which includes ethnic groups, infants, children, pregnant or lactating women, elderly, and those with genetic predispositions or certain diseases, remains scarce.

41.2 Selenium Occurrence

Selenium occurs in various forms and is fairly ubiquitous in the environment, though the amounts in which it is found can vary widely [5, 6]. Selenium can exist stably in the environment as inorganic species, which includes elemental selenium, selenide, selenite, and selenate. Organic species, which includes the methylated selenium compounds, selenoamino acids, and the selenium-containing proteins, are found in biological systems of living matter [7]. Frost [8] described the dynamic balance and interconversions among the inorganic and organic forms. Although selenium can be detected in the air in the aerosol phase, this contribution is considered to be insignificant to human health [9], except in areas where the burning of fossil fuels contributes significantly [10]. Exceptions also include industrial settings of glass, chemical, or electronics manufacturing, where occupational exposures can increase substantially. Another significant source of selenium exposure can include supplement use for human and farm animal diets, which is the largest pharmaceutical and agricultural use of selenium, although their overall contribution to exposures remains controversial. Overall, the main body sources for most humans are through the inorganic forms in water and soil, though their contribution to human selenium status are generally very minor, and, importantly, dietary exposure to mainly organic forms in plants and animals.

41.2.1 Water

The primary bioavailable water-soluble forms of selenium in water are inorganic selenate and selenite ions. Currently, the amount of selenium is regulated by the US EPA under the Safe Drinking Water Act, and federal standards allow up to 50 μg/L in drinking water [11]. At typical levels, the selenium content in drinking water contributes only slightly to the daily intake [12], ranging in values from 0.12 to 0.44 μg/L [13]. Exceptions are historic instances where drinking water levels of

selenium may have been elevated during drought, as occurred in rural southeastern Colorado in 1975, in highly seliniferous areas of the Midwestern US, where values from 50 to 330 μg/L in wells had been reported [14], or in a Northern Italy municipality, where unusually high selenium content was measured in public tap water in 1990 [15]. Selenium is detectable in sea water in amounts from 0.09 to 0.11 μg/L [16]. Subsequently, aquatic life forms, including prokaryotic cells, algae, sea weeds as well as marine invertebrates and vertebrates have the opportunity to take up this mineral and can become part of human consumption when entering the food chain. Bioaccumulation of selenium has been reported to result in adverse effects such as deformities and reduced survival of various species of fish and water fowl [17, 18].

41.2.2 Soil

In the terrestrial environment, the weathering of rocks and soils is the primary source of selenium as this element occurs naturally in the Earth's crust. It can often be found in the presence of the sulfide ores of heavy metals [19]. Whereas most rocks, including granite, sandstone and limestone, contain selenium at relatively low levels, some phosphatic rocks, coal, and other organic-rich deposits have been found to have higher levels of selenium with typical ranges from 1 to 20 mg/kg. Amounts exceeding 600 mg/kg have been reported for seleniferous parent materials such as some black shales [20]. Seleniferous soils are widespread in parts of North and South America, China, and Russia. Some locations within the North American great plains area are infamously known for their high soil selenium content, where toxicity in livestock (a.k.a., alkali disease) has been recorded as far back as 1860 [21], and also again in the 1930s [22]. In such areas, plants, including certain species of *Astragulus*, may accumulate up to 3,000 μg selenium per gram and, though often not palatable to livestock, may potentially be toxic [23]. Recent studies by Bajaj et al. [24] reported soil and water levels of selenium in India. Toxic concentrations of selenium (45–341 μg/L) were detected in groundwater in two villages in Punjab province. Prevailing intensive irrigation practices are thought to have contributed to the selenium accumulation in both topsoil and groundwater. On the contrary, selenium-poor soils are also found within the same countries, indicating strong regional differences. Anthropogenic sources of selenium to the soil include fossil fuel burning and industrial waste from metal smelters and steel plants, which contributes about 3,500 metric tons of selenium per year in the US [19]. Furthermore, the addition of sodium selenite to fertilizer and/or animal feed is a common practice in areas where the soil is selenium-poor, including areas in southwestern Oregon, and in countries such as Finland and New Zealand [25, 26].

Chemical speciation determines selenium solubility and therefore its bioavailability and potential for transport in the environment [27]. Selenium absorption by plants mainly depends, among other factors, on the pH of the soil, as selenite dominates in acidic soils whereas the oxidized form selenate dominates in alkaline soils. Inorganic selenium occurs in three soil-phases, fixed, adsorbed and soluble, and

only the adsorbed/soluble forms of selenium are thought to be available for plant uptake [1]. Because selenate represents the more soluble form [2], it is more easily acquired by plants. Recent findings by Oram et al. [28] provide evidence that show that within the rhizosphere, enhanced selenium bioavailability occurs via oxidation of reduced soil selenium to more soluble selenium species.

41.2.3 Dietary Sources of Selenium

In humans, the primary source of selenium intake is food [29] (Fig. 41.1). The US Food and Drug Administration (FDA) Total Diet Study, which was conducted from March 1991 to January 1999, detected selenium in about 50% of all food items [30], with levels that were highly variable. Additionally, animal and human studies have shown that the bioavailability of selenium compounds from food is highly varied [31, 32], depending on the source and form of selenium. The amounts listed in these subsequent paragraphs, unless noted otherwise, were obtained through the USDA tool at http://www.nal.usda.gov/fnic/foodcomp/search/, which is based on Release 23 of the USDA National Nutrient Database for Standard Reference.

41.2.3.1 Grains

In the US, because of its high consumption, wheat is one of the primary sources of dietary selenium, with the major available form found in grains being selenomethionine. Because the availability and chemical species of selenium in soils is the main influence of selenium uptake by plants, the content of selenium in wheat and corn used for cereals, breads, and other food products can vary widely. For example, on average, breakfast cereals and wheat toast breads, which are some of the most common forms of wheat consumed in the US, contain below three (e.g., cooked cream of wheat) to over 100 μg/100 g (e.g., puffed wheat), respectively. Selenomethionine is the most predominant selenium species in wheat (~55%) but other forms, such as selenocysteine and selenite/selenate, are detectable in substantial amounts (up to 20%, respectively). It is important to note that even though the total selenium content in wheat can vary as much as 500-fold due to geographical differences and fertilization techniques, selenomethionine levels are maintained at about 55% of the total [33]. These findings suggest that controls exist in plants that balance the types and amounts of specific selenocompounds that can occur.

41.2.3.2 Fruits, Vegetables, and Fungi

Unless fortified or grown on selenium-rich soil, fruit, and vegetables typically contain only small amounts of selenium, typically <0.1 μg/100 g in tomatoes and 2.3 and 7.2 μg/100 g in asparagus and lima beans, respectively. However, some "accumulator"

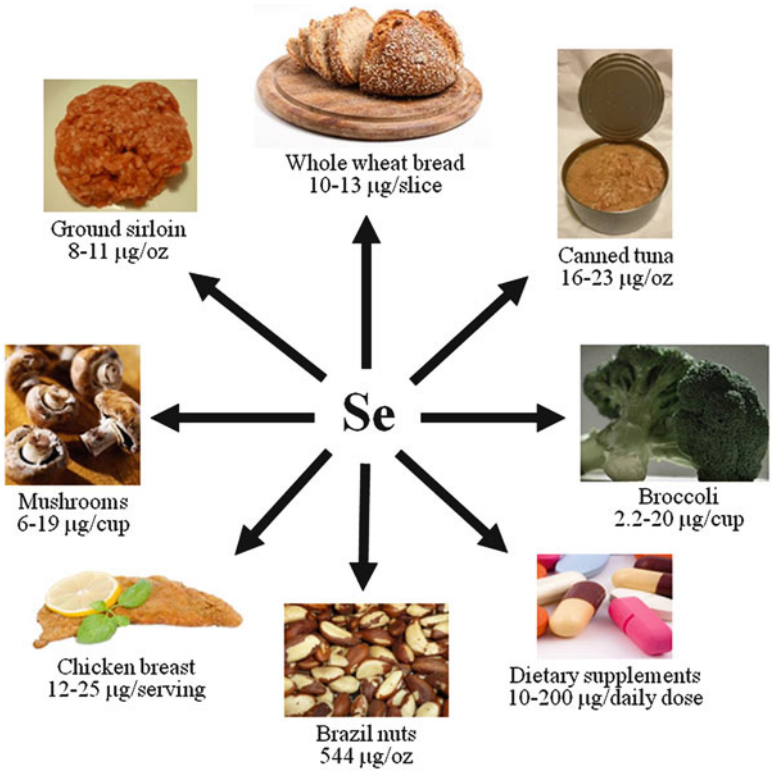

Fig. 41.1 Common food sources of selenium. The selenium content in foods depends on the genetics of the organism and on the amount of selenium in the soil or added to the fertilizer and feed

vegetables [34], including onions, wild leeks, garlic and broccoli, can be grown under selenium-enriched conditions, which increases the selenium content up to 50-fold or higher due to their ability to accumulate selenium. Highly selenium-enriched garlic has been reported to contain selenium concentrations as high 1,355 µg/100 g [35]. This may also shift the predominant selenium species found in these plants from mostly selenomethionine to a higher content of selenium-methylselenocysteine and γ-glutamyl-selenium-methylselenocysteine [36, 37]. For example, broccoli grown under selenium-enriched conditions contains a higher amount of selenium-methylselenocysteine (>40%) than selenomethionine [38]. Other plants that can accumulate selenium include various species of algae, multiple *Brassica* species, and, infamously, Brazil nuts [29]. Brazil nuts have a reported average selenium content of 1,470–1,917 µg/100 g, with most of it present as selenomethionine [39]. Fungi, such as mushrooms and yeast, can also accumulate selenium in substantial amounts and may contain more than 20 selenium-containing compounds, including organic forms such as selenocysteine, selenomethionine, selenium-methylselenocysteine, and selenium-adenosylselenohomocysteine, as well as some inorganic forms [40].

41.2.3.3 Animal Products

The human selenium intake through meat consumption comprises a relatively large proportion of the overall intake, and thus, next to wheat, meat consumption is the other primary source of dietary selenium. In the US, the average selenium content of domestic beef is 20–35 μg selenium/100 g tissue, chicken has 10–24 μg/100 g, lamb has 20–30 μg/100 g, and pork has 20–40 μg/100 g. However, the cut of the meat is important in estimating bioavailable selenium. Organ meats usually contain more selenium than muscle meats. Poultry liver has an estimated 60–80 μg selenium/100 g tissue, whereas cooked kidneys from beef, veal, lamb, and especially pork, have an estimated 100–311 μg/100 g tissue. Among dairy products, cow's milk (whole) on average is estimated to contain 3.7 μg/100 mL, whereas higher amounts are observed in cheeses ~14 μg/100 g. The animal's selenium content, and therefore the form of selenium consumed by humans, also depends on the animal's diet. Selenium supplementation of cattle, hogs, and chickens is common practice in the US and elsewhere [41], and can contribute to high muscle and organ selenium concentrations. Regional differences due to geographical variations in soil and subsequent plant materials also contribute to varying selenium concentrations in animal products for human consumption. The predominant selenium species in animal meat for human consumption are thought to be mostly selenomethionine (up to 60%) and selenocysteine (up to 50%) in meat. However, meat from animals supplemented with inorganic selenium in the form of selenite or selenate will contain selenium primarily in the form of selenocysteine, whereas meat from animals that received feed containing selenomethionine subsequently contain both selenocysteine and selenomethionine [42].

Eggs on average contain about 26 μg selenium/100 g. However, chicken feed is highly supplemented and selenium-enriched eggs have been introduced to many markets worldwide [41, 43]. Therefore, much higher values can be achieved and can therefore contribute significantly to human exposures. The predominant selenium species in eggs, much like in meat, are thought to be selenomethionine (up to 60%) and selenocysteine (up to 50%) [44, 45].

On average, the selenium content of fish is estimated to be about 27.2 μg/100 g. However, the ranges can be very large: from about 12 μg/100 g in freshwater catfish, to 44 μg/100 g and over 70 μg/100 g in marine mackerel and canned tuna, respectively. The predominant selenium species in fish are thought to be selenomethionine and selenite/selenate [44, 45]. Because heavy metals, e.g., mercury, are known to decrease selenium absorption via chelation and precipitation, it is possible that the selenium in mercury-containing fish may not be fully bioavailable [46].

41.2.3.4 Dietary Supplements

In the Western world, dietary supplement use in humans is very common. An estimated 52% of the US population is thought to consume dietary supplements regularly [47] including 18–19% who report using dietary supplements containing

Table 41.1 RDAs and ULs for selenium, in microgram per day, for infants, children, and adults as published in the 2000 IOM report on Dietary Reference Intakes [50]

Age	RDA	UL
0–6 months[a]	15	45
7–12 months[a]	20	60
1–3 years	20	90
4–8 years	30	150
9–13 years	40	280
14–18 years	55	400
19–70 years	55	400
>70 years	55	400
Pregnant female	60	400
Lactating female	70	400

[a]There is insufficient information on the micronutrient selenium to establish a RDA for children under the age of 1 year. Instead, an Adequate Intake (AI) is calculated that is based on the amount of selenium consumed by healthy infants who are fed breast milk [50]

selenium [48], and it has grown into a multibillion dollar industry. Because of a lack of regulation and FDA oversight, there are many different supplement formulations available, which vary in amount of selenium included. Thus, the actual content of selenium in supplements (including multivitamin/multimineral supplements and specific selenium supplements) varies between 10 and 200 μg/daily dose [49]. Further complicating this estimate are the various forms of selenium that are typically found in dietary supplements: selenomethionine, selenite or selenium-enriched yeasts are frequently added, which may result in differential absorption and bioavailability. Selenium is also available in "high selenium yeasts" which may contain very large amounts that are up to 2 mg/g. A daily dose of 200 μg/day, as found in many dietary supplements, constitutes nearly four times the Recommended Dietary Allowance (RDA) for adults (55 μg/day) and 50% of the tolerable Upper intake Level (UL) of 400 μg/day [50] (Table 41.1). The UL is likely to be reached in people who consume a selenium-rich diet in addition to taking dietary supplements. Until recently, exposures to dietary supplements have been evaluated inadequately and thus knowledge about their significance in determining the selenium status of humans remains controversial [51, 52].

41.3 Human Selenium Requirements

Human basic nutrient requirements define the dietary intake level at which specified criteria of nutrient adequacy are met, and risks of deficit or excess are prevented [53]. There is a fine line between the basic human selenium requirements and the

amounts sufficient to maintain near-optimal functions of the many biochemical and physiological mechanisms in which selenium is involved.

Selenium is an essential dietary micronutrient, and in general is absorbed very efficiently by humans. Both the inorganic forms selenite and selenate, and the organic selenomethionine are thought to be absorbed at levels greater than 80%; however, the bioavailability of selenium is complicated by presence of other dietary components, and the various metabolic pathways involved [54]. Usually, the biological response to nutrient adequacy is assumed to follow a Gaussian distribution [55]. Like with many other nutrients, the biological response is very individual-based, and may depend on gender [56], genotype [57, 58], selenium status, and presence of disease, among many other factors. A recent review by Mathers et al. [59] suggests that genetics is likely only a minor determinant of the selenium status of individuals. Regardless, it has been argued that a U-shaped curve should be applied when it comes to risk of disease and intake, indicating that there is a profile for predicting the risk–benefit consequences of supplementation with dietary selenium [60]. This may be especially applicable in light of differential responses to selenium supplementation due to human gene polymorphisms in selenium-containing proteins [61, 62].

The nutrient-specific RDA, as used by the Food and Nutrition Board of the US National Academy of Sciences, is defined by the average daily intake that meets the basic nutrient requirements of 97.5% of the apparently healthy US population. Details and criteria used regarding the development of dietary standards have been discussed, in depth, previously [63]. As discussed in Chap. 33, adequate data for sex-specific intake recommendations are still lacking. Also scarce are data regarding selenium intake, status and requirement of ethnic segments of the population, including African-Americans [64]. Because other chapters in this book discuss the micronutrient selenium in cancer prevention, diabetes, and other health related diseases, this chapter focuses primarily on the normal adult selenium status and requirement.

41.3.1 Selenium Requirement

In 1980, the National Research Council (NRC) had established an estimated safe and adequate daily dietary intake for selenium in humans. Based on extrapolations from animal studies, the recommendation for adults was set from 50 to 200 μg/day. Repletion studies in selenium-deficient regions of China found that approximately 40 μg selenium per day achieved maximal activity of plasma glutathione peroxidase [65]. Subsequently, in 1989, the Dietary Reference Intake (DRI), reflecting corrections for body weight and subject variability in the US population, was established for selenium, with a RDA of 70 μg for men and 55 μg for women [46, 66] in accordance with the World Health Organization. In the year 2000, this RDA was adjusted to 55 μg/day for both men in women [50], which is slightly lower than in the United Kingdom and Australia/New Zealand [67], but substantially higher than

the recommendation of 25 and 35 μg/day set for men and women, respectively, in countries such as Japan [68]. The changing selenium requirements during human development are addressed by the current RDAs (Table 41.1).

41.3.2 Selenium Status

The Third National Health and Nutrition Examination Survey (NHANES III) assessed plasma selenium as a marker for selenium nutritional status in over 17,000 participants and thus the database provides a recent estimate of selenium status in the US population. The median and mean serum selenium levels for all individuals, regardless of gender, ethnicity, or supplement use, were 1.58 and 1.56 μmol/L, respectively [69]. The NHANES III study provided compelling evidence that over 99% of the adult participants were selenium replete and were consuming selenium at or more than the RDA of 55 μg/day. Thus, routine supplementation does not appear warranted in the USA to meet RDA needs [70]; admittedly, others believe that higher exposures may have as of yet unsubstantiated health benefits [71].

Selenium concentrations in human tissues can serve as long-term biomarkers and toenail, hair, and urine samples provide noninvasive access to assay individual selenium status [72]. A more physiological and commonly used approach is provided by assaying selenium concentration in serum or whole blood, which also reflects recent changes in dietary intake. Additionally, serum selenoproteins, such as glutathione peroxidases and selenoprotein P [73, 74], can be useful biomarkers due to their dose-dependent expression, as described in detail in Sunde et al. [73] and Chap. 16 in this book, respectively. Selenoprotein P accounts for over 60% of the selenium in human plasma, and it has been suggested that the quantification of a combination of selenoproteins may be the most beneficial indicator of selenium status [75, 76].

41.3.3 Selenium Deficiency

There are three specific diseases that have been linked to severe selenium deficiency, all of which are described primarily in children of selenium-poor areas in China and southeast Siberia: (1) Keshan Disease, which was first described in Chinese medical literature more than 100 years ago, results in cardiac anomalies and congestive heart failure; (2) Kashin–Beck Disease (described in Chap. 45), a selenium-responsive bone and joint disease which results in osteoarthropathy and joint necrosis; and (3) Myxedematous Endemic Cretinism (described in Chap. 29), which results in mental retardation and has been reported from selenium/iodine-deficient areas in central Africa [77]. Prophylactic oral administration of selenium and improvements of the nutritional status in Chinese rural communities have been effective in prevention

of these diseases in many cases. All of these have been described in great detail elsewhere (e.g., [78, 79]). It should be noted that selenium may be a cofactor in the etiology of these diseases, as other dietary or infectious agents have been implicated.

