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Polypeptide chain termination and stop codon readthrough on eukaryotic ribosomes

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Abstract During protein translation, a variety of quality control checks ensure that the resulting polypeptides deviate minimally from their genetic encoding template. Translational fidelity is central in order to preserve the function and integrity of each cell. Correct termination is an important aspect of translational fidelity, and a multitude of mechanisms and players participate in this exquisitely regulated process. This review explores our current understanding of eukaryotic termination by highlighting the roles of the different ribosomal components as well as termination factors and ribosome-associated proteins, such as chaperones.

Abbreviations *aa-tRNA:* Amino-acyl tRNA · *eLF:* Eukaryotic translation initiation factor · *IF:* Prokaryotic translation initiation factor · *eEF:* Eukaryotic translation elongation factor · *EF:* Prokaryotic translation elongation factor · *eRF:* Eukaryotic translation termination factor (release factor) · *RF:* Prokaryotic translation release factor · *RRF:* Ribosome recycling factor · *Rps:* Protein of the prokaryotic small ribosomal subunit · *Rpl:* Protein of the eukaryotic large ribosomal subunit · *S:* Protein of the prokaryotic small ribosomal subunit · *L:* Protein of the prokaryotic large ribosomal subunit · *PTC:* Peptidyl transferase center · *RNC:* Ribosome-nascent chain-mRNA complex · *ram:* Ribosomal ambiguity mutation · *RAC:* Ribosome-associated complex · *NMD:* Nonsense-mediated mRNA decay

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Introduction

The eukaryotic translation machinery must recognize the site on a messenger RNA (mRNA) where decoding should begin and where it should end. The selection of the translation start site is generally given by the first AUG codon encoding the amino acid methionine. During initiation soluble translation initiation factors (eukaryotic translation initiation factors [eIFs] in eukaryotes and prokaryotic translation initiation factors [IFs] in prokaryotes) bind the mRNA, deliver the initiator Met-tRNA, and assemble to form a complete 80S ribosome from the 40S and 60S subunits. By progressing along the mRNA in the 5′- to -3′ direction the ribosome decodes the information and translates it into the polypeptide chain. During this process, repeated delivery of amino-acyl tRNA (aa-tRNA) to the ribosome, peptide bond formation, movement of the mRNA, and the growing peptidyl-tRNA is mediated by both soluble elongation factors (eukaryotic translation elongation factors [eEFs] in eukaryotes and prokaryotic translation elongation factors [EFs] in prokaryotes) and the activity of the ribosome. The final step in the translation process occurs when one of the three termination codons occupies the ribosomal A-site. Translation comes to an end and soluble release factors (eukaryotic translation termination factors [eRFs] in eukaryotes and prokaryotic translation termination factors [RFs] in prokaryotes) facilitate hydrolytical release of the polypeptide chain (for recent reviews, see Inge-Vechtomov et al. 2003; Kisselev et al. 2003; Wilson and Nierhaus 2003; Kapp and Lorsch 2004). Errors during all stages of translation are common and translational fidelity is an important issue for the cell. This review will focus mainly on problems related to the translation termination process in yeast.

Errors in translation termination: premature termination and readthrough

Proteins arising from untimely translation termination will be C-terminally truncated in case of premature translation termination, or will carry a C-terminal extension in case of stop codon readthrough. It is obvious that the effect on protein function can be significant in both cases, and it has been suggested that abortive termination may be more harmful to a cell than missense substitutions, which are only rarely detrimental (Kurland 1992; Dong and Kurland 1995). It has long been known that in yeast a high level of stop codon readthrough can be deleterious (Liebman and Sherman 1976).