41.3.4 Selenosis/Toxicity

Selenosis, a condition defined by blood selenium levels greater that 100 μg/dL, can result in symptoms including gastrointestinal upsets, hair loss, white blotchy nails, garlic breath odor, fatigue, irritability, and mild nerve damage [80]. Historically, alkali disease in livestock has been documented and continues with the 2009 report about 21 polo ponies that accidentally were injected with a vitamin/mineral supplement dose containing selenium levels at 1,000 times higher amounts than needed. This treatment resulted in liver selenium concentrations that were 20 times more than normal. The horses began to die shortly before a match in the United States Polo Open due to selenium toxicity [81], raising issues about stress releasing the selenium from cellular sites.

Selenosis in humans is a rare event outside accidental industrial exposures. An exception was an episode of human selenium poisoning that occurred due to a manufacturing error of a dietary supplement, resulting in a product that contained 182 times the amount of selenium as declared on its label. It should be noted that even in this case there was wide variability in the symptoms that occurred in those taking the same amounts. Significant adverse effects occurred within a few days to weeks after consumption and included effects on hair, nails, and liver [82], similar to what has been described in high selenium areas of China.

In order to prevent the risk of potential selenosis in humans, the Institute of Medicine of the National Academy of Sciences provides a DRI and has set tolerable upper intake levels for selenium and other nutrients (Table 41.1) [50]. The tolerable upper intake levels for selenium of 400 μg are also well below the Lowest Observed Adverse Effect Levels (LOAEL) of 910 μg [82].The RDAs for adults in the general US population have been in place largely unchanged for the past 2 decades and are well below the No Observed Adverse Effect Levels (NOAEL) of 200 μg.

41.4 Conclusions

Much controversy still exists regarding the established levels of selenium intake for the general population especially in light of subgroups potentially more sensitive to selenium intake and status. Some argue that benefits of selenium supplementation remain uncertain and that the general population should not consume selenium supplements for disease prevention [2] and that even a low-dose chronic overexposure of selenium may contribute to increased health risks [83] rather than benefits. Others

argue the opposite in that the current RDAs for selenium should be revised upwards to realize the health benefits that selenium can provide for humans and animals. It appears that a broad and indiscriminate use of selenium supplementation across the general population would benefit at least some segments of the population, but could potentially result in adverse health outcomes in others. Thus, information regarding those among the general population that may potentially benefit vs. those who may be sensitive to selenium status is critically important and will need to be addressed in much more detail. This then hopefully will result in better and more individualized recommendations for selenium intake and supplementation in the future.

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Chapter 42
Selenium and Adverse Health Conditions of Human Pregnancy

Margaret P. Rayman

Abstract There is evidence that selenium affects a number of adverse pregnancy health conditions. While higher selenium status has been associated with a lower risk of miscarriage and preterm birth, the level of evidence is stronger for a benefit of higher selenium intake/status in preeclampsia and autoimmune thyroid disease characterized by raised thyroid peroxidase antibodies. The ability of selenium to reduce oxidative stress, endoplasmic reticulum stress, and inflammation, to protect the endothelium, to control eicosanoid production, to regulate vascular tone, and to reduce infection is likely to be important in the context of these conditions.

42.1 Introduction

This chapter is an attempt to pull together what is known about the role of selenium in adverse health conditions of human pregnancy. Though there are one or two randomized trials, most of the evidence for an involvement of selenium in pregnancy outcome is only case-control or cross-sectional in nature, allowing only an association, rather than causal involvement, to be inferred. Despite the paucity of strong evidence, given the encyclopedic roles of selenoenzymes, it would be surprising if they did not influence pregnancy. We know for instance that selenoprotein P (SEPP1) is expressed in the placenta suggesting that it may play a role in the transplacental transport of selenium to the fetus [1]. Furthermore, a SNP in selenoprotein S (SEPS1) has been shown to affect the risk of the pregnancy syndrome, preeclampsia [2]. The pregnancy conditions for which most evidence for involvement of selenium exists are preeclampsia, miscarriage, preterm birth (birth before 37-weeks gestation), and autoimmune thyroid disease. These will be addressed in turn below.

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42.2 Preeclampsia

Preeclampsia, a major complication in 2–8% of pregnancies [3], is associated with high maternal and perinatal morbidity and mortality; there is currently no cure other than early delivery of the baby [4]. Surviving infants are likely to be small-for-dates and premature, factors that may jeopardize their development and health even into adulthood [3, 5]. Preeclampsia portends an increased risk of maternal coronary heart disease, hypertension, and stroke in later life, further emphasizing the significance of this condition [6].

It is important to have some understanding of the syndrome of preeclampsia in order to appreciate how selenium might affect its risk or progression. Figure 42.1 summarizes the etiology of the oxidative and endoplasmic reticulum (ER) stress, dysfunctional maternal endothelium, and excessive maternal systemic inflammatory response that result in the characteristic clinical signs of preeclampsia, i.e., hypertension, proteinuria, and sudden edema [7–18].

There are indications that low selenium status may increase the risk of preeclampsia. A selenium-free diet caused a preeclampsia-like syndrome in pregnant rats with significantly increased blood pressure, proteinuria, placental oxidative stress, and significantly lower pup weight than in selenium-adequate controls [19]. Supplementation of Chinese women deemed to be at risk of pregnancy-induced hypertension with 100 μg selenium/day prevented pregnancy-induced hypertension and gestational edema, two of the signs of preeclampsia [20]. Significantly lower levels of plasma selenium, plasma and placental glutathione peroxidase (GPx), and placental thioredoxin reductase (Txnrd) have been found in preeclamptic women than in matched healthy controls [19, 21–25].

In a case-control study in Oxford where selenium status is quite low [26], toenail samples were collected from 53 preeclamptic women and 53 matched pregnant controls of average gestational age 34 weeks [27]. Because of the time taken for the toenails to grow (up to 12 months), analysis of toenail clippings allows the determination of trace element status before the development of symptoms and probably largely before pregnancy. We found that the toenail selenium concentration in women with preeclampsia was significantly lower than that in matched pregnant controls ($P = 0.001$) (Table 42.1). If in the bottom tertile of toenail selenium, women were significantly more likely to have preeclampsia [odds ratio (OR), lowest tertile vs. rest, 4.4; 95% confidence interval (CI) 1.6–14.9]. Within the preeclamptic group, lower selenium status was significantly associated ($P = 0.029$) with more severe disease, as measured by delivery before 32 weeks [27]. There were no significant differences in toenail concentration of other elements measured – zinc, copper, or iron [27] – precluding an effect due to the acute-phase response [28, 29].

42.2.1 How Might Selenium Affect the Risk of Preeclampsia?

As selenium supplementation is known to accentuate the Th1 response [30], it is at first somewhat surprising that higher selenium status may reduce the risk of preeclampsia, since the latter is characterized by a Th1 bias that is considered undesirable

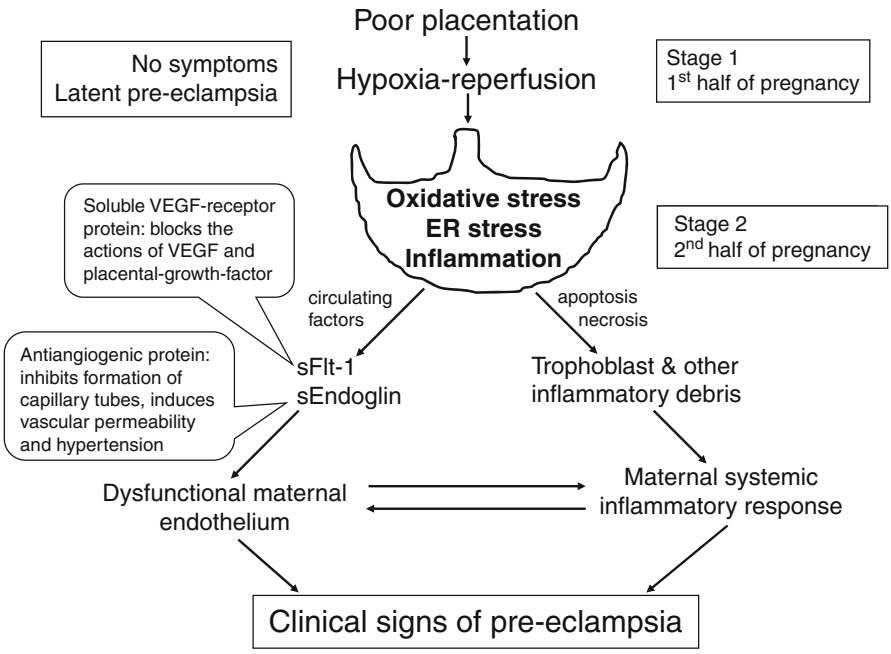

Fig 42.1 Schematic representation of the etiology of preeclampsia. Deficient placentation occurring during the first half of pregnancy frequently precedes the development of preeclampsia [7]. Shallow trophoblast invasion and inadequate spiral arteriole remodeling result in a placenta that is not effectively perfused, resulting in localized areas of ischemia and placental oxidative stress [7, 8]. This results in increased apoptosis and necrosis of the syncytiotrophoblast layer lining the intervillous space [9]. This placental debris is then transported into the maternal circulation where it contributes to a maternal systemic inflammatory response and endothelial activation, the latter causing the characteristic symptoms of hypertension, proteinuria, and sudden edema [7, 10–12]. Furthermore, in response to placental hypoxia and inflammation, soluble vascular endothelial growth factor (VEGF) receptor protein (also known as soluble fms-like tyrosine kinase 1, sFlt-1), which blocks the actions of VEGF and placental-growth-factor (PlGF), is released into the circulation where it damages endothelial integrity [13]. Other characteristics of the condition are higher circulating levels of soluble adhesion molecules produced by the inflamed endothelium [14], and of the potent inflammatory mediator, peroxynitrite, that causes vasoconstriction, platelet aggregation, and thrombus formation [15, 16] and a prostacyclin/thromboxane ratio that favors vasoconstriction [17] (modified from Redman and Sargent [18])

for successful pregnancy [31]. Other beneficial effects of selenium must therefore outweigh this Th1 effect. Regulatory T cells (Treg) are known to reduce the risk of preeclampsia and miscarriage by increasing immune tolerance [32]. Though there are no published human data on how selenium might affect the Treg population, there is one study in mice that suggests that supplementation with selenium can upregulate Tregs [33]. We also know that selenium can reduce oxidative stress, endoplasmic reticulum (ER) stress and inflammation, protect the endothelium, control eicosanoid production, and regulate vascular tone [34–36]. These effects of selenium/selenoproteins are explained below.

Table 42.1 Distribution of subjects in preeclampsia and control groups by tertile of toenail selenium [27]

Group	Number of subjects	Low selenium 0.492 mg/kg[a]	Mid selenium 0.588 mg/kg[a]	High selenium 0.707 mg/kg[a]
Preeclampsia	53	26	13	14
Control	53	9	22	22

[a]Mean toenail selenium concentration

The GPxs reduce lipid hydroperoxides (or H_2O_2) to harmless alcohols (or H_2O) thus reducing oxidative stress [34]. Both Txnrd, the most highly expressed selenoenzyme in endothelial cells, and GPxs have been shown to protect endothelial cells from oxidants including oxidized LDL [34–40]. SEPP1, which is recruited to the endothelium in areas of inflammation, can scavenge the powerful inflammatory agent peroxynitrite, thereby reducing oxidative stress and inflammation and shielding endothelial membranes from its attack [41–43]. By controlling the cellular levels of reactive fatty acid hydroperoxides, GPxs can modulate the production of biologically active eicosanoids by the cyclooxygenase (COX) and lipoxygenase (LOX) pathways [34]. In selenium deficiency, endothelial cells produce enhanced amounts of pro-apoptotic 15-hydroperoxyeicosatetraenoic acid (15-HPETE) and proaggregatory thromboxane and decreased amounts of antiaggregatory prostacyclin thereby contributing to vascular dysfunction, a feature of preeclampsia [44, 45].

Selenium aids in the shunting of arachidonic acid toward endogenous anti-inflammatory mediators as an adaptive response to protect cells against proinflammatory gene expression induced by oxidative stress (Fig. 42.2) [35, 36, 46–57]: thus selenium supplementation in macrophages increases the production of 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) (by COX-1), an endogenous inhibitor of a key kinase of the NF-κB cascade, IκB-kinase β (IKKβ) [46]. This results in decreased activation of NF-κB and downregulates expression of inflammatory genes such as COX-2, TNF-α, IL-6, and vascular cell adhesion molecule-1 (VCAM-1) [46, 47]. In a second selenium-dependent anti-inflammatory mechanism acting through 15d-PGJ_2, selenium-supplemented macrophages activate the peroxisome proliferator-activated nuclear receptor-γ (PPAR-γ), repressing inflammatory gene expression [48, 49]. In inflammation, endothelial cells produce adhesion molecules that recruit leukocytes to the site of injury where they can penetrate and inflame the vasculature, activating NF-κB and increasing COX-2 expression [34, 58]. Selenium supplementation or phospholipid hydroperoxide GPx overexpression was able to block the cytokine-induced expression of adhesion molecules in endothelial cells [59–61], whereas in selenium deficiency, enhanced mRNA expression and neutrophil adherence were observed (Fig. 42.2) [62].

Heme oxygenase-1 (HO-1) is an inducible enzyme that is upregulated in oxidative stress with cytoprotective and anti-inflammatory functions linked to its removal of the pro-oxidant, heme, and its production of the antioxidant bilirubin and the vasodilatory, anti-inflammatory, carbon monoxide (CO) [50, 51]. The HO system has been linked to antioxidant effects, successful placentation, inhibition of soluble vascular endothelial growth factor (VEGF) receptor protein (otherwise known as

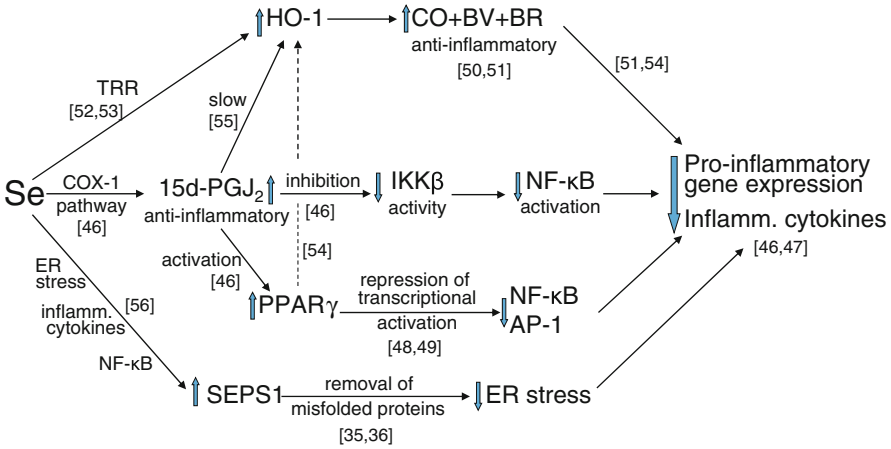

Fig. 42.2 Mechanisms by which selenium reduces inflammation. Selenium may reduce inflammation resulting from oxidative and ER stress by several possible mechanisms [35, 36, 46–56], which are shown in the figure. *ER* endoplasmic reticulum; *SEPS1* selenoprotein S; *15d-PGJ2* 15-deoxy-Δ12,14-prostaglandin J2; *IKKβ* IκB-kinase β; *TRR* thioredoxin reductase; *BV* billiverdin; *BR* billirubin (modified from Rayman [57] with permission from Humana/Springer, New York)

fms-like tyrosine kinase 1, sFlt-1) release, uterine quiescence, placental hemodynamic control, regulation of the apoptotic and inflammatory cascades in trophoblast cells, and protection against abortion [50–51, 63–65]. Txnrd is required for the expression of HO-1 in endothelial cells during pro-oxidant challenge [52] and selenium has been shown to upregulate HO-1 by a number of pathways that involve selenium or the thioredoxin/Txnrd system either directly or indirectly, resulting in reduced expression of proinflammatory genes [52–55] (Fig. 42.2).

Perhaps, the most important role of selenium in affecting the risk of preeclampsia, which is above all an inflammatory condition [31], is its role in SEPS1. SEPS1 is an ER membrane protein involved in the control of inflammation and ER stress [35, 36]. It helps remove stressor-induced misfolded proteins from the ER [35, 36], preventing their accumulation and the subsequent stress response that leads to activation of NF-κB, proinflammatory cytokine gene transcription, and the inflammation cascade (see Fig. 42.2). Genetic variation in SEPS1 has been shown to influence the inflammatory response [66]. Impairment of SEPS1 is directly associated with increased cellular cytokine production and release [66]. During the acute-phase response in mice, SEPS1 appears to be expressed at the expense of SEPP1 in the liver but the increased synthesis depends on selenium status being sufficiently high [36]. The ER is the location of at least six other selenoproteins, some of which may also contribute to the reduction of placental ER stress [35].

SEPS1 has been implicated specifically in preeclampsia risk. A retrospective study in a large Norwegian case-control cohort compared maternal genotype and allele frequencies of the *SEPS1* g-105G>A polymorphism in preeclamptic ($n = 1{,}139$) and control ($n = 2{,}269$) women [2]. Women with preeclampsia were 1.34 times more

likely to have the GA or AA genotype ($P=0.0039$; 95% CI, 1.09–1.64) and 1.22 times more likely to carry the A allele ($P=0.023$; odds ratio, 1.22; 95% CI, 1.02–1.46) showing that the A allele of this polymorphism is a significant risk factor for preeclampsia in that population.

Despite the many potential pathways outlined above by which selenium could affect the risk of preeclampsia, other than the Chinese trial in pregnant women at risk of pregnancy-induced hypertension [20], to date, there has been no randomized, placebo-controlled trial of selenium in a pregnant population with a view to affecting the risk of preeclampsia. My colleagues and I are currently running a randomized, placebo-controlled trial (SPRINT) of low-dose selenium (60 μg/day as high-selenium yeast) in 200 UK pregnant women (primiparae) of relatively low selenium status to see if we can reduce markers of risk of preeclampsia.

42.3 Miscarriage

First-trimester pregnancy loss affects up to 15% of clinically recognized pregnancies but 2–4% of couples will suffer recurrent losses, often with no identifiable cause [67].

Idiopathic miscarriage has been shown to be associated with selenium deficiency in veterinary practice [68]. Only one human study has reported on selenium status in spontaneous miscarriage: significantly lower serum selenium was found in 40 UK women who miscarried in the first trimester compared to 40 pregnant women of similar gestational age attending the same antenatal clinic [69]. In a later study, the same investigators found significantly lower serum selenium in 25 women who miscarried in the first trimester (52.1 μg/L) than in 12 (nonpregnant) recurrent aborters (67.1 μg/L) and 25 nonpregnant controls (76.6 μg/L) [70]. The selenium concentration in the first-trimester miscarriage group was significantly lower ($P=0.011$) than that in the recurrent miscarriage group and the selenium concentration in the recurrent miscarriage group was significantly lower than that in the control group ($P=0.014$). In another UK study, there was evidence of selenium deficiency in 26 women with a history of recurrent miscarriage compared with a control group of 18 women with a good reproductive performance [71]. The authors comment that the difference was seen in hair samples but not serum samples and therefore might not represent a simple nutritional deficiency. Similarly, an Indian study found significantly lower red-cell selenium in 20 (nonpregnant) women with three or more unexplained recurrent pregnancy losses than in a similar number of nonpregnant controls with no history of miscarriage [mean (SD) 119.55 (32.94) vs. 150.85 (37.63) μg/L, respectively; $P<0.01$] [72].

However, not all studies are in agreement with these findings: whole-blood and plasma concentrations of selenium were no different in Polish women who had just miscarried than in women of the same gestational age with viable pregnancy, though their red-cell and plasma GPx activities were significantly lower [73]. A Scottish study found lower selenium levels in nonpregnant women suffering recurrent

miscarriage than in controls, but the difference did not reach significance [74]. However, the choice of control group can be criticized in this study as it did not exclude women who had suffered a miscarriage.

In interpreting the results of these studies, it is important to be aware that selenium status falls in pregnancy [75], partly because of plasma volume expansion, but excessive inflammation, a probable part of the picture in miscarriage [76], will also lower circulating selenium [29, 77]. Though we cannot say that low selenium status is a causative factor in miscarriage, there are certainly credible mechanisms by which selenium inadequacy could be associated with pregnancy loss, as described below.

42.3.1 How Might Selenium Affect the Risk of Miscarriage?

If low selenium status were indeed a risk factor, what might be the explanation? Miscarriage has a number of features in common with preeclampsia [31]. Both are likely to be associated with failure, or partial failure, of immunoregulatory mechanisms that prevent rejection of paternal alloantigens; both involve defective placentation, impaired placental perfusion, and excessive placental oxidative stress; both are characterized by excessive inflammation [31, 76]. At the extreme, the outcome is pregnancy loss; if the pregnancy continues, the result may be preeclampsia [31]. Thus, many of the mechanisms discussed above in relation to the effect of selenium on the risk of preeclampsia will also be relevant to its potential effect on miscarriage.

It is probably worth emphasizing here the importance of HO-1 in relation to miscarriage: physiological pathways that protect the fetus from rejection are thought to be similar to those leading to allograft acceptance. HO-1 protects against rejection in transplantation models due to its antioxidant, anti-inflammatory, and cytoprotective functions [32]. Upregulation of HO-1 expression diminished fetal rejection and abortion rates in a murine abortion model [32]. Furthermore, HO-1 upregulation may also augment the levels of Tregs, improving immune suppression [32]. As explained above, selenium/Txnrd may upregulate the expression of HO-1 by a number of pathways [52–55] (see Fig. 42.2), which would be protective against abortion.

42.4 Preterm Birth

Preterm birth, defined as birth before 37-weeks gestation, occurs in 5–13% of pregnancies and is the most important cause of perinatal morbidity and mortality [78]. Short- and long-term health sequelae include cerebral palsy, respiratory distress syndrome, neurodevelopmental impairment, difficulties with schooling, and behavioral problems [79].

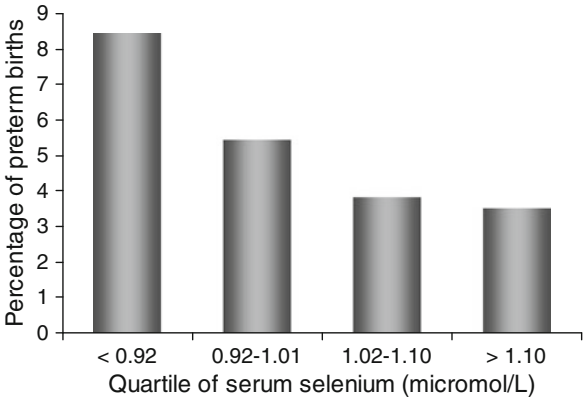

Fig. 42.3 Percentage of preterm births by quartile of serum selenium in a Netherlands study of 1,129 women followed prospectively from 12-weeks gestation (Rayman et al. [80])

In a Netherlands study of 1,129 pregnant women followed prospectively from 12-weeks gestation, 60 women (5.3%) had a preterm birth [80]. The commonest causes of preterm delivery were preterm premature rupture of membranes (PPROM, $n=21$) and preeclampsia ($n=13$), together accounting for 57% of the preterm births. Those who delivered preterm had significantly lower serum selenium at 12-weeks gestation than those who delivered at term [mean (SD): 75.8 (11.1) and 80.5 (10.3) μg/L, respectively, $P=0.001$] [80]. The percentages of women with preterm birth by quartile of serum selenium at 12 weeks were significantly different ($\chi^2=8.01$, d$f=3$, $P<0.05$) (Fig. 42.3). Even after adjusting for the occurrence of preeclampsia, which is associated both with selenium status (see above, and [27]) and with preterm birth, women in the lowest quartile of serum selenium at 12-weeks gestation had twice the risk of preterm birth as the rest (adjusted OR 2.18; 95% CI 1.25–3.77). These results suggest that low selenium status in early gestation may increase the risk of preterm premature rupture of membranes, a major cause of preterm birth [78], as seen in this population.

The above study does not show that low selenium status *caused* preterm birth. Both preterm birth and low plasma selenium may have been joint outcomes, for instance, of increased inflammation [80]. Plasma selenium concentration decreases in proportion to the magnitude of the inflammatory response while the concentration of plasma SEPP1, a component of plasma selenium, declines with inflammatory activity and cytokine production [29, 77]. However, the significant reduction also seen in the incidence of premature (though not exclusively preterm) rupture of membranes with selenium supplementation in a small, randomized controlled trial in Iran suggests that selenium status may indeed be relevant [81].

42.4.1 How Might Selenium Affect the Risk of Preterm Birth?

If causative, what mechanisms might account for the relationship between low first-trimester selenium and preterm birth? Selenium status in the Netherlands is

relatively low [82]. Low selenium status could increase the risk of preterm birth because selenium (probably as selenoproteins) has a number of protective effects that are directly relevant to pathways implicated in preterm birth or its subcategories, preterm, premature rupture of membranes and preeclampsia, as explained below. These pathways include infection, inflammation, defective placentation, placental ischemia-reperfusion, oxidative stress, the presence of antithyroid antibodies, and premature extracellular matrix degradation of fetal membranes [78, 83–85].

Selenium is required for an adequate immune response [86], and therefore, low selenium status in either the mother or the fetus is a risk factor for infection, a major cause of preterm birth [78]. Inflammation may be an underlying factor linking many of these pathways as suggested by the fact that polymorphisms that increase the magnitude or duration of the inflammatory response were associated with an increased risk of preterm birth, while those that decrease the inflammatory response were associated with lower risk [87]. Selenium is capable of attenuating the excessive inflammatory response associated with adverse pregnancy outcomes by a number of mechanisms that have been explained above (see Fig. 42.2) [2, 34, 36, 46–57]. Defective placentation and placental ischemia-reperfusion are both counteracted by HO-1, which is upregulated by a number of pathways that involve selenium or the thioredoxin/Txnrd system, either directly or indirectly (see Fig. 42.2) [52–55]. Oxidative stress is counteracted by the GPxs [34], by SEPP1 (scavenging peroxynitrite [34, 41]), and by the antioxidant effects of the products of HO-1 (biliverdin and/or bilirubin) [50, 51, 63]. Higher selenium status or supplementation with selenium appears to be able to reduce the titer of thyroid peroxidase antibodies (TPO-Ab), the most common form of antithyroid antibodies [88] (see next section). Finally, selenium species have been shown to decrease the ratio of matrix metalloproteinases to tissue inhibitors of matrix metalloproteinases [89]: this ability may potentially reduce the risk of fetal membrane rupture, a characteristic feature of preterm birth [85].

42.5 Autoimmune Thyroid Disease

Autoimmune thyroid disease, the most common endocrine disorder in women of reproductive age, has a prevalence ranging between 5 and 20% [90]. It represents the main cause of hypothyroidism in pregnant women [90]. While the incidence of gestational hypothyroidism is some 2.4%, thyroid autoantibodies are present in 55–80% of these women [90]. The commonest form of autoimmune thyroid disease (Hashimoto's thyroiditis) is characterized by the presence of complement-fixing autoantibodies to thyroid peroxidase (TPO-Ab) [88]. Some 6% of pregnant women have TPO-Abs [91] though the titer shows a tendency to decrease toward term, reflecting the downregulation of the immune system during gestation [92]. Although TPO-Abs are regarded as an epiphenomenon of autoimmune thyroid disease and are not regarded as harmful, they do tend to correlate with progressive thyroidal damage and lymphocytic inflammation [93]. Furthermore, an elevated TPO-Ab titer

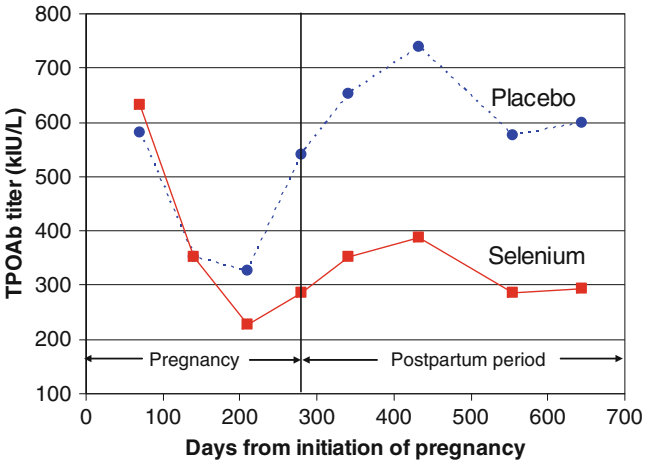

Fig. 42.4 Thyroid peroxidase antibody (TPO-Ab) titers in 151 TPO-Ab-positive Italian women randomized to selenium (200 μg/day as selenomethionine) or placebo during pregnancy and the postpartum period. Values are significantly different between groups at delivery (280 days) and in the postpartum period (modified from Negro et al. [98])

is associated with poor obstetric outcome including an increased risk of miscarriage [94], perinatal mortality [95], placental abruption [91], and preterm premature rupture of membranes [96].

Selenium is important to the thyroid. Not only is it a component of the iodothyronine deiodinase selenoenzymes that convert thyroxine (T_4) to tri-iodothyronine (T_3) and reverse T_3 (rT_3), but is also a component of GPx3 which protects thyroid cells from the hydrogen peroxide that is generated there to be used by TPO for the synthesis of T_4 and T_3 from iodide and the tyrosyl residues of thyroglobulin [97]. This protective function may be the basis of the beneficial effect of selenium supplementation on autoimmune thyroiditis [88]. A recent systematic review and meta-analysis found that selenium supplementation (200 μg/day selenomethionine or sodium selenite) for 3 months significantly decreased TPO-autoantibody titers [88]. More important in the context of pregnancy is the beneficial effect observed in a randomized controlled trial of selenium supplementation in reducing thyroid inflammatory activity and the risk of postpartum thyroid disease in TPO-Ab-positive women in Italy [98]. During pregnancy and the postpartum period, 151 TPO-Ab-positive women were randomized to selenium (200 μg/day as selenomethionine) or placebo. Both groups displayed a significant reduction of TPO-Ab during gestation, but the reduction was significantly greater in the selenium-supplemented group ($P=0.01$) and remained so in the postpartum period ($P=0.01$) (see Fig. 42.4). Importantly, there was a significant reduction in the incidence of postpartum thyroid disease and hypothyroidism in the selenium-supplemented group (28.6 vs. 48.6%, $P<0.01$ and 11.7 vs. 20.3%, $P<0.01$, respectively) [98].

Reinforcing these findings is our own prospective cohort study in 1,129 pregnant women from the Netherlands followed from early gestation until delivery, in

whom selenium status and thyroid parameters were assessed (Pop, Rayman et al. unpublished work). We found that low maternal selenium status in early gestation was related to markedly elevated TPO-Ab titers and to the persistence of elevated titers throughout gestation.

42.5.1 How Might Selenium Affect the Risk of Autoimmune Thyroid Disease in Pregnancy?

Pregnancy represents a considerable challenge to the thyroid as a woman has to increase her production of T_4 by 50% to maintain maternal euthyroidism and transfer thyroid hormone to the fetus early in the first trimester, before the fetal thyroid is functioning [99]. The increased synthesis of thyroid hormones required in pregnancy triggers a rise in the production of hydrogen peroxide that is used by TPO in the multistep synthesis of T_4 from iodide and thyroglobulin [97]. As hydrogen peroxide is damaging, any excess must be removed for the protection of the thyroid, largely by GPx3, which is highly expressed in the thyrocytes [97]. Thus, the requirement of the thyroid for selenium in pregnancy probably increases above the non-pregnant level and may allow differential effects of selenium status to be seen, at least in populations of low or relatively low selenium status such as those of Italy and the Netherlands, as described above.

TPO-Abs tend to correlate with lymphocytic inflammation of the thyroid. Hence, apart from the specific role of selenium in GPx3, the anti-inflammatory effects of selenium, probably as selenoenzymes, might play a role in downregulating TPO-Abs during gestation. These effects can occur through a number of different pathways that downregulate proinflammatory gene expression and ER stress, as already outlined above [2, 34, 36, 46–57] (see Fig 42.2).

42.6 Concluding Comments

The studies described above have mostly been carried out in countries with low or relatively low selenium intake or status. Thus, these findings cannot be extrapolated to countries with a higher selenium intake.

Though credible mechanisms have been described above by which selenium might affect the risk of adverse pregnancy outcomes, the volume of quality evidence is still low; the most convincing data are for an effect of selenium on autoimmune (TPO-Ab) thyroid disease and on preeclampsia. Randomized controlled trials in pregnant women, such as the one we are running in the UK, are required for proof. However, it is becoming increasingly difficult to recruit subjects to such trials in developed countries, as the percentage of pregnant women taking supplements that contain selenium is now very high (63% in our Oxford study). This figure is not

surprising given the plethora of specialized supplements marketed to pregnant women and the fact that in a recent US general population survey (NHANES 2003–2006), 53% of females sampled reported supplement usage and one-third of the population used a multivitamin-multimineral supplement; such supplements almost always contain selenium [100]. Randomized controlled trials of any nutrient in pregnancy may soon be a thing of the past making it impossible to prove or disprove any nutrient-related hypothesis.

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Part IV
Mouse Models for Elucidating the Role of Selenium and Selenoproteins in Health

Chapter 43
Mouse Models for Glutathione Peroxidase 4 (GPx4)

Marcus Conrad

Abstract The selenoperoxidase glutathione peroxidase 4 (GPx4 – also frequently referred to as phospholipid hydroperoxide glutathione peroxidase, PHGPx) is one of the eight glutathione peroxidases in mammals, but the only one known to be essential for early mouse development. GPx4 is emerging as one of the most central selenoproteins, and thus has attracted considerable interest in recent years. Key insights into GPx4 function came from the numerous transgenic and knockout mouse studies performed mainly during the last couple of years, which are summarized here. These investigations not only firmly established a crucial role for GPx4 in male fertility and neuroprotection, but also indicated a major regulatory role of GPx4 in oxidative stress-induced cell death signaling. Beyond this, lipid hydroperoxides (LOOH), downstream of GPx4 inactivation, have been recently shown to control receptor tyrosine kinase (RTK) signaling, thus adding a new layer of complexity to the multifaceted roles of GPx4 in cell signaling and disease development.

43.1 Introduction

GPx4 was discovered in 1982 by Ursini's laboratory [1] as a monomeric selenoperoxidase of mammals that efficiently inhibits lipid peroxidation and that is homologous to the previously known tetrameric Sec-containing cytosolic glutathione peroxidase (GPx1). Both enzymes use glutathione (GSH) as a reductant, but

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only GPx4 reduces lipid hydroperoxides (LOOH) in membranes besides small hydroperoxides such as H_2O_2 or free fatty acid hydroperoxides. GPx4 is one of the eight mammalian glutathione peroxidases (GPx) (GPx7 and GPx8 are just predicted) whereby only GPx1, 2, 3, 4, and 6 (in humans) carry selenocysteine (Sec) in their redox-active site. GPx4 is unusual, not only for its structural and catalytic traits, but also for its multifaceted functions in physiological processes e.g., sperm development and neuroprotection and in signaling pathways such as apoptosis regulation (see Sect. 43.6) and also in controlling receptor tyrosine kinase (RTK) signaling (see Sect. 43.7).

GPx4 is monomeric, unlike the most abundant mammalian GPx, GPx1, which forms tetramers. Despite its relatively small size, GPx4 has a broad substrate specificity not only for its substrates such as (phospho)LOOH, but also with regard to its cofactor GSH. Under physiological conditions GSH, present in somatic cells up to 10 mM, is the favored electron source. But under very low GSH concentrations, as physiologically evident in maturing sperm cells, GPx4 also accepts electrons from protein thiols. Thereby, GPx4 turns into a moonlighting enzyme and introduces disulfide bridges into sperm proteins in vivo (see Sect. 43.3). Three *GPx4* isoforms originate from a single gene (Fig. 43.1a), a cytosolic (also called the short form), a mitochondrial (also called the long form), and a nuclear form. While the short form is expressed ubiquitously in embryonic and adult tissues in varying degrees and confers a vital function [2], expression of the other two forms is restricted to testis where they exert significant functions during male gametogenesis (see Sect. 43.3).

Similar to GPx1, GPx4 kinetics follows a ping-pong mechanism. The selenolate is first oxidized by the hydroperoxide and then reduced back by GSH. Thereby, the formation of an enzyme-substrate complex, if any, does not limit the overall reaction rate [3]. Reduction requires two consecutive steps where the mixed selenenylsulfide between GSH and the Sec moiety is reduced by the second GSH [3]. The reactions of selenoperoxidases are among the fastest ever detected for bimolecular reactions. For instance, the rate constant for the reaction with phosphatidylcholine hydroperoxide (k_{+1}), is around 10^7 Mol^{-1} s^{-1} for GPx4. Owing to its extremely fast reactivity toward various peroxides, GPx4 protects many cell types and tissues from deleterious insults such as pro-oxidants, DNA-damaging agents, glucose depletion, and irradiation (reviewed in [4]). This chapter aims at summarizing data mainly obtained by gain-of-function and loss-of-function studies involving GPx4 in mice and cells derived from these mouse models.