Premature translation termination can be caused by substitutions or frame shift mutations in DNA, which lead to the synthesis of a mRNA containing a premature stop codon within the coding region. As a result, truncated protein versions are produced that frequently cause gain-of-function or dominant-negative effects. As one molecule of aberrant mRNA is translated into many polypeptide molecules, its rapid disposal is of vital interest. For this purpose the eukaryotic cell harbors a specific machinery that degrades stop codon-containing mRNA in a process termed nonsense-mediated mRNA decay (NMD). The NMD machinery is directly linked to translation termination factors (compare also below; for recent reviews see Gonzalez et al. 2001; Baker and Parker 2004; Lykke-Andersen 2004). A second possibility for premature translation termination are so-called processivity errors, which interrupt elongation of a polypeptide in the absence of a stop codon. Processivity errors can result from false termination at sense codons, frame shifts of the ribosome into an out-of-phase stop codon, and "drop off" the ribosome from the mRNA. The frequency at which bacterial ribosomes prematurely abort translation is close to 2×10^{-4} (Dong and Kurland 1995).

Opposite to premature translation termination is stop codon readthrough. The frequency of stop codon readthrough in yeast varies within a broad range. Depending on the stop codon and its context values between approximately 10^{-4} and 10^{-1} have been determined (Bonetti et al. 1995). Readthrough results in the synthesis of C-terminally extended polypeptides. Depending on the nature of the 3′-region, different consequences for the translation machinery may arise. In case the downstream region contains a stop codon, the C-terminally extended version of the polypeptide can be released in a normal termination process. In the absence of a downstream stop codon, however, the ribosome will be stalled at the end of the mRNA resulting in a ribosome-nascent chain-mRNA complex (RNC). A specific machinery, which has only recently been discovered, degrades the so-called non-stop mRNA contained in the RNC (Maquat 2002; van Hoof et al. 2002; Vasudevan et al. 2002). How the eukaryotic ribosome is rescued from the nascent polypeptide covalently attached to the tRNA at the ribosomal P-site is unclear. For all we know, the elegant tmRNA disposal system of eubacteria is not conserved in eukaryotes. Bacterial tmRNA acts both as a tRNA and an mRNA and, in a process known as trans-translation, adds a short peptide tag to truncated proteins. Trans-translation in bacteria plays at least two physiological roles: releasing ribosomes stalled upon mRNA, and targeting the resulting truncated proteins for degradation by proteases (Karzai et al. 2000; Withey and Friedman 2002).

While readthrough is usually a detrimental process, in some cases it can help to suppress problems, e.g. arising from premature stop codons present on the DNA level. This type of readthrough, also termed nonsense suppression, leads to the generation of a fraction of the full length protein in addition to the shortened version. Omnipotent suppressors cause nonsense suppression of all three termination codons. In this process, a near cognate tRNA successfully competes with the termination factors such that amino acid incorporation rather than premature termination of translation occurs. Omnipotent suppression can be caused by mutations in various factors involved in the process of translation termination. Nonsense suppression can also result from an aa-tRNA that decodes a termination codon (suppressor tRNA); in this case only one of the termination codons is efficiently suppressed (Hawthorne and Leupold 1974; Stansfield and Tuite 1994).

The eukaryotic translation termination factors eRF1 and eRF3

In eukaryotes, translation termination is mediated by two essential release factors eRF1 (in yeast encoded by *SUP45*) and eRF3 (in yeast encoded by *SUP35*), which act as class I and II factors respectively (Frolova et al. 1994; Stansfield et al. 1995b; Zhouravleva et al. 1995). eRF1 and eRF3 interact both in vitro and in vivo and form a heterodimeric complex (Stansfield et al. 1995b; Paushkin et al. 1997; Frolova et al. 1998; Ito et al. 1998; Eurwilaichitr et al. 1999).

In contrast to bacteria, which possess two class I release factors (RF1 and RF2) with different stop codon specificities, eRF1 functions as an omnipotent release factor in eukaryotes. eRF1 is thought to recognize stop codons in the A-site and stimulate peptide release through interaction with the peptidyl transferase center (PTC; Fig. 1). The actual cleavage of the nascent polypeptide from the P-site tRNA, however, is catalyzed by ribosomal RNA (Caskey et al. 1971; Arkov et al. 1998, 2000; Song et al. 2000; Polacek et al. 2003). eRF1 consists of three distinct domains. Domain 1 is implicated in stop codon recognition (Bertram et al. 2000; Song et al. 2000; Ito et al. 2002). Crosslinking studies showed that domain 1 specifically contacts stop codons in the A-site (Bulygin et al. 2002; Chavatte et

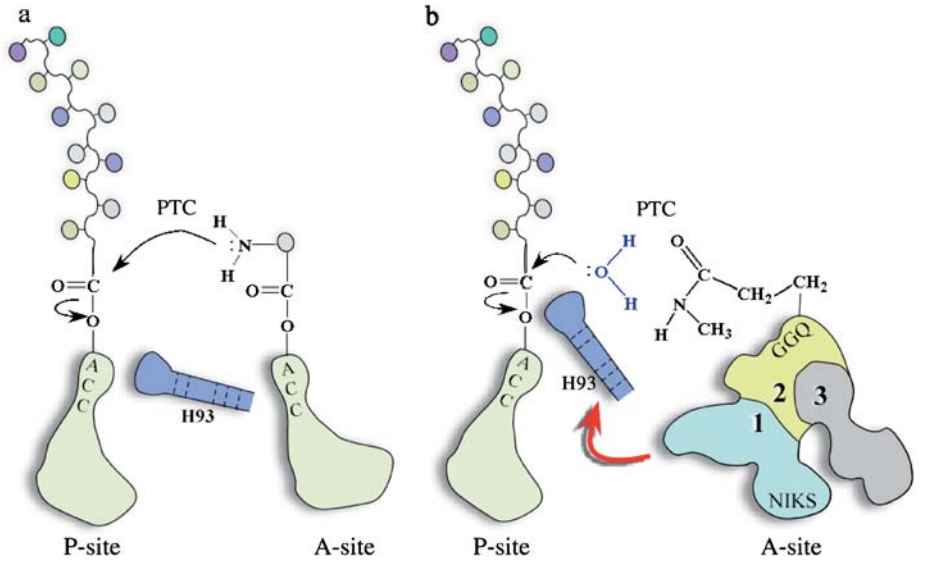

Fig. 1 Reactions catalyzed at the peptidyl transferase center (*PTC*). **a** The ribosomal PTC catalyzes the formation of a peptide bond. The α-amino group of the A-site-bound amino-acyl tRNA attacks the carbonyl carbon atom of the ester bond linking the peptidyl residue to the 3′ end of the P-site-bound tRNA. The result is a new peptide bond and the transfer of the peptidyl residue to the amino acid of the A-site-bound tRNA. During peptide bond formation the ribosome must employ mechanisms to exclude water from the PTC, as otherwise premature peptidyl-tRNA hydrolysis can occur. During peptide bond formation, A2602 of helix 93 (*H93*) of the large subunit rRNA allows proper positioning of tRNA molecules. **b** The PTC catalyzes hydrolysis of the peptidyl-tRNA bound in the P-site by a catalytic water molecule. Translation termination depends on the inclusion of a water molecule in the PTC and on the action of translation termination factor eRF1. Domain 1 of eRF1, containing the NIKS motif, localizes to the A-site of the decoding center. Domain 2 containing the GGQ motif is thought to contact the PCT. Shown is the methylated form of yeast Q182 (Q185 in human eRF1) within the GGQ motif, which may play a direct role in peptide release by positioning the catalytic water molecule for attack of the ester bond. However, a more auxiliary function of eRF1 as modulator of PTC activity has also been suggested. Helix 93 of the PTC plays a critical role during hydrolysis of peptidyl-tRNA and reorients itself upon eRF1 binding (*red arrow*; Song et al. 2000; Frolova et al. 2002; Polacek et al. 2003; Heurgue-Hamard et al. 2005). For details, see text