43.2 Trangenic Mouse Models for GPx4

Already in 1997, Bosl and colleagues demonstrated that the simultaneous loss of selenoprotein expression in mice due to knockout of Sec tRNA gene (*trsp*) causes early embryonic lethality around gestation, implying that at least one or several selenoproteins are prerequisites for early embryonic development in mice [5]. In the same year it was also noticed that mice lacking GPx1 develop normally and are

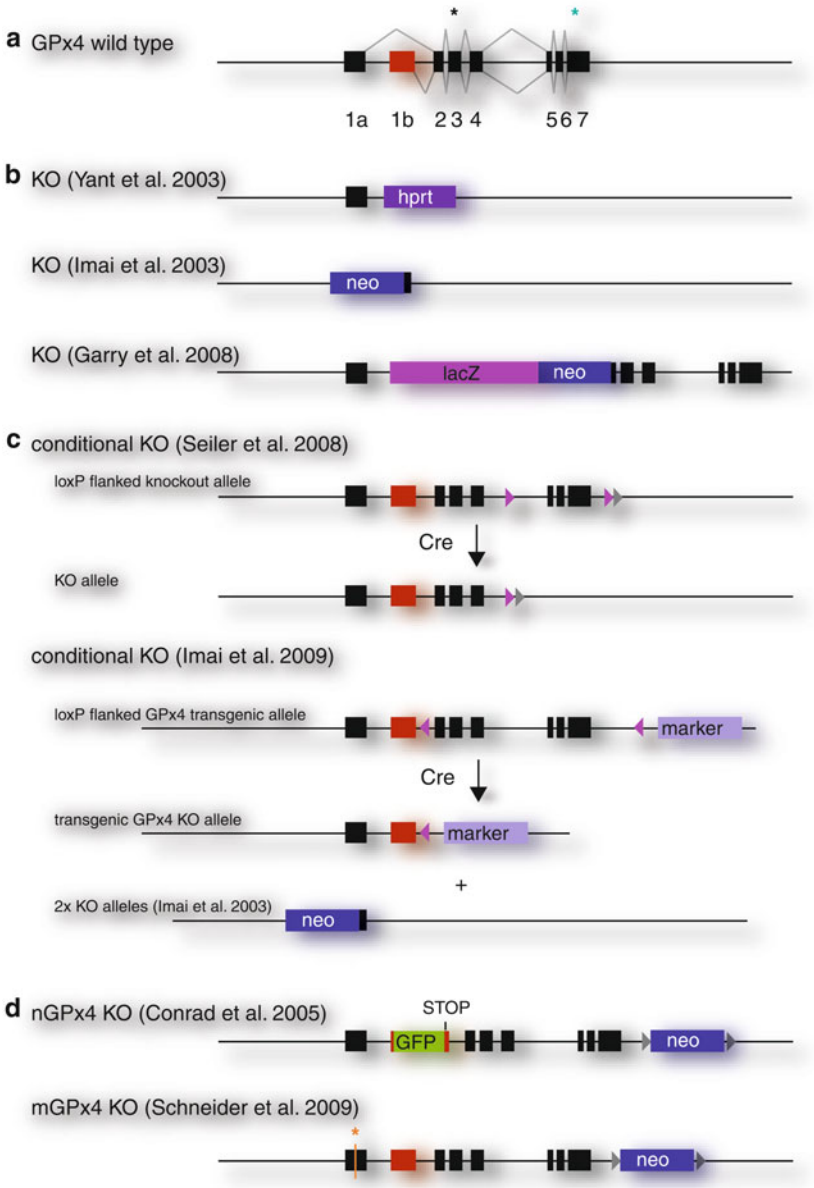

Fig. 43.1 Schematic overview of Glutathione Peroxidase 4 (GPx4) knockout mouse models. (**a**) The wild-type GPx4 gene consists of 7 "classical" exons (*filled boxes*) and one exon, which is almost exclusively expressed in late sperm cells (*red box*, (**b**)). Splicing, which generates the different GPx4 isoforms, is indicated by *lines*. *Black* and *green asterisks* point to the localization of the UGA codon and the SECIS element, respectively. The constitutive *GPx4*$^{-/-}$ models are shown in (**b**) and the conditional GPx4 knockout models in (**c**). (**d**) Knockout mouse models for nuclear and mitochondrial GPx4 isoforms are shown (*gray triangles* indicate frt sites and magenta triangles loxP sites. *hprt* hypoxanthine-guanine phosphoribosyltransferase; *neo* neomycin phosphotransferase; *lacZ* β-galactosidase; *GFP* green fluorescent protein)

apparently healthy, and fertile under normal housing conditions [6]. Subsequent knockout studies with gastro-intestinal glutathione peroxides (GPx2), deiodinase 2, SelP or even compound mutant mice for GPx1 and GPx2 failed to identify a single selenoprotein being responsible for the lethal phenotype as observed in *trsp* knockout mice (reviewed in [7]).

In 2003, two laboratories almost simultaneously reported on the embryonic lethal phenotype of knockout for an individual selenoprotein, namely GPx4 [8, 9]. Targeted removal of either exons two to seven of the *GPx4* locus [8] or the entire *GPx4* gene locus [9] was shown to cause embryonic death around E7.5 (Fig. 43.1b), whereas one copy of *GPx4* apparently was sufficient to allow normal embryonic development, male fertility, and an almost normal life span compared to wild-type mice. Further knockout studies of individual selenoproteins disclosed that loss of either cytosolic thioredoxin reductase (Txnrd1) or mitochondrial thioredoxin reductase (Txnrd2) was embryonic lethal, albeit at later stages [10, 11]. Garry and colleagues published the third knockout model for GPx4 where a part of exon 1, intron 1, and a small part of exon 2 were replaced by a lacZ/neo cassette [12] (Fig. 43.1b). Embryonic lethality of GPx4 null mice could be confirmed with this model and lung fibroblasts with only one *GPx4* allele were more sensitive toward oxidative stress including treatment with hydrogen peroxide, cadmium chloride, and cumene hydroperoxide [12]. Despite these efforts, the underlying causes of embryonic death of GPx4 deficiency remained to be elucidated.

To bypass embryonic lethality of GPx4 knockout mice and to study the molecular mechanisms of GPx4 in cell signaling pathways, a conditional knockout mouse for GPx4 was developed by our laboratory [13]. Thereby, the last three exons of the *GPx4* gene including the SECIS element were flanked by loxP sites which can be targeted by Cre recombinase (Fig. 43.1c). Ubiquitous Cre-mediated deletion of the loxP-flanked allele and subsequent intercross of $GPx4^{+/-}$ mice yielded the same embryonic lethal phenotype as already observed for the aforementioned constitutive knockout approaches. This implies that removal of the last three exons is sufficient to disrupt GPx4 function [13]. Phenotypes obtained by tissue-specific inactivation of GPx4 and with inducible $GPx4^{-/-}$ cells are described in the following subsections.

Finally, Imai and colleagues generated another conditional knockout mouse model [14], where they took advantage of their previously established knockout model [9]. Thereby, a transgenic mouse model was generated where exons 2–7 were flanked by loxP sites. Expression of this transgene is driven by a 5 kBp upstream promoter region and this construct was able to rescue the embryonic lethal phenotype of *GPx4* null mice [14]. To achieve tissue-specific GPx4 inactivation, the conditional GPx4 transgenic allele was crossbred on the $GPx4^{-/-}$ background, along with spermatocyte-specific Cre-expressing mice [14]. This model not only allowed to recapitulate some of the cellular findings described by our model [13], but also showed that GPx4 is essential for the early stages of spermatogenesis [14] (see Sect. 43.3). More specific knockout models for the specific GPx4 variants are described in Sect. 43.3 (Fig. 43.1d).

As the early constitutive knockout approaches for GPx4 did not allow investigation of GPx4 functions in adult tissue in homozygous mutant mice, various studies

have been conducted with *GPx4*$^{+/-}$mice and cells derived thereof (reviewed in [4]). For instance, cells with only one *GPx4* allele are sensitized to oxidative stress challenges such as hydroperoxides, paraquat, and γ-irradiation [8, 15]. Similarly, overall survival of *GPx4*$^{+/-}$ mice is reduced in response to relatively high doses of γ-irradiation, whereas, perhaps unexpectedly, one allele provides some survival advantage as *GPx4*$^{+/-}$ mice have a slightly longer life span compared to wild-type counterparts [16]. The underlying reasons are altered pathologies including delayed occurrence of fatal lymphoma and reduced severity of glomerulonephritis. In terms of tumor development, one may hypothesize that GPx4 may act in a way like an oncogene by protecting developing tumors from apoptosis.

Besides these knockout studies, various gain-of-function models for GPx4 have been created with forced expression of the short or the long form of GPx4 either in the entire body or in certain tissues of the mouse (reviewed in [4]). In all cases, *GPx4* transgenic mice or cells isolated thereof were protected from many deleterious insults such as diquat-induced liver damage [17], paraquat- and hyperoxia-induced retinal degeneration and function [18], ischemia/reperfusion-triggered cardiac dysfunction [19], and reduced atherosclerotic lesions in the aorta of *GPx4* transgenic mice when crossed on the *ApoE*$^{-/-}$background [20] (see also Sects. 43.5 and 43.6).

The combination of GPx4 transgenic mice and *GPx4*$^{-/-}$ mice also allowed to unequivocally assign, which of the three GPx4 isoforms is the vitally important one for early mouse development. While mice transgenic for the mitochondrial form failed to rescue *GPx4*$^{-/-}$ mice from embryonic lethality, it emerged that overexpression of just the short form is sufficient to compensate embryonic lethality [2]. This supported the findings from Imai's laboratory, which showed that expression of cytosolic GPx4 with the Sec left intact rescues cell death induced by GPx4 inactivation in mouse embryonic fibroblasts (MEFs) [14]. By contrast, overexpression of the mitochondrial form was only partly protective and overexpression of the nuclear form had no rescuing effect.

43.3 The Versatile Functions of GPx4 in Male Gametogenesis

For many decades, Se was recognized as an important factor for male fertility [21, 22]. Spermatozoa from animals maintained on a Se-deprived diet are immotile and display major lesions, such as breaks in the neck and hairpins between the principal piece and the mid-piece and giant heads [23, 24]; yet it has remained unknown for quite some time, which selenoenzyme and which molecular function limits mammalian spermatogenesis. A major breakthrough in selenoprotein research with regard to male mammalian fertility was achieved in 1999 by a joint effort of Ursini's and Flohé's laboratories. GPx4 was found to make up the major structural component of the mitochondrial capsule, which confers the structural stability for the mid-piece of mature spermatozoa [25]. Thereby, GPx4 occurs in a catalytically inactive form cross-linked to high molecular mass complexes with other capsular proteins

most likely through disulfide and selenenyldisulfide bridges [26]. The mechanism of GPx4 inactivation is thought to be related to GSH depletion (GPx4 is promiscuous also for its reducing substrate), which physiologically occurs during germ cell maturation and which is a basic requirement to enable the many oxidative steps during sperm maturation [27]. Yet these studies did not allow to discriminate, which of the different isoforms is responsible for male fertility. The nuclear form of GPx4 was first described only by 2001 [28]. The sperm-nuclei-specific selenoenzyme [28], an N-terminal variant of GPx4 [28, 29], was initially believed to be the crucial form for male fertility. But, as shown later, specific knockout of the nuclear form of GPx4 had hardly any impact on male fertility (Fig. 43.1d), despite increased levels of free thiols in sperm nuclei of knockout animals due to lack of thiol peroxidase function of GPx4 as discussed under Sect. 43.1 [30].

It was not until 2009, when it was shown for the first time that lack of a distinctive GPx4 isoform, mitochondrial GPx4 (mGPx4), causes male infertility [31]. To generate mGPx4 null mice [31], an in-frame stop codon was inserted amid the mitochondrial and the cytosolic start codons, leading to disruption of the mGPx4 with no impact on the expression of cGPx4. This was deemed necessary as it was hypothesized that the cytosolic form is the essential isoform for murine embryogenesis. Like *nGPx4*$^{-/-}$ mice, *mGPx4*$^{-/-}$ mice are fully viable [31]. The major finding of this study, however, was that male knockout mice are infertile and isolated sperm failed to fertilize oocytes in vitro due to the severe impairment of sperm motility and progressivity. Isolated *mGPx4*$^{-/-}$ spermatozoa revealed severe morphological abnormalities particularly in the midpiece of mature spermatozoa (Fig. 43.2), reminiscent of sperm derived from Se-deprived rodents. Hence, these studies provided unequivocal proof that mGPx4 confers the vital role of Se in male fertility. On the contrary, mitochondrial GPx4 emerged as being dispensable for apoptosis regulation, which is different from previously drawn conclusions, where an essential role in apoptosis regulation had been postulated for mGPx4. Since neither nuclear GPx4 nor mitochondrial GPx4 plays a major role in embryonic development, it was concluded that the cytosolic variant must be the vital form for early embryogenesis. Thus, conditional disruption of GPx4 in tissues other than testis will disclose the function of the cytosolic form. In fact, direct experimental proof that the short form of GPx4 is the essential form for embryo development was reported by Liang and colleagues [2] (see Sect. 43.2). Although transgenic expression of the short form of GPx4 alone is sufficient to rescue embryonic lethality when crossbred with *GPx4*$^{-/-}$ mice [2], sperm from these mice phenocopy *mGPx4*$^{-/-}$ spermatozoa [31].

Spermatocyte-specific disruption of GPx4 using *pgk2-Cre* transgenic mice and transgenic mice carrying a loxP-flanked *GPx4* allele on a *GPx4*$^{-/-}$ background (Fig. 43.1c) also causes male infertility due to oligospermia, severe structural abnormalities of isolated sperm, and impaired mitochondrial respiration and thus mitochondrial membrane potential of epididymal spermatozoa [14]. Unfortunately, since Cre expression of *pgk2-Cre* transgenic mice is restricted to late spermatogenic cells including spermatocytes, final proof regarding an essential role for GPx4 in early spermatogenetic cells including germinal cell stem awaits further study.

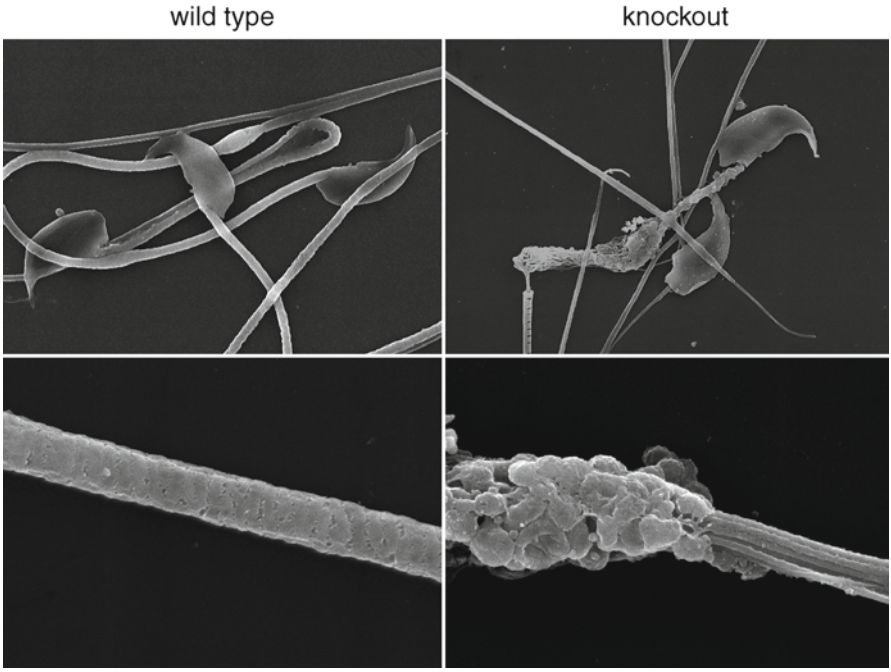

Fig. 43.2 Targeted disruption of the mitochondrial form of GPx4 causes male infertility. Scanning electron micrographs of epididymal sperm of *mGPx4* knockout mice (right panel) reveal many structural aberrations like bends in the midpiece of mature sperm and sliding of mitochondria along the axoneme (the figures have been adopted from [31])

43.4 GPx4 Prevents Neurodegeneration

Se levels are rather low in brain, but they are remarkably stable even after prolonged Se-deficiency in contrast to most other organs [32]. Comparative genome expression analysis revealed that all 24 selenoproteins are in fact expressed in mouse brain and, more specifically, enriched in neurons of brain regions including hippocampus, olfactory bulb, cerebral and cerebellar cortex [33]. Mice lacking the Se transport protein selenoprotein P (SelP) display seizures and ataxia when kept on a Se low diet [34]. Notably, the neurological phenotypes could be reverted by liver-specific SelP transgenic expression alone, indicating that hepatically derived circulating SelP is responsible for the priority supply of the element to the brain [35]. Simultaneous loss of all selenoproteins in neurons, achieved by Cre-mediated knockout of *Trsp* in cortical and hippocampal neurons, caused widespread neurodegeneration and impaired differentiation and function of cortical inhibitory parvalbumin-positive interneurons [36]. Previously, we showed that conditional disruption of GPx4 in functional neurons using the loxP-flanked (floxed) *GPx4* mice and the *CamKIIα-Cre* transgenic mice leads to ataxia, hyperexcitation, and seizures [13]. Due to the dramatic overall phenotype of the mice, neuron-specific GPx4 knockout

mice had to be euthanized around 2 weeks after birth. The manifested pathological changes included the occurrence of numerous pyknotic cells, an increase in TUNEL + cells, and loss of NeuN + cells in the CA3 region of the hippocampus.

Cortical neuronal cultures could only be generated from *GPx4*$^{-/-}$ mice when vitamin E was included in the cell culture medium [13], whereas vitamin E supplementation did not rescue cell death in *Trsp*$^{-/-}$ neurons [36]. This strongly argues that at least one more selenoprotein besides GPx4 is essential for neuronal survival and function, which complies with the findings that neurodegeneration in cortex and hippocampus was more advanced in *Trsp* than in *GPx4* mutant mice [36]. In this context it is noteworthy that brain-specific disruption of Txnrd1 causes massive cerebellar hypoplasia, which seemed not to be the consequence of neuronal dysfunction but rather due to strongly reduced expansion of Bergmann glia cells [37]. Nonetheless, these studies firmly established the essential neuroprotective role for GPx4.

43.5 GPx4 and the Cardiovascular System

In addition to the studies performed in testis and brain, gain-of-function studies revealed that GPx4 overexpression is also protective in the cardiovascular system, particularly when mice were challenged with stress-inducing conditions like ischemia/reperfusion or a high fat-containing diet. More specifically, when mice transgenic for the human GPx4 gene (a genomic fragment encompassing the intact human *GPX4* gene and approximately 30 and 20 kbp of up- and downstream flanking sequences [17]) were crossbred with *ApoE*$^{-/-}$ mice, the extent of lesion formation and tissue levels of F2-isoprostanes were clearly decreased in the aorta of GPx4 transgenic mice [20]. These results comply with the long-standing observation that oxidatively modified low density lipoproteins (LDL) are a major risk determinant and a causing agent in atherosclerosis development [38]. While systemic overexpression of GPx4 was shown to be highly beneficial in terms of disease outcome [20], it remains to be shown which cell type (e.g., monocytes/macrophages, endothelial cells, pericytes) and which enzyme(s) (like NADPH oxidase, NO synthase, myeloperoxidase, xanthine oxidase and/or 12/15-lipoxygenase (12/15-LOX)) is responsible for LDL modification in the development of atherosclerosis. Hence, cell-type-specific disruption of GPx4 will provide a unique tool to shed light into the role of oxidative stress and lipid peroxidation in the complexity of this disease.

Systemic overexpression of mitochondrial GPx4 improves cardiac contractile function following global ischemia/reperfusion insults of the heart and preserves mitochondrial respiration particularly that of complexes III and IV [19]. Also, lipid peroxidation products including malondialdehyde and 4-hydroxyalkenals were found to be decreased in challenged hearts of transgenic mice.

Recent investigations with subcutaneous tumors originated from c-myc and ha-ras transformed *GPx4*$^{-/-}$ MEFs revealed that GPx4 is dispensable for tumor growth; however, tumor angiogenesis appeared to be altered in knockout tumors [39]. The vascular phenotype of GPx4 null tumors was characterized by an increase in

microvessel density and a reduced number of smooth muscle cell-covered vessels. Pharmacological inhibition of 12/15-LOX successfully reversed the phenotype and led to normalization of vessel morphology.