al. 2002) and it has been speculated that a conserved motif contained in an exposed loop of domain 1 might function as an omnipotent "anticodon" element (Frolova et al. 2002; Nakamura and Ito 2003). Domain 2 contains the conserved and essential GGQ motif, which presumably facilitates the entry of a water molecule to the PTC (Fig. 1). It was proposed that the GGQ motif, which is exposed in a loop at the tip of domain 2, mimics an aminoacyl group at the 3′-CCA acceptor stem of a tRNA (Fig. 1). However, the exact mechanism of stop codon recognition and peptidyl-tRNA hydrolysis remains unclear, and A2602 in helix 93 of the PTC also plays a critical role (Frolova et al. 1999; Song et al. 2000; Seit-Nebi et al. 2001; Klaholz et al. 2003; Polacek et al. 2003). Recently, it was found that glutamine in GGQ of *S. cerevisiae* eRF1 is methylated by a previously uncharacterized S-adenosylmethionine-dependent methyltransferase. The need for the methyltransferase to optimize the growth of yeast suggests that methylation affects functionality of eRF1 (Heurgue-Hamard et al. 2005). Domain 3 at the C-terminus of eRF1 mediates association with the C-terminal domain of eRF3 and possibly other, less well-characterized factors (Fig. 2, and compare below).

Eukaryotic eRF3 is a ribosome- and eRF1-dependent GTPase (Zhouravleva et al. 1995; Frolova et al. 1996). The protein shares a number of features with its bacterial homolog RF3.

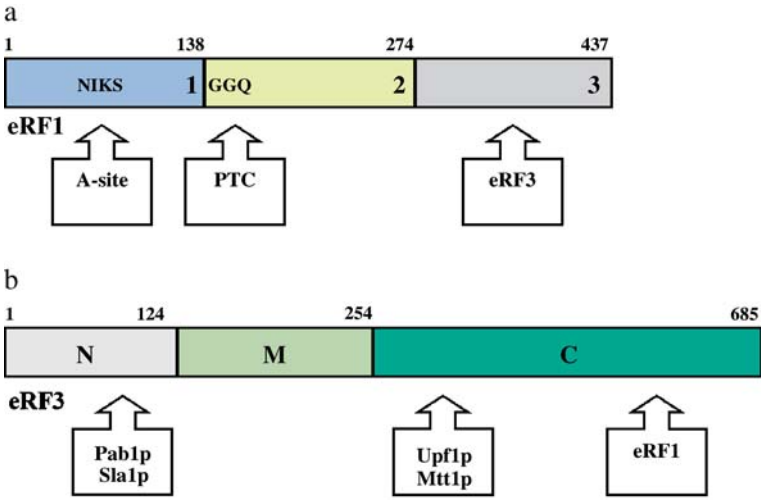

Fig. 2 Domain architecture of the two subunits of eukaryotic translation termination factor. **a** Yeast eRF1 (Sup45p) is an essential protein of 49 kDa consisting of three functional domains. During the termination process domain 1 localizes close to the ribosomal A-site. Domain 2 contains the conserved GGQ motif, which is thought to participate in the catalysis of polypeptide release. Domain 3 interacts with eRF3 and possibly other regulatory factors. **b** Yeast eRF3 (Sup35p) is an essential GTPase of 76 kDa consisting of three major domains. The N-domain is dispensable for cell viability and is responsible for the prion-like properties of yeast eRF3. The M-domain and the eF1α-like C-domain contain regions of homology to GTPase proteins. Pab1p and Sla1p associate with the N/M-domain. Upf1p, Mtt1p, and eRF1 interact with the C-domain of eRF3. Additional proteins interact with eRF3; however, their interaction has been characterized in less detail. For details see text

However, significant differences between the two class II release factors exist: eRF3 is an essential protein while RF3 is not, eRF3 contains additional domains not present in RF3, and forms a stable complex with eRF1 while bacterial RF3 stably interacts with neither RF1 nor RF2 (Buckingham et al. 1997; Kisselev et al. 2003). eRF3 consists of three major domains (Fig. 2). The C-terminal domain is responsible for translation termination activity and is essential for viability (Ter-Avanesyan et al. 1993; Zhouravleva et al. 1995). It is also the domain to which eRF3 binds and stimulates its partner protein eRF1. The domain shows sequence and structural similarity to elongation factor eF1α/EF-Tu (Kong et al. 2004). Functional studies suggest that Mg^{2+} concentrations higher than 0.3 mM weaken GDP binding but strengthen GTP binding to the C-terminal domain, implying that intracellular Mg^{2+} conditions favor GTP binding. This mechanism would be consistent with the absence of a GTP exchange factor for eukaryotic eRF3 proteins (Kong et al. 2004). The N-terminal region of eRF3 is dispensable for the termination process, but is implicated in the binding of poly(A) binding protein (PABP) and other factors involved in the translation process (Fig. 2; Hoshino et al. 1999b; Inge-Vechtomov et al. 2003). This domain is the main determinant for the transition of yeast eRF3 to an insoluble, non-functional form termed [*PSI*$^+$], which displays prion-like characteristics (Ter-Avanesyan et al. 1993; DePace et al. 1998). The yeast prion [*PSI*$^+$] has been instrumental in elucidating the mechanism of prion formation, inheritance, and species barrier (for recent reviews, see Chien et al. 2004; Wickner et al. 2004).

Ribosome recycling factor (RRF) and elongation factor-G (EF-G) are required to recycle the prokaryotic ribosome back to a new round of initiation after termination (Nakamura and Ito 2003). No recycling factor has been identified so far in the cytoplasm of eukaryotic cells. To explain this difference it has been postulated that eukaryotic eRF3 has a dual function:

assisting translation termination by eRF1 (in bacteria performed by the non-essential RF3) and recycling ribosomes (in bacteria performed by the essential RRF; Buckingham et al. 1997).

The ribosome is actively involved in maintenance of translational fidelity

Structures of the 30S subunit of the eubacterium *Thermus thermophilus* (Wimberly et al. 2000), the 50S subunits of the archaebacterium *Haloarcula marismortui* (Ban et al. 2000), the eubacterium *Deinococcus radiodurans* (Harms et al. 2001), and the 70S ribosome of *T. thermophilus* (Yusupov et al. 2001) have been solved by X-ray crystallography. So far, neither the 60S nor the 40S eukaryotic ribosomal subunits are available in atomic detail. Our current understanding of the eukaryotic ribosome structure comes from a combination of cryoelectron microscopy, crystallography data on individual components, and homology modeling based on the prokaryotic counterparts. Cryo-electron microscopy (cryo-EM) of the yeast 80S ribosome revealed remarkable conservation with bacterial ribosomal structures, yet also significant differences. Expansion segments of the rRNAs and additional proteins, mainly at the surface of the 80S particle, are thought to serve as docking sites for factors involved in processes downstream of translation. In addition, more complex intraribosomal networks have evolved to modulate the dynamics of the eukaryotic ribosome (Dube et al. 1998; Morgan et al. 2000; Spahn et al. 2001; Doudna and Rath 2002; Stark 2002).

In yeast, the small subunit is composed of the 18S rRNA (1789 nucleotides) and 32 ribosomal proteins. Compared with *E. coli*, the small subunit of yeast contains 11 more proteins and only 15 of the proteins have homologous bacterial counterparts (Spahn et al. 2001). The 18S rRNA of yeast is 256 nucleotides longer than the 16S rRNA of *E. coli*, but its overall tertiary structure is well conserved (Dube et al. 1998; Spahn et al. 2001). The 18S rRNA of the small subunit is essential for decoding the genetic information and helices 18, 24, 27, 34, and 44 form the highly conserved decoding region (Fig. 3; Carter et al. 2000; Wimberly et al. 2000; Velichutina et al. 2001). The large ribosomal subunit of yeast is built of 25S rRNA (3392 nucleotides), 5.8S rRNA (158 nucleotides), 5S rRNA (121 nucleotides), and 45 ribosomal proteins. The 23S rRNA of *E. coli* (2904 nucleotides), represents a combination of 25S plus 5.8S rRNA, which is 646 nucleotides shorter. In addition, the large subunit of yeast contains 12 more proteins than the large subunit of *E. coli* and 14 proteins more than the large subunit of *H. marismortui*. Despite these differences, the overall structure of the 25S rRNA fits well into the atomic model of the large subunit from *H. marismortui* (Ban et al. 2000; Spahn et al. 2001).