43.6 GPx4 and Oxidative Stress-Induced Cell Death Signaling

Numerous in vitro and in vivo over-expression studies of GPx4 established a very powerful antiapoptotic role for GPx4 in many cell types and tissues (reviewed in [4]). To be able to study the molecular and cellular mechanisms of cell death signaling, we established a 4-OH-tamoxifen (Tam)-inducible *GPx4* ex vivo knockout system from loxP-flanked GPx4 mice [13]. Tam-inducible GPx4 disruption was associated with massive lipid peroxidation and cell death in knockout cells, both of which could be effectively prevented by the lipophilic antioxidant α-Tocopherol [13]. These findings could be confirmed by the second conditional knockout model generated by Imai and colleagues [14]. Further cellular investigations revealed that lipid peroxides are not secondary to oxidative stress, but rather deliberately produced by 12/15-LOX in our model system [13]. Not only could cell death induced by GPx4 disruption be compensated by 12/15-LOX-specific inhibitors, but isolated *12/15-LOX*$^{-/-}$ cells became resistant to experimental GSH depletion. This set of data showed that the cell death progression downstream of GSH depletion or GPx4 inactivation requires functional 12/15-LOX. Furthermore, activation of apoptosis inducing factor (AIF), a pro-apoptotic molecule, was another downstream event in this cell death cascade [13] (Fig. 43.3). Recently, Culmsee's laboratory reported that glutamate treatment of immortalized HT-22 hippocampal neurons causes cellular GSH deprivation, 12/15-LOX dependent lipid peroxidation, Bid activation, which in turn sparks further lipid peroxidation, AIF activation, and cell death [40]. This implies that activation of Bid downstream of 12/15-LOX and upstream of AIF translocation may represent another step in this cell death signaling cascade. Hence, the identification of a distinct cell death signaling pathway of how oxidative stress is sensed via the GSH/GPx4 system and translated into a 12/15-LOX dependent lipid peroxidation that finally activates Bid and AIF (Fig. 43.3), opens promising cues to systematically explore therapeutic interventions in the cure of degenerative diseases [41].

43.7 GPx4 as a Novel Regulator of Receptor Tyrosine Kinase Signaling

Signaling through RTKs including the PDGF β-receptor (PDGFβR) is negatively controlled by protein tyrosine phosphatases (PTPs). Inhibitory and reversible oxidation of the active-site cysteine of PTPs has emerged as a novel general mechanism for PTP regulation [42, 43]. PTP oxidation has been shown after activation of ROS-inducing cell surface receptors, such as RTKs, GPCRs, integrins, B- and T-cell

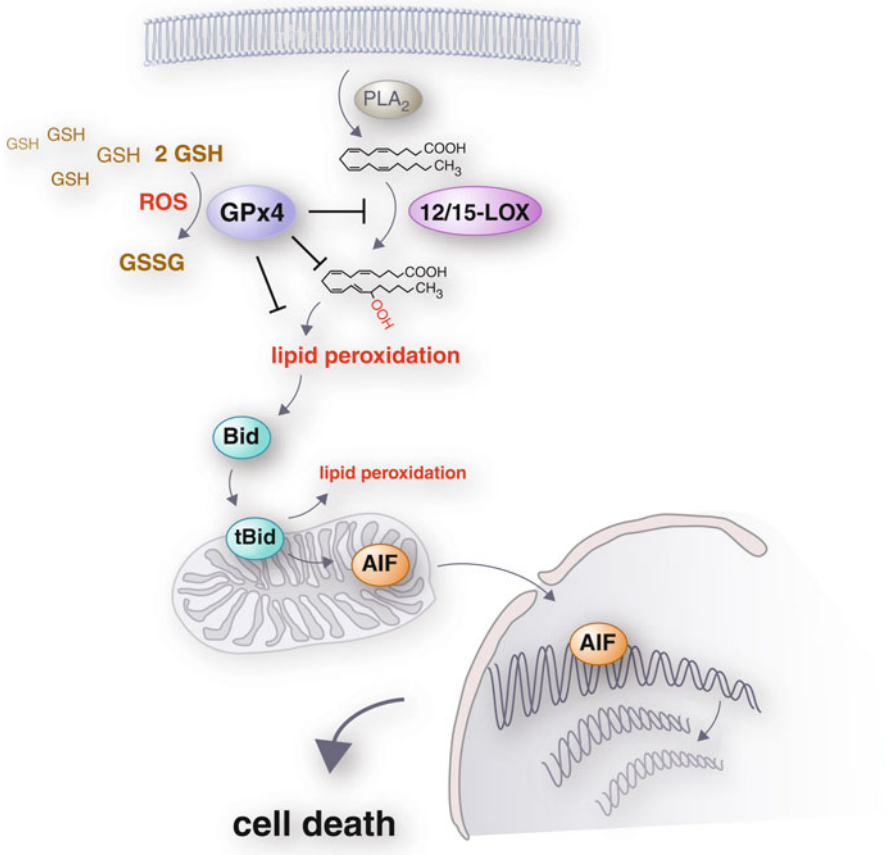

Fig. 43.3 An oxidative stress-induced cell death signaling pathway. Low GSH levels, as evident in many degenerative diseases, cause impaired GPx4 function and aberrant 12/15-lipoxygenase (12/15-LOX) activity. LOOH may induce truncation and activation of Bid (tBid). This, in turn, causes a second peroxide signal which triggers apoptosis inducing factor (AIF) activation and caspase-independent cell death (adopted and modified from [49])

receptors. Manipulation of the expression levels of reducing enzymes such as peroxiredoxin II (PrxII), cytosolic glutaredoxin, and GPx1 has also been shown to affect RTK signaling and PTP oxidation in vitro and in vivo [44–47]. Yet in most of these cases, PTP oxidation could be reverted by the addition of soluble thiol-containing antioxidants such as *N*-acetyl-cysteine (NAC) or DTT, and H_2O_2 has been implied as the main mediator of PTP oxidation.

To investigate the effects of peroxidized lipids on PTP oxidation, we took advantage of the Tam-inducible *GPx4*$^{-/-}$ system [13]. Inducible GPx4 depletion led to a marked increase in cellular PTP oxidation in response to PDGFβR stimulation [48]. *GPx4*$^{-/-}$ cells displayed increased PDGFβR phosphorylation, which was sensitive to vitamin E and 12/15-LOX inhibition but not to NAC or high concentrations of

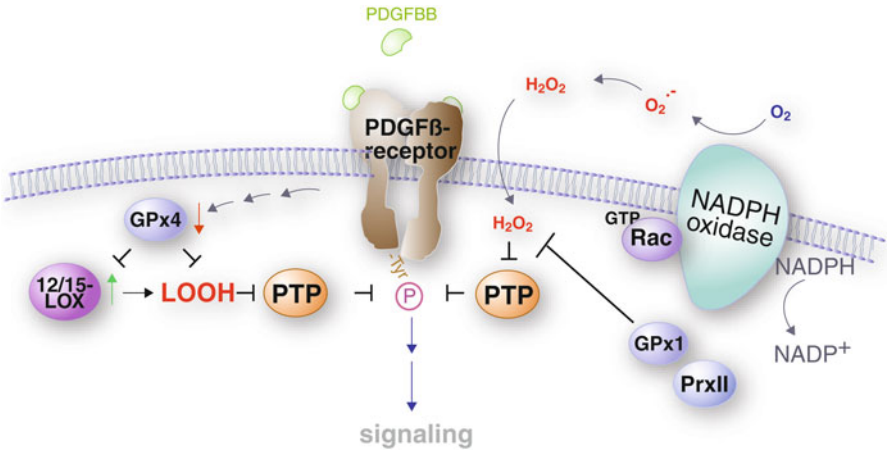

Fig. 43.4 A novel pathway describing how lipid hydroperoxides (LOOH) regulate receptor tyrosine kinase (RTK) signaling. The well-established concept of PTP regulation by oxidation is shown on the *right*. Stimulation of the receptor triggers NADPH oxidase dependent formation of superoxide anion (O_2^-) and H_2O_2. H_2O_2 enters the cell and oxidizes the catalytically active thiolate of PTP's. Glutathione peroxidase 1 (GPx1) and peroxiredoxin II (PrxII) have been shown to control H_2O_2 levels, and thus RTK signaling output. We propose that LOOH effectively oxidize and transiently inactivate PTP's (*left*). As GPx4 efficiently removes peroxidized lipids and counteracts 12/15-LOX, GPx4 may be regarded also as an important regulator of RTK signaling

diphenyliodonium, an effective NADPH oxidase inhibitor. Analyses of downstream signaling including cellular studies revealed that phospholipase C γ1 activation and lamellipodia formation was enhanced in *GPx4*$^{-/-}$ cells. These results were most consistent with investigations on individual phosphorylation sites within the PDGFβR, which disclosed that individual sites within the receptor appear to be differently affected by lipid peroxides. Finally, the efficacy of purified LOOH (i.e., 15-HPETE, a 12/15-LOX metabolite) to oxidize recombinant PTP's in vitro was compared with H_2O_2, the classical inducer of PTP oxidation. These studies demonstrated that lipid peroxides are highly efficient inducers of PTP oxidation [48], thus uncovering a previously unrecognized pathway controlling RTK activity (Fig. 43.4).

43.8 Concluding Remarks

Due to the utilization of transgenic approaches and ex vivo investigations, enormous progress has been made forward to a better understanding of the role of GPx4 in sperm development and neuroprotection. The direct comparisons of tissue-specific GPx4 knockout mice with mice lacking selenoprotein synthesis in the corresponding tissues provided initial evidence that GPx4 must be regarded as one of the most central selenoproteins at least in neurons and skin (see Chap. 44). Moreover, inducible *GPx4* knockout cell systems have provided unique tools to obtain a clearer

picture regarding the molecular mechanisms of GPx4 in cell signaling pathways like apoptotic signaling and RTK signaling. Hence, it will be interesting to see in which other tissues and cellular processes GPx4 is centrally involved.

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Chapter 44
Mouse Models that Target Removal or Overexpression of the Selenocysteine tRNA[Ser]Sec Gene to Elucidate the Role of Selenoproteins in Health and Development

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Abstract Several mouse models targeting the selenocysteine (Sec) tRNA[Ser]Sec gene for removal or overexpression have been generated and they include: (1) mice carrying a conditional knockout of the Sec tRNA gene; (2) transgenic mice encoding wild type or mutant Sec tRNA transgenes; and (3) conditional knockout/transgenic or standard knockout/transgenic mice carrying wild type or mutant Sec tRNA transgenes. These models have provided powerful tools for elucidating the roles of selenoproteins in development and health.

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44.1 Introduction

Selenium is an essential element in the diet of humans and other mammals as well as many other life forms. This element has been implicated in numerous health benefits and cellular functions. For example, some of the health benefits attributed to selenium include its roles in preventing cancer, heart disease and other cardiovascular disorders, inhibiting viral expression, impeding the inception of AIDS in HIV positive individuals, as well as having roles in male reproduction, mammalian development, and boosting immune function ([1] and this book).

Selenium is incorporated into protein as the amino acid selenocysteine (Sec), which is the 21st amino acid in the genetic code [2–4]. The biosynthesis of Sec was established recently in eukaryotes and archaea [5, 6]. Sec synthesis occurs in eukaryotes, unlike any other known amino acid, in that it is carried out on its tRNA, designated $tRNA^{[Ser]Sec}$ [7]. The number of selenoprotein genes in mammals has been established and found to be 24 in rodents and 25 in humans [8].

One of the key features about selenium-containing proteins, which are also designated selenoproteins, is that their expression is dependent on Sec $tRNA^{[Ser]Sec}$. If the expression levels of this tRNA are altered, or the primary structure of Sec $tRNA^{[Ser]Sec}$ is changed, the levels and relative amounts of individual selenoproteins also change [9, 10]. Selenoproteins have been identified in recent years as the agents responsible for many of the health benefits attributed to selenium. These studies have been accomplished largely through the targeted removal or manipulation of the Sec $tRNA^{[Ser]Sec}$ gene (*Trsp*) and by the targeted removal of individual selenoproteins. Mouse models generated by knockout of specific selenoproteins are covered in Chap. 43 (and references therein), while mouse models generated by introducing *Trsp* transgenes into the genome of mice or from knockout of *Trsp* (designated Δ*Trsp*) are the focus of this chapter.

44.2 Transgenic Mouse Models

The Sec $tRNA^{[Ser]Sec}$ population in mammalian cells and tissues contains two isoforms, designated methylcarboxymethyl-5′-uridine (mcm^5U) and methylcarboxymethyl-5′-uridine-2′-*O*-methylribose (mcm^5Um) [11]. These two Sec $tRNA^{[Ser]Sec}$ isoforms differ by a single methyl group on the 2′-*O*-ribosyl moiety at position 34 (Fig. 44.1 [12] and references therein). This methyl group is abbreviated Um34. The synthesis of Um34 is highly dependent on the primary, secondary, and tertiary structure of mcm^5U [13]. We have used this feature to generate transgenic mouse models encoding mutant $Trsp^t$ transgenes that do not make the mcm^5Um isoform as discussed below in Sects. 44.3 and 44.4.

Transgenic mouse models involving Sec $tRNA^{[Ser]Sec}$ have been generated, wherein the genome encodes wild type or mutant Sec $tRNA^{[Ser]Sec}$ transgenes ($Trsp^t$) that vary in the number of transgene copies and in the position of the muta-

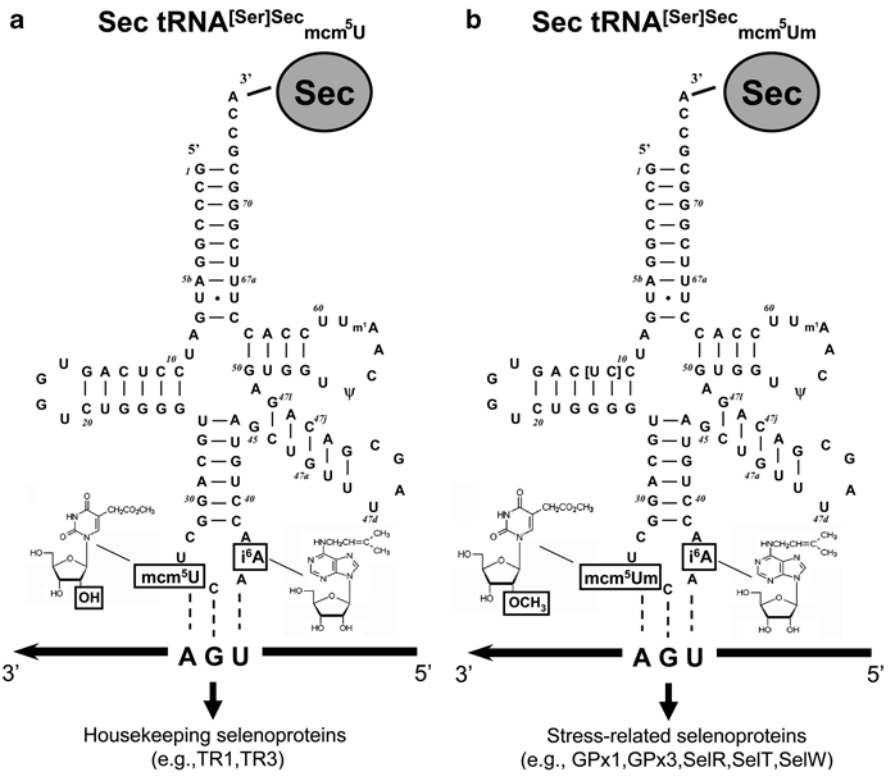

Fig. 44.1 The primary structures of bovine liver (**a**) mcm^{5}U and (**b**) mcm^{5}Um isoforms are shown in a cloverleaf model. Sec tRNA$^{[Ser]Sec}$ sequences in mammals are 90 nucleotides long. They contain base modifications at positions 34 (mcm^{5}U [see text]), 37 (i^{6}A [see text]), 55 (pseudouridine; ψ) and 58 (*N1*-methyladenosine; m^{1}A). The two isoforms differ from each other by a single methyl group on the 2′-*O*-ribosyl moiety at position 34 and the presence or absence of this methyl group plays a major role in Sec tRNA$^{[Ser]Sec}$ conformation [11, 12] and in selenoprotein synthesis, wherein mcm^{5}U is involved in housekeeping selenoprotein synthesis and mcm^{5}Um is essential for stress-related selenoprotein synthesis (see text). The base at position 34 has been mutated to A (T34→A34) or to G at position 37 (A34→G37) and the consequences of these mutations are that they do not synthesize Um34, causing a virtual loss in stress-related selenoprotein expression (see text) in addition to having lost the highly modified base at the respective position. The A at position 34 is converted to inosine at this site resulting in a change in the decoding properties of this isoform as discussed in the text

tion within the tRNA [9, 10, 14]. Two mutant *Trsp*^{t} mouse lines were prepared where the mutation occurred either in *Trsp*^{t} at position 37 (A37→G37) or at position 34 (T34→A34) (Fig. 44.1). Both mutations in the corresponding *Trsp*^{t} result in a mutant Sec tRNA$^{[Ser]Sec}$ product that cannot form the Um34-containing isoform, designated Sec tRNA$_{mcmUm}^{[Ser]Sec}$ [10, 13, 14]. As shown in Fig. 44.1, position 37 normally contains isopentenyladenosine (i^{6}A) and position 34 normally contains mcm^{5}U [11]. Interestingly, mcm^{5}U is associated with housekeeping

selenoprotein expression (e.g., expression of thioredoxin reductase 1 (TR1)) and mcm^{5}Um is essential in stress-related selenoprotein expression (e.g., expression of glutathione peroxidase 1 (GPx1)) [14]. Transgenic mice that are Δ*Trsp* and are dependent on the G37 transgene for selenoprotein expression cannot synthesize stress-related selenoproteins. Housekeeping selenoproteins are essential to the animal's survival and are less sensitive to selenium status, while stress-related selenoproteins are not essential to the animal's survival and are highly regulated by selenium status [14]. The phenomenon of higher levels of stress-related selenoprotein expression in a selenium enriched environment and lower levels of stress-related expression in a selenium deficient environment correlates with changes in the Sec tRNA$^{[Ser]Sec}$ population [11, 12]. That is, the overall levels of the Sec tRNA$^{[Ser]Sec}$ population decrease during selenium deprivation, but the mcm^{5}Um isoform expression is severely reduced, whereas the mcm^{5}U isoform is reduced less [12].

In transgenic mice encoding either wild type or G37 mutant transgenes, the number of transgene copies ranged from as few as two to as many as 40 [9]. On the other hand, no more than 12 transgene copies were produced from the A34 mutant in a wild type *Trsp* background or more than two in a liver Δ*Trsp* background [14]. The A at position 34 in tRNA$^{[Ser]Sec}$ is converted to inosine that in turn wobbles to decode U/C/A in the 3′-position of codewords. Thus, this mutant Sec tRNA$^{[Ser]Sec}$ decodes the cysteine codons UGU and UGC, in addition to UGA [14]. Since Sec is expected to be inserted into selenoproteins in response to Cys codons that are located near the Sec-UGA codon ([15] and references therein), a likely explanation for the low copy number of mutant A34 transgenes inserted into the mouse genome is that Sec is inserted into housekeeping selenoproteins in response to Cys codons near the Sec-UGA codon. It should be again noted that mcm^{5}Um is not synthesized in the mice carrying either of the two *Trsp* mutant transgenes in a Δ*Trsp* background and therefore no stress-related selenoproteins are synthesized.