Eleven subunit–subunit interactions have been identified between the 40S and 60S subunits of the yeast ribosome and once the two ribosomal subunits have assembled, they form a communicating ensemble (Gabashvili et al. 1999, 2003; Spahn et al. 2001). For example, the PTC of the large subunit has to be coordinated with the decoding center of the small subunit. After all, the distance between the two most important functional sites of the ribosome is approximately 75 Å (Nakamura and Ito 2003; Ma and Nussinov 2004). Interaction between these sites that are far apart can be achieved either via transmission of conformational changes within and between the subunits or via ribosome-associated factors connecting the different sites. Both principles operate during protein synthesis (Rospert 2004).

The accurate selection of aa-tRNAs cannot be fully accounted for by differences in the interaction energies between cognate or near/non-cognate duplexes (the "decoding problem" is summarized in Ogle et al. 2003). Only recently, a combination of structural, kinetic,

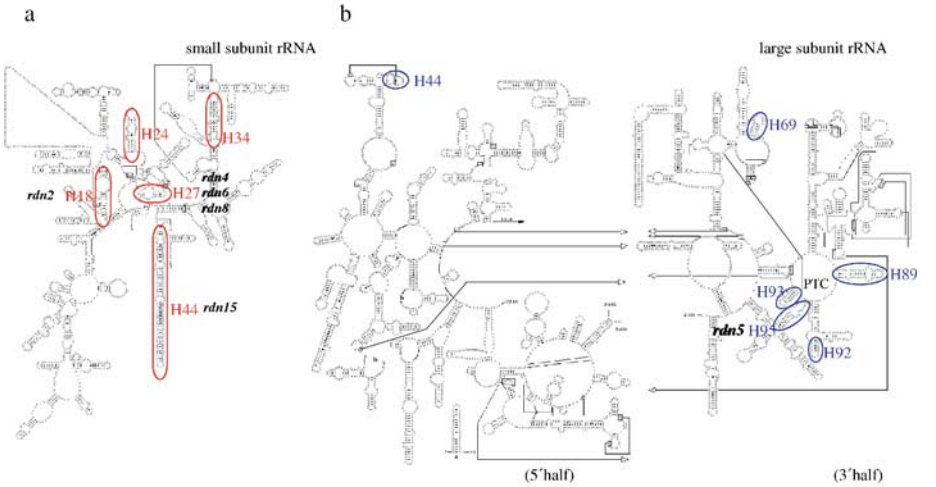

Fig. 3 Secondary structure of the ribosomal rRNA of *Saccharomyces cerevisiae*. http://www.rna.icmb.utexas .edu (Cannone et al. 2002). The numbering of nucleotides is according to *E. coli*. Helices (*H*) discussed in the text are highlighted and localization of yeast *rdn* mutations are indicated. **a** Secondary structure of the small subunit 18S rRNA. Helices discussed in the text are labeled in *red*. **b** Secondary structure of the 25 rRNA. Helices discussed in the text are labeled in *blue*. Helix 44 is part of the L7/L12 stalk; helix 95 contains the sarcin–ricin loop. For details, see text