The *Trsp^t* transgenic mouse models and their uses in elucidating impact on selenoprotein synthesis and on variations in selenoprotein expression in relation to various health issues are shown in Fig. 44.2. The first transgenic mouse models involving a tRNA transgene were generated with either wild type or G37 mutant *Trsp^t* [9]. Transgenic mice carrying up to 40 wild type transgenes that enhanced the overall levels of the Sec tRNA$^{[Ser]Sec}$ population many fold were found to have little or no effect on enhancing selenoprotein expression in the organs examined, suggesting that the level of Sec tRNA$^{[Ser]Sec}$ normally occurring in these tissues was not limiting in the synthesis of this class of proteins ([9, 10, 14] and references therein). However, the higher levels of G37 mutant Sec tRNA$^{[Ser]Sec}$ synthesized from the higher corresponding transgene copy numbers affected selenoprotein synthesis in both a tissue and protein specific manner [9]. That is, the expression of selenoproteins did not appear to be affected in testes, whereas the expression of stress-related selenoproteins was substantially downregulated in liver (GPx1), while the expression of some housekeeping selenoproteins was increased (e.g., TR3). The Sec tRNA$^{[Ser]Sec}$ population is normally

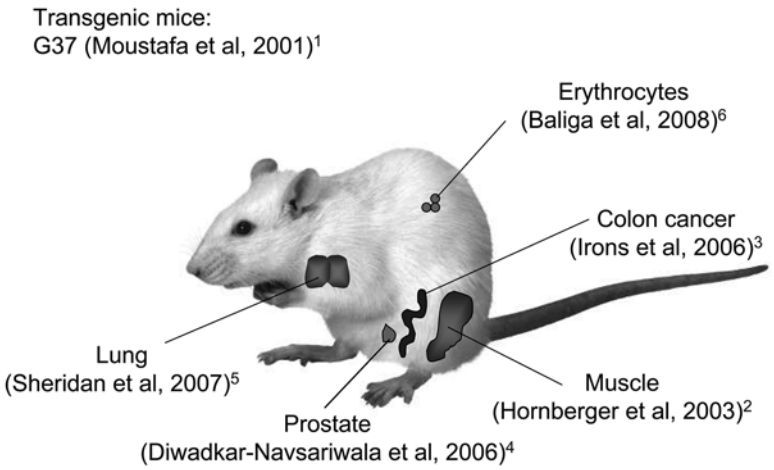

Fig. 44.2 G37 mutant *Trsp*^{t} transgenic mouse models. Each mouse line shown in the figure encodes G37 mutant *Trsp*^{t} in all tissues and organs. Descriptions of each mouse model, major findings and number of transgenes encoded by the transgenic mouse that are designated by the superscript number in the figure are: (1) first transgenic mouse generated that encoded a tRNA transgene; levels of stress-related selenoproteins decreased in a protein and tissue-specific manner as discussed in the text; the number of transgenes carried by the transgenic mice were 2–4, 8–16, or 20–40 depending on whether the mice were heterozygous or homozygous [9]; (2) increased skeletal muscle adaptation after exercise enhanced growth, and muscles exhibited increased site-specific phosphorylation on both Akt and p70 ribosomal S6 kinase before ablation compared to control mice [39]; (3) first demonstration that selenoproteins reduce colon cancer incidence; colon was targeted with azoxymethane and mice had a greater number of azoxymethane-induced aberrant crypt foci (a preneoplastic lesion for colon cancer) than the corresponding control animals [16]; (4) first demonstration that selenoproteins reduce prostate cancer incidence; mice carried a prostate cancer driving *C3(1)/Tag* transgene and exhibited accelerated development of lesions associated with prostate cancer progression compared to control mice [17]; (5) lung was targeted by administration of influenza virus; changes in the immune system due to infection, wherein mice manifested greater chemokine levels, higher IFN-γ, and slower viral clearance than control mice [40]; and (6) mice manifested higher micronuclei formation in erythrocytes following exposure to X-rays than control animals [41]

three- to fourfold higher in testes than in liver, which most certainly accounts for the relative differences in tissue specificity with respect to selenoprotein synthesis detected in the G37 transgenic mice. Regarding the variation observed in stress-related and housekeeping selenoproteins in liver of G37 *Trsp*^{t} mice, it was subsequently found that the high levels of G37 mutant Sec tRNA$^{[Ser]Sec}$ impaired the expression and ability of the mcm^{5}Um isoform to synthesize stress-related selenoproteins [10, 14].

The G37 mutant *Trsp*^{t} mouse model has been used to demonstrate that stress-related selenoproteins are involved in colon [16] and prostate cancer protection [17], and that this subclass of proteins has roles in prevention of several other disorders (Fig. 44.2 and corresponding legend).

44.3 Standard and Conditional *Trsp* Knockout Models

Knockout of *Trsp* (Δ*Trsp*) is embryonic lethal [18, 19] and the fetus dies after only a few days following fertilization [18]. Therefore, this knockout mouse could not be used to study the role of selenoproteins in development of various tissues and organs or their function in mature tissues and organs unless the knockout could be rescued with wild type or mutant transgenes (see Sect. 44.4 below). To further pursue the effect of *Trsp* loss in development and health, we generated the conditional *Trsp* knockout mouse using *loxP-Cre* technology [19]. Our groups and other investigators have examined the fate of selenoprotein loss in both developing and fully developed organs and tissues (Fig. 44.3). These studies demonstrated that selenoproteins function in many different aspects of development and disease prevention. Interestingly, selenium was also known for many years to play roles in several of the same systems and/or disorders. Several of the conditional *Trsp* knockout mice shown in Fig. 44.3 are further discussed below.

Selenium was known for many years to have a role in boosting the immune system [20], and the targeted removal of *Trsp* in T cells [21] and macrophages [22–24] has shown that selenoproteins are responsible, at least in large part, for proper immune function. Selenoprotein loss in T cells resulted in decreased pools of mature T cells, in T cell dependent antibody responses and an oxidant hyperproduction that suppressed T cell proliferation in response to T cell receptor stimulation [21]. Selenoprotein-less macrophages also had increased oxidative stress and manifested a reduced migration in a protein gel matrix and an abnormal expression of extracellular matrix-related genes [23, 24]. The loss of selenoprotein expression in macrophages and the transcription factor, *Nrf2*, caused reduced viability and an increase in oxidative stress and susceptibility to hydrogen peroxide, compared to the targeted removal of either corresponding gene within the same mouse [22]. The removal of only selenoproteins in macrophages caused an increased expression of genes involved in oxidative stress and detoxification enzymes [22].

In addition, selenium has been known for many years to have a role in heart disease prevention [25], and the loss of selenoproteins in myocytes of heart muscle was found to result in death of affected mice at about 12 days after birth from myocardial failure [26]. The *Cre* recombinase gene that was responsible for removing floxed *Trsp* was under the control of the muscle creatine kinase gene (*MCK*) promoter which is expressed in myocytes at birth and is fully active by days 10–12. The targeted removal of *Trsp* resulting in death of the mice from cardiac failure shortly after the Sec $tRNA^{[Ser]Sec}$ gene is lost strongly suggests that selenoproteins are responsible, at least in part, for selenium's role in heart disease prevention.

Selenium deficiency has also been known to be associated with a number of neurological phenotypes [27], but whether selenoproteins have a role in preventing neurological disorders or in neurological development was not known. Wirth et al. [28] knocked out *Trsp* in neurons and found that mice which were selenoprotein-less in neuronal tissue died at about 2 weeks of age and had manifested several features. For example, they (1) lost postural control, (2) developed seizure-like

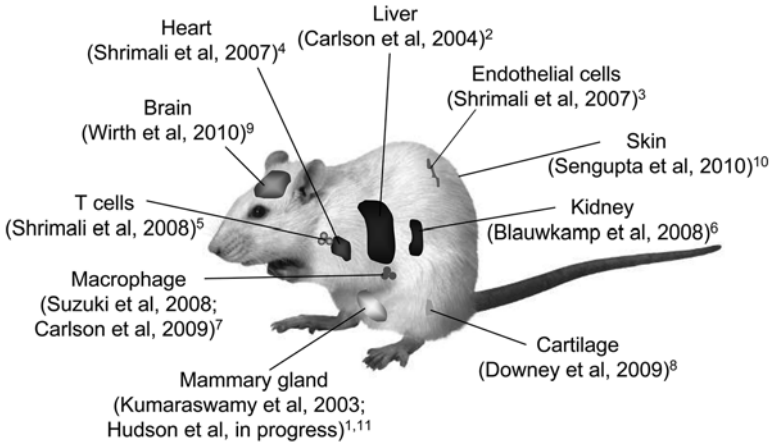

Fig. 44.3 *Trsp* conditional knockout mouse models. Each of these mouse models used *loxP-Cre* technology to target the removal of *Trsp* in a specific tissue or organ. The *Cre* promoter, the targeted organ or tissue and the major findings of each study that are designated by the superscript number in the figure are: (1) *MMTV-Cre* and *Wap-Cre* promoters used independently; mammary gland; first description of the conditional knockout of a tRNA gene [19]; (2) *Alb-Cre* promoter; liver; newborns died between 1 and 3 months due to severe hepatocellular degeneration and necrosis, brain selenium levels were maintained in the absence of liver-derived SelP and hepatic Dio1 is not essential to maintain plasma thyroid hormone levels; selenoproteins have a role in proper liver function [31]; (3) *TieTek2-Cre* promoter; endothelial cells; embryonic lethal, wherein 14.5 day-old embryos were smaller in size, more fragile, poorly developed vascular system, underdeveloped limbs and heads; selenoproteins have a role in endothelial development and function [26]; (4) *MCK-Cre* promoter; heart and skeletal muscle; selenoproteins have a role in preventing heart disease as discussed in the text [26]; (5) *LCK-Cre* promoter; T cells; selenoproteins have a role in the immune system as discussed in the text [21]; (6) *NPHS2-Cre* promoter; kidney; podocyte selenoprotein loss did not cause increased oxidative stress or enhanced nephropathy [42]; (7) *LysM-Cre*; macrophage; enhanced oxidative stress and transcriptional induction of cytoprotective antioxidant and detoxification enzyme genes, accumulation of ROS levels and impaired invasiveness, and altered expression of extracellular matrix and fibrosis-associated genes; selenoproteins have a role in immune function [22–24]; (8) *Col2a1-Cre*; cartilage (osteochondroprogenitor); mouse model for Kashin-Beck disease ([43] and see Chap. 45); (9) *Tα1 antigen-Cre*; neuron specific (brain); selenoproteins (and specifically GPx4) have a role in brain function and development as discussed in the text [28]; (10) *K14-Cre*; skin; selenoproteins have a role in skin and hair follicle development as discussed in the text [30]; and (11) *MMTV-Cre*; mammary tissue; a decrease in Brca1 expression and an increase in p53 expression in mice suggests that the mice might be more susceptible to breast cancer (Hudson, Carlson, Hatfield, and Green [manuscript in preparation])

behavior, (3) suffered from cerebellar hypoplasia with Purkinje cell death and decreased granule cell proliferation, and (4) manifested interneurons in the cerebral cortex and hippocampus that did not develop parvalbumin expression, wherein extensive neuron-degeneration was observed in these two brain regions. Interestingly, the knockout of only glutathione peroxidase 4 (GPx4) had similar cerebellar and interneuron phenotypes as mice with total selenoprotein loss demonstrating that many of the defects caused by *Trsp* knockout were the result of the removal of a single selenoprotein [28].

Selenium has been implicated in protecting numerous defects in skin that include cancer, restoration of hair loss, and the effects of UV-induced damage; furthermore, its topical application, along with other antioxidants, is known to improve various aspects of the skin surface ([29, 30] and references therein). The targeted removal of selenoproteins in epidermis has shown that this protein class is responsible for proper development and function of skin. Targeting *Trsp* removal in the epidermis resulted in premature death of affected offspring that died with a mean lifespan of 10 days [30]. These mice manifested stunted growth with wrinkled and fragile skin, sparse hair resulting from loss that increased with age and reduced intradermal fat. Although the initiation of hair follicle formation appeared normal, hair follicles underwent a premature repression in affected mice. Histological analysis of the hair follicles showed their reduced number and growth retardation. An analysis of the epidermal tissue revealed moderate epidermal hyperplasia and an acute focal coagulative necrosis of the epidermis. Keratinocytes isolated and cultured from affected mice manifested an impaired ability to attach and proliferate, compared to their normal counterparts; however, other antioxidants such as vitamin E improved attachment and survival [30]. A subsequent study demonstrated that the targeted removal of only GPx4 manifested similar effects in early epidermal and hair follicle development as the loss of all selenoproteins [Sengupta, Lichti, Carlson, Ryscavage, Conrad, Chatterjee, Gladyshev, Yuspa, and Hatfield, manuscript submitted]. Similar to *Trsp* knockout mice, *GPx4* knockout mice had abnormal hair follicles, a hyperplastic epidermis and were slightly smaller in size. However, at approximately 4 weeks of age, most of these abnormalities ceased and the majority of the affected mice had a similar lifespan as their wild type siblings. These findings revealed that selenoproteins, and specifically GPx4, have an essential role in epidermal development and function, including hair follicle morphogenesis.

Additional details that involve the above mouse models along with a brief discussion of other models are presented in Fig. 44.3 and its legend.

44.4 Transgenic/Conditional and Transgenic/Standard *Trsp* Knockout Models

Alternative mouse models, in addition to transgenic *Trsp*^{t} and standard and conditional *Trsp* knockout mouse models described above, were developed to study the impact of the Sec tRNA$^{[Ser]Sec}$ population generated from mutant and wild type transgenes on selenoprotein synthesis in the absence of a wild type *Trsp* background (Fig. 44.4). These models involved combining the transgenic and *Trsp* removal models by (1) rescuing the Sec tRNA$^{[Ser]Sec}$ population in the standard knockout with a wild type or mutant transgene [10] or (2) replacing the Sec tRNA$^{[Ser]Sec}$ population in the conditional knockout with a wild type or mutant transgene [14]. As noted above, the knockout of *Trsp* was embryonic lethal [18, 19], but selenoprotein expression was rescued with as few as 2 or as many at 40 copies of *Trsp*^{t} or G37 *Trsp*^{t} [10]. With high copy number *Trsp*^{t} mice in a Δ*Trsp* background, wherein the Sec tRNA$^{[Ser]Sec}$

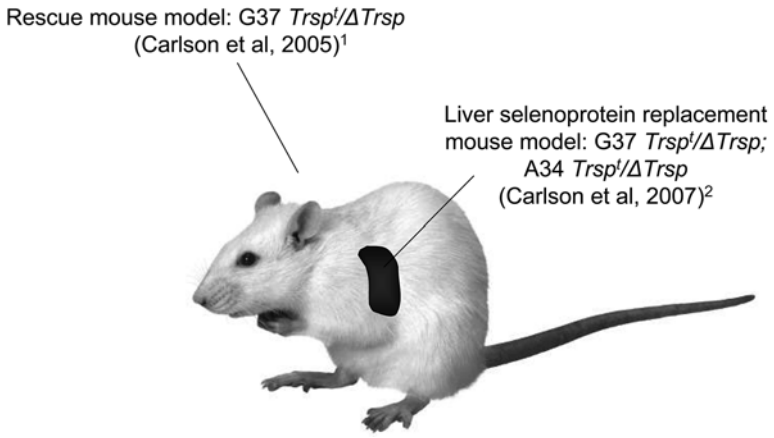

Fig. 44.4 Standard *Trsp* knockout/G37 transgenic (Δ*Trsp*/G37*Trsp*^{t}) and conditional *Trsp* knockout/mutant transgenic (liver Δ*Trsp*/G37*Trsp*^{t} and Δ*Trsp*/A34*Trsp*^{t}) mouse models. In the rescue mouse model, the standard knockout mouse, which is embryonic lethal was rescued with the mutant G37 transgene demonstrating that mcm^{5}Um is essential in stress-related selenoprotein expression as discussed in the text [10]. In the replacement of selenoprotein expression in the liver Δ*Trsp* mouse model, the targeted removal of selenoprotein expression in liver was carried out with the *Alb-Cre* promoter and floxed *Trsp* and the housekeeping selenoprotein population was replaced with either G37*Trsp*^{t} or A34*Trsp*^{t} ([14] and see text)

population was enriched many fold, selenoprotein levels in the tissues and organs examined appeared to be very similar as the corresponding wild type mouse providing further evidence that the Sec tRNA$^{[Ser]Sec}$ population is not limiting under normal conditions. Using G37 *Trsp*^{t} to rescue mice encoding Δ*Trsp*, only housekeeping selenoproteins were synthesized [10, 13]. This finding demonstrated that the mcm^{5}Um isoform, which is not synthesized in these mice, is essential for the expression of stress-related selenoproteins (see also Fig. 44.1). The G37 *Trsp*^{t}/Δ*Trsp* mice appeared phenotypically very similar to the corresponding wild type mice, but manifested reduced male fertility and difficulties with female pregnancy [10]. This study also demonstrated that stress-related selenoproteins are not essential to survival. Interestingly, we were unable to rescue the Δ*Trsp* mouse with the A34 mutant transgene, suggesting that the selenoprotein products synthesized from the A34 mutant Sec tRNA$^{[Ser]Sec}$ may be deleterious to the health of the animal [10].

The loss of selenoprotein expression and partial replacement with only housekeeping selenoproteins in G37 *Trsp*^{t}/Δ*Trsp* or A34 *Trsp*^{t}/Δ*Trsp* conditional knockout mice afforded us an opportunity to examine possible different effects of either mutant isoform on housekeeping selenoprotein expression in the absence of stress-related selenoproteins [14]. Since mice survived *Trsp* removal in hepatocytes [31], liver was used as the system for developing these mouse models. As noted above, the mutant A34 transgene could not be used to rescue Δ*Trsp* mice and only ~14 copies of A34 *Trsp*^{t} could be used in generating *Trsp*^{t}/*Trsp* transgenic mice [14]. Housekeeping selenoproteins that were replaced in liver in the *Trsp* conditional

knockout mice by either mutant transgenes were similar, although some minor differences were noted [14]. Interestingly, only 1–2 copies of the A34 mutant transgene could be introduced into the genome of these conditional Δ*Trsp* mice, whereas many more of the G37 transgene could be inserted. This novel model provided a means of determining why so few copies the A34 transgene could be used in generating transgenic mice as further discussed below; and we are actively characterizing selenoproteins generated from the A34 Sec tRNA$^{[Ser]Sec}$ transgene product to assess where Sec may be inserted in response to Cys codons, UGU/UGC, within the Sec-UGA-SECIS coding region.

44.5 Other Mouse Models Involving *Trsp*

Two additional mouse models involving *Trsp* and selenoprotein expression have been developed and both engage one of the upstream regulatory elements that is essential in proper transcription of *Trsp*, designated the distal sequence element (*DSE*) [32, 33]. The *Trsp* regulatory region has been reviewed in detail elsewhere [34], and each of the regulatory elements discussed herein and references to the original work are found in this review. The *DSE* is located approximately 200 bp upstream of the gene, is composed of an activator region (*AE*) and an octomer sequence. A transcription factor, designated the Sec tRNA$^{[Ser]Sec}$ gene transcription factor (STAF), binds to the *AE* stimulating transcription of *Trsp*. One of the mouse models involving *DSE* disrupts this regulatory region by inserting a 3.2-kb fragment between *DSE* and another regulatory element near the coding sequence of *Trsp*, designated the proximal sequence element, located approximately 30 bp upstream of the gene [32]. The insertion of this fragment resulted in embryonic death due to the poor transcription of *Trsp* and enormous reduction in *Trsp* transcripts. However, since the insertion sequence was flanked by *loxP* sites, it could be removed by the *Cre*-recombinase, restoring normal levels of *Trsp* transcription [32]. Heterozygous mice encoding wild type *Trsp* and the inserted sequence alleles showed that the enhancer activity of the *DSE* region was tissue dependent; heart expressed *Trsp* normally and thus was not dependent on both wild type alleles but several other tissues, such as hepatocytes, required both *DSE* alleles properly distanced from the gene. The other investigated tissues expressed selenoproteins in varying levels [32].

In another mouse model involving *DSE* in *Trsp* transcription, the STAF binding site or *AE* region was removed and transgenic mice lacking the *AE* region were prepared that were also Δ*Trsp* such that the mice were dependent on the mutant transgene for survival [33]. The levels of selenoprotein expression were unchanged or slightly elevated in heart and testes but varied in other tissues examined, wherein approximately 60% loss in selenoprotein expression was observed in kidney and liver, approximately 70% in spleen and lung, and approximately 80% in brain and skeletal muscle. The level of the mcm^{5}Um isoform was dramatically reduced in all tissues examined and selenoprotein synthesis was affected most dramatically in the examined tissues and organs wherein Sec tRNA$^{[Ser]Sec}$ levels were most severely

reduced. Interestingly, mice that were dependent on the mutant transgene lacking *AE* manifested a neurological phenotype similar to mice without a selenoprotein P (*SelP*) gene (see [35] and references therein). The *AE*$^{-}$ mice and *SelP*$^{-}$ mice phenotypically showed growth retardation, tissue calcification, smaller spleens and liver, and brain defects. These data suggest that STAF controls selenoprotein synthesis by increasing *Trsp* transcription in an organ/tissue-specific manner by regulating Sec tRNA$^{[Ser]Sec}$ modification [33].

44.6 Concluding Remarks

Roles of selenium in cancer and heart disease prevention, development, boosting immune function, inhibiting viral expression, and in enhancing male fertility have been known for many years. However, only in more recent years have mouse models involving the loss or modulation of selenoprotein expression by knockout, conditional knockout or overexpression of *Trsp* described herein or of individual selenoprotein genes (Chap. 43 and references therein) been developed to demonstrate unequivocally that selenoproteins have major roles in these health benefits (see also [36]). Such studies as these and numerous in-depth studies characterizing individual selenoproteins in vitro [37] have shifted the focus of the long held debate of whether small molecular weight selenocompounds or selenoproteins play major roles in health and development (reviewed in [38]) onto selenoproteins as the key components in providing these benefits. As further studies involving the role of small molecular weight selenocompounds in health are developed, it will be interesting to see if specific functions can be ascribed to these components, including perhaps some as major players.

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Chapter 45
Selenoproteins in Skeletal Development and Disease: Lessons from *Trsp* Deletion in Murine Bone and Cartilage Progenitor Cells

Charlene M. Downey and Frank R. Jirik

Abstract The use of global gene disruption to study the roles of specific selenoproteins in skeletal development and homeostasis can be handicapped by the difficulty in determining whether or not any observed phenotype is attributable to losses of gene function specifically in chondrocytes, osteoblasts, or osteoclasts. Indeed, the potential effects of global gene knockouts on the brain, kidney, endocrine glands, and hematopoietic system, tissues involved in the regulating skeleton growth and function, can confound phenotypic analyses. Tissue-specific gene alterations, in contrast, have the ability to reveal information about selenoprotein gene function within cells directly involved in skeletal growth and homeostasis. The dramatic phenotype resulting from disruption of *Trsp* in osteo-chondroprogenitor cells, for example, illustrates how bone and cartilage development are critically dependent on normal selenoprotein function.

45.1 Introduction

The free radical[1] theory of disease, articulated by Harman in the mid-1950s [1–3], postulates that cells living in aerobic environments are exposed to chronic oxidative stress. Reactive oxygen species (ROS), a normal by-product of oxidative phosphorylation and various other enzymatic reactions, is thought to lead to an accumulation of damage to various cellular constituents that over time produce a progressive loss of tissue function [3]. This process is especially evident in cell populations that

[1] Note: hydrogen peroxide, peroxynitrite, aldehydes such as 4-hydroxy-2-nonenal (HNE), and other species stemming from lipid peroxidation [8], while not free radicals, nevertheless play important roles in macromolecule damage.

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are not readily renewable and that are exposed to high oxygen tensions, such as neurons [2]. Other cell types, such as muscle, osteocytes, and chondrocytes that are turned-over relatively slowly in adults, may also be vulnerable to cumulative ROS-mediated damage. Because not all damage caused by ROS (and reactive nitrogen species, RNS) can be repaired, a gradual accumulation of oxidative damage occurs that is thought to be an important mechanism underlying the aging process and age-dependent diseases. Not only are DNA and proteins subject to injury, but polyunsaturated fatty acids and lipoproteins are also highly prone to oxidative damage [2]. The role of lipid peroxidation during tissue aging is supported by data showing increasing levels of lipid peroxidation markers as animals age, and also by studies demonstrating that caloric restriction can decrease age-dependent increases in lipid peroxidation [2, 4–8]. Lastly, mutations or other events that impair the activity of cellular antioxidant defense mechanisms can result in increased levels of ROS, potentially leading to an acceleration of age-associated degenerative diseases, such as osteoporosis and osteoarthritis, in which reactive species are thought to play a pathogenic role.

45.2 Cellular Antioxidant Defenses

Attesting to dangers posed by aerobic life, all cells contain a wide range of molecules whose functions are, in general, either to protect against or to repair ROS-mediated damage to cellular constituents. Mitochondria are the principal source of ROS in cells, owing to superoxide anion radical generation stemming from electron transport chain "leakiness" ($O_2 + e^- \rightarrow O_2^-$). To cope with ROS, in addition to the small molecule antioxidants (e.g., urate, glutathione, ascorbate, vitamin E), a range of proteins exist for detoxifying ROS, such as superoxide dismutases, catalase, thioredoxins, glutathione peroxidases, and thioredoxin reductases. The latter two molecules belong to the selenoprotein family.

45.3 Selenoproteins

Selenium is an essential dietary micronutrient that is associated with various organic molecules, including the 21st amino acid, selenocysteine (Sec), required for the function of selenoproteins [9]. There are 25 human and 24 murine selenoprotein genes [10], approximately one-third of which function as antioxidants. These include glutathione peroxidases 1–6 [11] that are responsible for protecting cells against ROS/RNS-mediated damage [12]. Some of these are tissue specific, while glutathione peroxidases 1 and 4 (GPx1 and GPx4) are ubiquitously expressed. Other members of the selenoprotein family include the thioredoxin reductases (such as Trxd1), which in conjunction with the thioredoxins regulate intracellular redox levels, thus controlling the activities of redox-sensitive signaling molecules and tran-

scription factors [11, 13]. Yet another selenoprotein family, the iodothyronine deiodinases (Diol and 2), catalyze the activation of thyroid hormone through conversion of thyroxine (T_4) into the active tri-iodothyronine (T_3) form that has many roles in growth and development, including the regulation of epiphyseal growth plate differentiation (see Sect. 45.8 below) [14–16]. Selenoprotein synthesis requires that Sec residues be inserted into the growing polypeptide chains via specific UGA codons, which code for selenocysteine, and require the participation of a group of proteins that recognize the Sec insertion sequence (SECIS) located in the 3′-UTR of selenoprotein mRNAs [17], allowing UGA to be recognized by the Sec tRNA (designated Sec $tRNA^{[Ser]Sec}$) encoded by the *Trsp* gene.

45.4 Conditional Mutagenesis of *Trsp*

Cre-*lox*P technology has been successfully used to study the consequences of selenoprotein loss using floxed *Trsp* gene ($Trsp^{fl/fl}$) excision in cardiac muscle, hepatocytes, lymphocytes, mammary epithelium, and neurons, with pathological changes being demonstrated in all of these tissues ([9]; also see Chap. 44). Although the tissue-specific removal of *Trsp* likely results in a global decrease in selenoprotein expression, it nonetheless allows some insight into the functional importance of this family in any given tissue. This approach may also serve to mimic the effects of severe selenium deficiency, but with the selenium "deficiency" being limited to a specific cell type. Once the importance of selenoproteins for a tissue has been established via phenotype analysis, it sets the stage for further studies aimed at determining the identity of the specific selenoprotein, whose deficiency accounts for some, or all, of the features observed in response to *Trsp* deletion. For example, a mouse model of skeletal disease, based on a tissue-specific deletion of *Trsp* in skeletal progenitor cells was described [18]. This mutant mouse demonstrated that selenoproteins have a critical role in the development and maintenance of the skeleton. These mice also represent a putative model for an environmentally-induced syndrome, known as Kashin-Beck disease (see Sects. 45.6–45.8 below) [18].

45.5 Kashin-Beck Disease

Kashin-Beck disease is thought to be an environmentally determined musculoskeletal syndrome affecting individuals in specific regions of Tibet, China, Siberia, and North Korea [19–21]. The disease, first evident in childhood, results in short stature, joint and limb deformities, and evidence of delayed skeletal ossification [22, 23]. These features have been attributed to impaired epiphyseal growth plate function and chondronecrosis [19, 21, 22, 24]. In addition, joint deformity and cartilage damage result in severe secondary osteoarthritis. Although several factors have been implicated in the pathogenesis of this disease, profoundly low serum selenium levels

represent one of the salient features of Kashin-Beck disease [19, 25, 26]. Interestingly, while individuals with Kashin-Beck disease show skeletal pathology, they are not reported to develop dysfunction of other organs or tissues. Thus, of all the tissues potentially affected in humans by severe selenium deficiency, at least in the geographic regions in question, it appears that cartilage may be the most vulnerable. In addition, the appearance of Kashin-Beck disease is often clustered within specific regions and/or families, and some geographical areas such as the Yunnan province in China and even Finland have very low levels of dietary selenium without the emergence of Kashin-Beck disease, suggesting that apart from selenium deficiency, there may be other contributing factors [27]. In keeping with this idea, a polymorphism in the GPx1 gene was reported to be a potential genetic risk factor for Kashin-Beck disease, perhaps providing an explanation for the sporadic emergence of this disorder [27]. However, the possibility remains that additional regionally determined environmental factors, besides selenium deficiency, play causative roles in this disease.

45.6 Deletion of *Trsp* in Skeletal Precursors Impairs Skeletal Growth and Leads to Premature Death

Since marked dietary deficiency of selenium and hence, of selenoproteins, is likely one of the primary factors implicated in the pathogenesis of Kashin-Beck disease [19, 25, 26], a *Col2a1* gene promoter-Cre recombinase transgenic was used to trigger osteo-chondroprogenitor-specific deletion of *Trsp*. Previous studies demonstrated that the global deletion of *Trsp* resulted in embryonic lethality [9], therefore, to circumvent this problem, mice having *lox*P-flanked (floxed) *Trsp* alleles were interbred with mice expressing the Cre recombinase under the control of the *Col2a1* promoter [18]. Compared to littermate controls, *Col2a1-Cre; Trsp*$^{fl/fl}$ mice demonstrated marked growth retardation by 1.5-2 weeks after birth, with the mutant mice exhibiting dwarfism, marked auricular (ear) hypoplasia, shortened snouts, decreased head size with frontal bossing, and shorter limbs and tails (Fig. 45.1). Interestingly, *Col2a1-Cre; Trsp*$^{fl/fl}$ mice were indistinguishable from controls within the first week after birth. By 3.5–4 weeks of age, however, the differences in body length and size between the two groups were dramatic, raising the possibility that an environmental factor, such as exposure to ambient oxygen, might be involved in disease pathogenesis. There was a also a high incidence of death in 4–5-week old *Col2a1-Cre; Trsp*$^{fl/fl}$ mice, with moribund animals demonstrating marked rib cage indrawing, suggestive of inspiratory obstruction (see Sect. 45.7 for the likely explanation). As suggested above, it is possible that the postnatal onset of runting in *Col2a1-Cre; Trsp*$^{fl/fl}$ mice was due to inhalation of ambient oxygen, with the ensuing increase in oxygen tension stressing the antioxidant defenses of chondrocytes and osteoblasts. In keeping with this idea, we have observed that in contrast to 3–4-week old mutant mice whose skeletons, and especially vertebrae, show grossly defective osteogenesis (Fig. 45.2), micro-computed tomographic imaging of the axial skeletons of 1 day old *Trsp*-deficient mice revealed evidence of enhanced osteogenic activity (see Sect. 45.8 below).

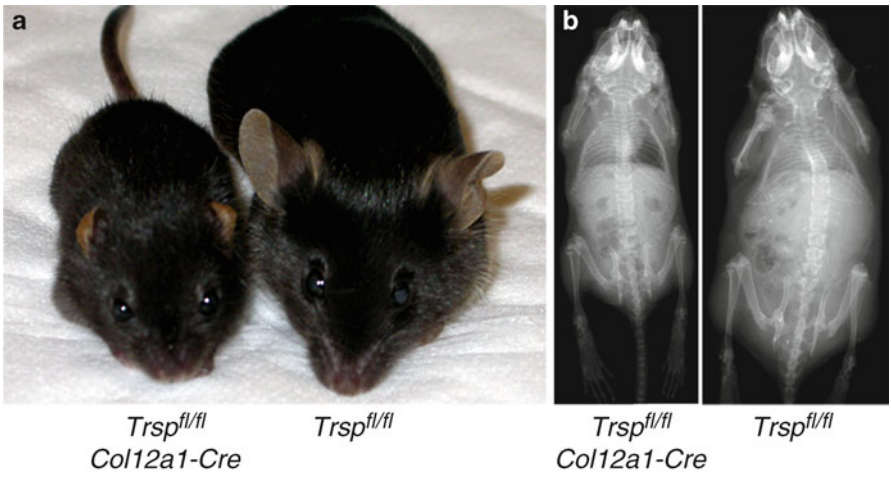

Fig. 45.1 *Trsp*$^{fl/fl}$*;Col2a1-Cre* mice exhibit stunted growth with striking differences in skeletal maturation. (**a**) Photograph of 4-week old mice demonstrating significant decrease in body length and size, dramatic shortening of snout, as well as the underdeveloped ears in the mutant *Trsp*$^{fl/fl}$*; Col2a1-Cre* mice compared with the *Trsp*$^{fl/fl}$ littermate controls. (**b**) Radiographic images of representative selenoprotein *Col2a1-Cre* mutant and control mice, emphasizing the dramatic overall size difference of the skeletons, and defect in osteogenic activity that is most evident in the spinal column of the *Trsp* mutant mouse

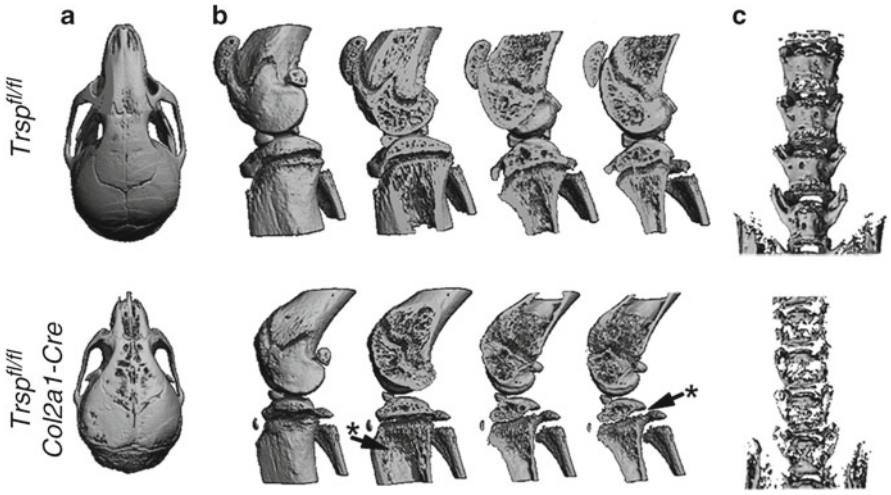

Fig. 45.2 Impaired bone development in *Trsp*$^{fl/fl}$*; Col2a1-Cre* mice. High-resolution 3-D micro-computed tomography (micro-CT) images of skull (**a**), knee (**b**), and lumbar spine (**c**) from 4 week old *Trsp*$^{fl/fl}$ control and *Trsp*$^{fl/fl}$*; Col2a1-Cre* mutant mice. (**a**) The skull of the mutant mouse demonstrates shortening of the snout and a more rounded cranium as compared to the skull from a control mouse. Incomplete ossification of the mutant skull frontal bones is suggestive of a defect in intramembranous ossification. (**b**) Knee images were created by taking saggital cut-planes through the reconstructed 3-D images to highlight the smaller size of the knees, as well as decreased trabecular bone formation in the marrow cavity, and increase in the size of the cartilaginous growth plate which appears as a space on the micro-CT image (both are marked by arrowheads) of the *Trsp* mutant. (**c**) Lumbar vertebrae demonstrate severely impaired development and ossification of the vertebral bodies in the *Trsp* mutant spine, as compared to the control spine

45.7 Abnormalities of Cartilage and Endochondral Ossification in *Col2a1-Cre; Trsp$^{fl/fl}$* Mice

Areas of chondronecrosis, a key pathological feature of Kashin-Beck disease [21, 22, 24], were observed in all cartilaginous tissues that were examined, including the proximal tibial growth plate, articular cartilage of the knee joint, as well as the auricular and tracheal cartilages (Fig. 45.3). In *Trsp*-deficient growth plates, there was decreased cell proliferation and increased apoptosis. There was a widespread defect in endochondral ossification, as seen in the proximal tibial growth plate, and most obviously in the vertebral column (Fig. 45.2). It should be noted that the frontal bones of the mutant mice showed thinning, suggesting that *Trsp* deficiency of osteoblasts also impaired intramembranous bone formation (Fig. 45.2).

Chondronecrosis accounted for the grossly hypoplastic ear and nasal cartilages (Fig. 45.1) of the mutant mice and the cartilaginous rings that support the trachea (Fig. 45.3). Premature death in *Col2a1-Cre; Trsp$^{fl/fl}$* mice appeared to be the result of respiratory distress secondary to the marked hypoplasia and chondronecrosis in *Col2a1-Cre; Trsp$^{fl/fl}$* tracheal cartilages (a form of tracheomalacia). Thus, cartilage development and survival was severely compromised in mice with deficient selenoprotein function.

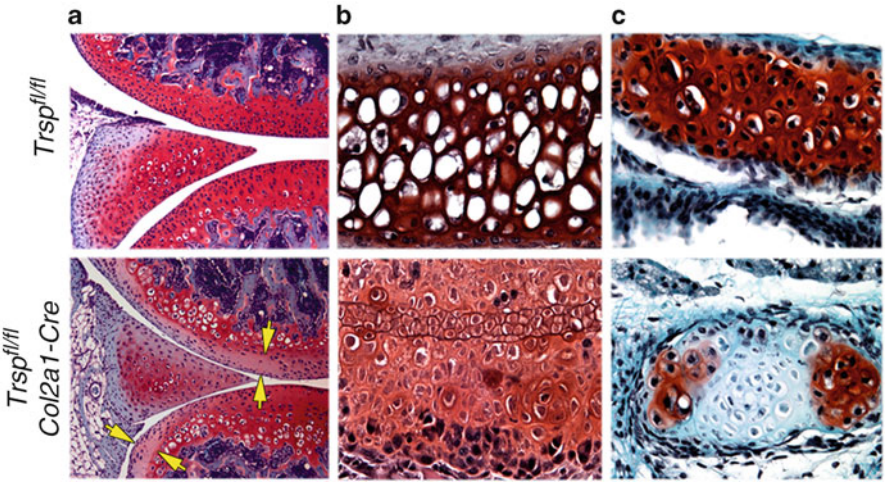

Fig. 45.3 Chondronecrosis was evident in all *Trsp* mutant cartilaginous tissues examined. Representative images of knee (**a**), ear (**b**), and tracheal (**c**) cartilages from *Trsp$^{fl/fl}$* control and *Trsp$^{fl/fl}$; Col2a1-Cre* mutant mice stained with hematoxylin, fast green and safranin O. The red staining of the glycosaminoglycan (GAG) extracellular matrix molecules surrounds the viable chondrocytes. (**a**) Necrotic areas in the articular cartilage of the knee are present in the mutant mice (*yellow arrowheads*). (**b**) Auricular cartilage of the *Trsp* mutant is grossly abnormal as a result of chondrocyte cell death. (**c**) Chondrocyte death accompanied by the loss of GAG staining is evident within the cartilaginous rings of a trachea obtained from a *Trsp* mutant animal

Since deletion of *Trsp* is predicted to lead to greatly diminished activity of multiple selenoproteins, it raises the question as to which selenoprotein(s) might be most important for normal cartilage and bone development to occur. For example, deficiency of glutathione peroxidase 4 leading to peroxidation of mitochondrial lipids could account for chondrocyte apoptosis in *Trsp* mutant mice (see Sect. 45.12). In view of the dramatic phenotype of *Trsp* mutants, it is possible that gene polymorphisms, capable of altering either the levels or activities of specific selenoproteins, might contribute to the extent of cartilage damage in human osteoarthritis.

45.8 Potential Role of Deiodinases in the Skeletal Phenotype of *Trsp* Mutant Mice

Is it possible that intracellular T_3 deficiency, resulting from decreased selenoprotein deiodinase activity, contributed to the phenotype of *Col2a1-Cre; Trsp*$^{fl/fl}$ mice? The thyroid secretes T_4 that is in turn converted by intracellular Dio1 or 2 within target tissues into bioactive T_3 [28, 29]. T_3, a hormone utilized by all tissues, is also required for normal growth plate development [30], and nuclear receptors for this ligand have been shown to be expressed in skeletal cells [14]. Although diminished Dio2 deiodinase activity could theoretically lead to a tissue-specific deficiency of T_3, genetic deletion of Dio2, either alone or in combination with Dio1 deiodinase, showed no overt evidence of impaired skeletal growth [31]. This is in keeping with data indicating that neither Dio1 nor Dio2 are expressed in rodent chondrocytes, and that Dio2 is only found in mature osteoblasts [32]. In contrast, a third deiodinase, Dio3, responsible for breaking down intracellular T_3 to T_2 in the skeleton (presumably to avoid accelerated bone maturation), is expressed in the chondrocytes and osteoblasts of young rodents. Indeed, mice lacking Dio3 exhibited generalized growth retardation that was attributed to perinatal thyrotoxicosis, which was subsequently compounded by severe hypothyroidism starting around the time of weaning [33]. One of the first deiodinases expressed during development, Dio3 is thought to regulate, and hence limit thyroid hormone availability to the developing skeleton [32]. This is important since thyrotoxicosis accelerates bone ossification as well as premature closure of epiphyseal growth plates, resulting in shortened stature [34]. Studies focused on the ubiquitously expressed thyroid hormone receptors (TRα and TRβ) have identified TRα as the main mediator of T_3 action in bone and can lead to a thyrotoxic phenotype when expressed alone [34, 35]. In view of these results, it is possible that osteo-chondroprogenitor-specific deficiency of Dio3 selenoprotein activity in *Col2a1-Cre; Trsp*$^{fl/fl}$ mice may have led to the accumulation of abnormally high concentrations of intracellular T_3 during the perinatal period. Raised T_3 levels would not only accelerate bone maturation, but would also contribute to oxidative stress [36]. The latter could aggravate macromolecule damage in cells already impaired in their selenoprotein-based antioxidant defenses, perhaps promoting cell death. The acceleration of osteogenic activity seen in neonatal mice (Fig. 45.4), however, was consistent with increased T_3 levels in the developing skeleton. It is

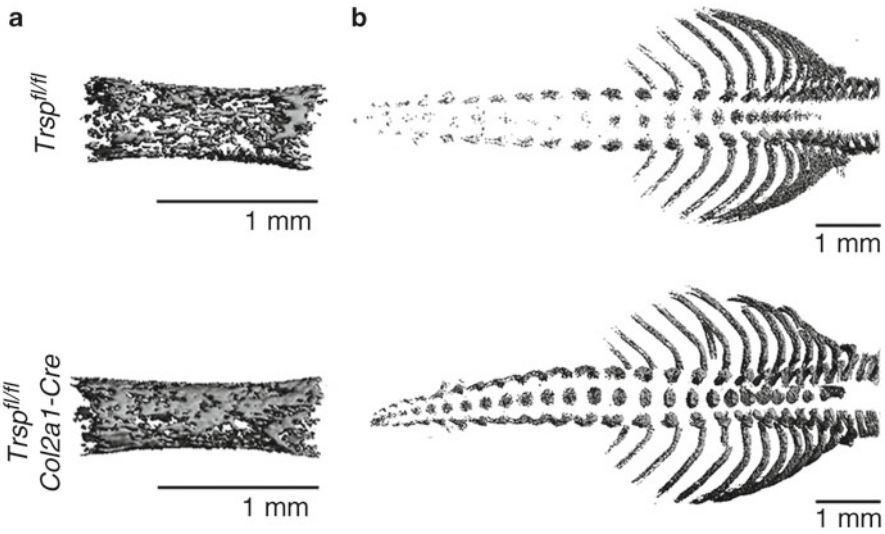

Fig. 45.4 *Trsp*$^{fl/fl}$*; Col2a1-Cre* mutant mice demonstrate increased axial skeleton and limb ossification at birth. High-resolution 3-D micro-CT images were taken at 1 day of age. (**a**) Femurs from representative mice demonstrate increased ossification in *Trsp*$^{fl/fl}$*; Col2a1-Cre* mutant mice, as compared with *Trsp*$^{fl/fl}$ controls. There was no significant difference in the size of the bones at birth. (**b**) Spine and rib cages from control and mutant mice demonstrating increased ossification in the *Trsp*-deficient skeleton, with little apparent difference in overall size of these structures. At this stage in development, the vertebral bodies were more developed in the mutant mice, as compared with controls

difficult, however, to attribute the marked chondronecrosis exhibited by 3–5 week old *Trsp*-deficient mice to abnormalities of thyroid hormone metabolism.

45.9 Potential Relationship Between Selenoprotein Deficiency and Osteoarthritis

There is considerable evidence that oxidative and nitrosative stress, either alone or in combination, have an important pathogenic role in osteoarthritis [37]. This degenerative joint disease can affect one or more joints, and is accompanied by low-grade inflammation [37]. Radiological signs of osteoarthritis are nearly universal in later life. Primary osteoarthritis appears to be the result of a decades-long loss of articular cartilage function and structure, stemming from a chronic imbalance between chondrocyte anabolism and catabolism. This process eventually results in cartilage matrix and cell loss, eventually culminating in bone-on-bone articulations and deformity. Herein, only the role of ROS (and RNS) in the pathogenesis of degenerative joint disease will be discussed.

There is considerable evidence that ROS are involved in degenerative joint disease [38–43]. Physiological levels of ROS appear capable of modulating normal chondrocyte activities such as matrix synthesis and remodeling, as well as cell

proliferation and activation. In contrast, when exposed to stimuli such as mechanical stress, variations in oxygen tension, or pro-inflammatory mediators that chondrocytes can elaborate, increased levels of ROS (and iNOS-derived NO) are generated [39, 40]. When the level of ROS produced exceeds cellular compensatory mechanisms, the result is oxidative stress-induced chondrocyte and cartilage matrix damage. Free radicals and other oxidants can lead to damage of chondrocyte lipids, proteins, proteoglycans [44, 45], and DNA (cellular and mitochondrial). Excess ROS generation can lead to chondrocyte apoptosis.

Oxidative stress appears to increase during normal aging as a result of progressive and cumulative damage to mitochondrial components, including lipid membranes and mitochondrial DNA. This in turn leads to increased generation of superoxide from dysfunctional electron transport chains. Mitochondrial DNA mutations have been associated with a variety of age-related diseases. Indeed, chondrocyte mitochondrial DNA mutations have been found in osteoarthritis, and cells from these patients demonstrated an increased sensitivity to ROS and RNS as compared to controls [38]. In addition, chondrocyte activation by extracellular matrix fragments, or in response to mechanical damage-induced release of latent growth factors from the matrix, may also stimulate superoxide generation via lipoxygenase stimulation, or via protein-tyrosine kinase receptor-mediated activation of small GTPases linked NADPH oxidase systems that generate superoxide [40].

While investigating the role of oxidant stress-induced senescence as a factor in the pathogenesis of osteoarthritis, Buckwalter's group [46] showed that senescent chondrocytes accumulate, as a function of age, in cartilage. In vitro chondrocytes can undergo senescence in response to repeated exposure to peroxide or to growth under supra-physiologic oxygen tensions. Mechanical shear stress applied to cartilage explants *in vitro* increases the generation of ROS. This suggests that such factors as excessive joint use, abnormal biomechanics, and cartilage surface defects that are known to predispose to osteoarthritis, all result in abnormal mechanical stresses to cartilage, and potentially, to ROS generation. Given the ability of ROS to induce senescence, chronic mechanical stress-induced ROS provide a potential mechanism behind the age-dependent decline in chondrocyte function. Nitric oxide may be even more important than ROS in the pathogenesis of osteoarthritis [47]. Interestingly, treatment of an osteoporosis-prone mouse strain with supplemental vitamins E, C, and selenium for 12 months, reduced arthritis severity [48]. It would be of considerable interest to evaluate the consequences of cartilage-specific selenoprotein, or *Trsp* gene disruptions in adult mice. It would be predicted that such animals would undergo rapidly progressive osteoarthritis.

45.10 Evidence for an Age-Dependent Increase in Oxidative Stress in the Pathogenesis of Osteoporosis

Age-related loss of bone mass and strength in humans is a problem that affects both sexes as they age, and interestingly, it is age, rather than bone mineral density, that appears to be the best predictor of fracture risk in either sex [49, 50].

Similar to humans, mice show progressive osteopenia with age, associated with decreased remodeling and bone formation rates, increased ROS generation, increased osteoclast and osteoblast apoptosis, and a progressive increase in p53 and $p66^{SHC}$ phosphorylation (consistent with increasing levels of oxidative stress) [49]. H_2O_2 suppresses osteoblastic differentiation and can promote osteoblast apoptosis [51]. Acutely, oxidative stress and activation of specific pro-apoptotic pathways are induced by gonadectomy [50]. Thus, ROS appear to be important to age-related bone deterioration [49].

Bone remodeling involves the combined activities of osteoblasts and osteoclasts within anatomical spaces known as basic multicellular units (BMU). Estrogen deficiency dramatically increases the number of BMUs, but with a shift in the bone anabolism/catabolism balance toward bone loss. The acute loss of bone postovariectomy is followed by a protracted period of slow bone depletion dominated by trabecular thinning, due in part to osteoblast apoptosis [51, 52]. It has been proposed that postmenopausal human bone loss and postovariectomy in certain rodent models are associated with increased levels of oxidative stress [50, 52, 53], and decreased antioxidant defenses [54]. Furthermore, by experimentally increasing, or decreasing glutathione levels in mice, it was possible to either attenuate, or promote, ovariectomy-induced bone loss, respectively [49, 55, 56].

45.11 Ovariectomy Leads to Oxidative Stress in Bone

Estrogen is able to attenuate oxidative stress by upregulating antioxidant defenses [57–60]. Furthermore, loss of estrogen increases oxidative stress in osteoblasts [49], and treatment with antioxidants is able to attenuate the osteoporotic effects of ovariectomy [55]. Interestingly, there is evidence that the expression of the selenoprotein GPx4 is upregulated by estrogen in some systems [61, 62]. We thus hypothesize that a loss of specific antioxidant defenses in osteoblasts would render them more susceptible to the effects of ROS generated as a result of aging or acute estrogen deprivation.

45.12 Glutathione Peroxidases and the Special Properties of GPx4

There are six GPxs: GPx1 (cytosolic, ubiquitous), GPx2 (cytosolic, gastrointestinal tract), GPx3 (secreted into plasma), GPx4 (membrane associated, ubiquitous; with cytosolic, nuclear, and mitochondrial isoforms), GPx5 (cytosolic, epididymis), and GPx6 (cytosolic, olfactory epithelium [11]). Of these, the most important are thought to be GPx1 and GPx4. Although loss of GPx1 is compatible with normal development (unless mice are challenged with an oxidative stress inducer) [11], loss of GPx4

is embryonic lethal [63, 64]. GPx4 function in fetal or adult mice must therefore be studied using the conditional mutagenesis approach [63, 65, 66]. For more detailed information on GPx4, see Chaps. 14 and 43.

Why is GPx4 so critical? The ubiquitously-expressed GPx4, also known as phospholipid hydroperoxide glutathione peroxidase (phGPx), is the only GPx that is able to reduce complex lipid hydroperoxides even when the latter are present within cellular biomembranes or lipoproteins. Age-associated lipid peroxidation products have been found in osteoblasts [49, 67], suggesting that levels of lipid peroxidation increase with age, perhaps as a result of declining GPx4 activity. In mitochondria, GPx4 prevents peroxidation of cardiolipin, thus preventing the release of cytochrome c [68, 69], an apoptotic stimulus.

Although GPx4 protects cellular membranes owing to its unique ability to reduce phospholipid hydroperoxides [70–73], it is also involved in eicosanoid synthesis, primarily via the control of lipoxygenases (LO) [49, 74, 75]. Because the latter enzymes require basal levels of lipid hydroperoxides for their activation, GPx4 activity can effectively influence LO activities, and as a consequence, leukotriene biosynthesis [76]. The final reaction product of LO involves a lipid peroxidation step and this may also be a substrate for GPx4. Recent studies of inactivation of *GPx4* in cells and mice have provided evidence that GPx4 effectively prevents oxidative stress-induced cell death as well as eicosanoid synthesis via its ability to negatively-regulate 5-LO and 12/15-lipoxygenase (12/15-LO) activities [63, 65, 77]. The12/15-LOs are capable of oxygenating ester lipids even when these are bound within membranes [78]. One of the important consequences of GPx4 deficiency relates to the release of apoptosis-inducing factor (AIF) from mitochondria [63, 65, 66]. In summary, loss of GPx4 can produce not only cell organelle/plasma membrane damage, and increased production of a range of LO products downstream of 5-LO and 12/15-LO, but also sensitization of cells to pro-apoptotic stimuli. Defective GPx4 could thus readily account for aspects of the skeletal phenotype observed in the *Trsp* mutant mouse.

45.13 GPx4 May Be Essential to Normal Osteoblast Development and Function

Considering the necessity to rapidly neutralize lipid peroxides before these can "infect" other membrane lipids, as well as the role of GPx4 in regulating the activities and products of LO, it could be hypothesized that loss of the gene encoding GPx4 in osteoblasts would sensitize these cells to ROS, possibly even to physiological levels of these, and especially during the oxidative stress caused by ovariectomy. Also relevant to osteoporosis, GPx4 depletes peroxidated lipids that are produced by, and required for activation of 5- and 12/15-LOs. Interestingly, the latter, encoded by the *Alox12* and *Alox15* genes, that have been implicated in the control of bone mineral density. Alox15, in particular, was shown to be a regulator of skeletal bone

mass in mice, since its inactivation (both by genetic means and by drugs that inhibit 12/15-LO) led to increased bone mass [79]. Interestingly, and perhaps contributing to bone loss during aging in mice, Alox15 expression increases with age [49]. The *Alox15* gene disruption rendered all tissues in the animal deficient for this enzyme, and decreased levels of this enzyme's PPAR-γ-stimulating reaction products [80] was proposed to account for increased osteoblast generation from marrow mesenchymal stem cells [79]. Since bioactive lipids generated by 12/15-LO and 5-LO also have inhibitory effects on committed osteoblasts [81], loss of these LOs would also be predicted to increase osteoblastic activity. Thus, the increased levels of endogenous bioactive lipids stemming from the lack of GPx4 in osteoblasts would be predicted to inhibit osteogenesis, thus promoting osteoporosis.

While increases in leukotrienes inhibit the generation and function of osteoblasts, they appear to have the opposite effect on osteoclast-mediated bone resorption. Not only are osteoclasts activated by ROS [82, 83], their development and functional activities are greatly augmented by 5-LO and 12/15-LO activity and specific leukotrienes [84–86]. In contrast, osteoclasts are suppressed by LO inhibitors or genetic disruption of genes encoding12/15-LOs [84, 87]. Since GPx4 has been shown to be a regulator of 5-LO and 12/15-LOs, it would be very important to examine the consequences of GPx4 deficiency in osteoclasts. If osteoclast survival was not compromised by the lack of GPx4, it is likely that the bone resorbing activity of these cells would be augmented.

45.14 Evidence for *GPx4* Polymorphisms in Humans

With respect to human osteoporosis, are there polymorphisms of *GPx4* that might impact the activity of this key antioxidant enzyme? In mice, GPx4 appears to be limiting when cells are exposed to oxidative stress. Thus, cells from mice lacking one *GPx4* allele rendered them sensitive to pro-oxidant stimuli or glutathione depletion [88, 89]. In contrast, transgenic mice that modestly overexpressed GPx4 were protected against pro-oxidant challenges [72, 90]. Possibly relevant to these observations, Villette et al. (2002) [91] examined the 3′ UTR of the *GPx4* gene in 66 healthy volunteers and identified a T C single nucleotide polymorphism (SNP) at position 718, near the predicted 3′-UTR selenocysteine insertion element sequence. The distribution of this SNP was in Hardy-Weinberg equilibrium, with 34% CC homozygotes, 25% TT homozygotes, and 41% TC heterozygotes. Interestingly, individuals of different genotypes exhibited significant differences in the levels of peripheral blood lymphocyte 5-lipoxygenase products, with CC homozygotes showing 36 and 44% more products than TT homozygotes and TC heterozygotes, respectively [91]. Thus, there are common polymorphisms that appear to affect *GPx4* activity levels in humans and that might plausibly modulate the pace of osteoporosis. Also, and perhaps indirectly implicating GPx4 activity as a factor in human osteoporosis, several studies have provided evidence of an association between this

disease and SNPs in the *ALOX12* and *ALOX15* genes [92–95]. Owing to its ability to regulate both the 5- and 12/15-LOs, it appears possible that *GPx4* might have a role in the pathogenesis of human osteoporosis.

45.15 Concluding Remarks

Many questions remain about the roles of selenoproteins in the development and the function of the skeleton, however, with currently available Cre-*lox*P technologies permitting gene alterations to be carried out in every skeletal cell type, it is now possible to begin to answer such questions. Herein, a rationale has been provided for investigating the roles of specific selenoproteins in the pathogenesis of common human diseases such as osteoarthritis and osteoporosis, and hopefully will serve to stimulate research into this important area.

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