and biochemical studies revealed how fidelity of translation is maintained in prokaryotes. Specificity of aa-tRNA selection requires kinetic proofreading, which can be viewed as a two-stage process. Initial selection takes place prior to GTP hydrolysis; a subsequent proofreading step is carried out after GTP hydrolysis, but before peptide bond formation. Different rejection rates for cognate or near/non-cognate complexes during both selection steps contribute to specificity (Rodnina and Wintermeyer 2001b; Rodnina et al. 2002). More recent kinetic data indicate that conformational coupling by induced fit enhances the stabilities of cognate codon–anticodon pairing and accelerates GTPase activation (Gromadski and Rodnina 2004; Wintermeyer et al. 2004). Structural studies strongly support a model in which the small subunit actively contributes to aa-tRNA selection. By induced fit, helices 18 and 44 of the decoding region come together to specifically contact the cognate codon–anticodon pair. As a consequence the 30S subunit traverses to a more closed conformation compared to the 30S subunit with an unoccupied A-site (Fig. 4; Ogle et al. 2003). This domain closure significantly contributes to the specificity of tRNA selection (Gabashvili et al. 1999; Ogle et al. 2001, 2002, 2003). Additionally, selection of aa-tRNA is accompanied by structural changes in regions other than the decoding region. These movements may either directly affect the architecture of the decoding region or alter communication between the small and the large ribosomal subunits (Rodnina et al. 2002).

Accurate selection of translation termination factors to ribosomes containing a stop codon in the A-site is less well understood. A picture is only beginning to emerge as the bacterial 70S ribosome and class I release factor RF2 atomic models have recently been fitted into cryo-EM structures. Via multiple interactions RF2 connects the ribosomal decoding site with the PTC and functionally mimics a tRNA molecule in the A-site. In the complex RF2 is close to helices 18, 44, and 31 of the 16S rRNA, small subunit ribosomal protein S12, helices 69, 71, 89, and 92 of the 23S rRNA, the L7/L12 stalk, and protein L11 of the large subunit (Arkov et al. 2000; Klaholz et al. 2003; Rawat et al. 2003). The L7/L12 stalk inter-

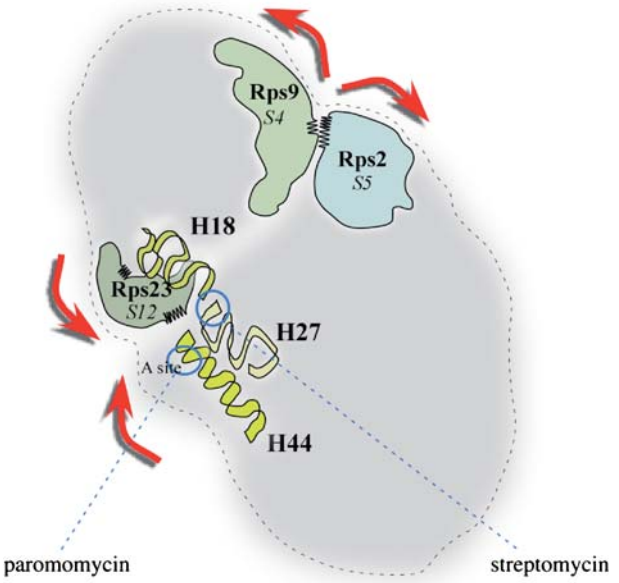

Fig. 4 Schematic section through the small ribosomal subunit of yeast (*gray*) exposing the decoding region. The model is based on structural work on the prokaryotic small ribosomal subunit. The homologous proteins of the *E. coli* decoding region are S4, S5, and S12 (Ogle et al. 2003). Movements within the small ribosomal subunit upon cognate tRNA binding (domain closure) are denoted by *red arrows*. Mutations in helices 18, 27, 44 and in ribosomal proteins Rps9p, Rps2p, and Rps23p (*wavy lines*) are most likely to affect translational fidelity in yeast by changing the energy barrier of domain closure. Mutations in Rps9 and Rps2 are predicted to localize to the contact zone of the two proteins and facilitate domain closure. Depending on their localization, mutations in Rps23p (*wavy lines*) either facilitate or aggravate domain closure. *Blue circles* point to the sites of paromomycin and streptomycin binding. An animated cartoon of the structural changes during domain closure and upon paromomycin and streptomycin binding is available at: http://alf1.mrc-lmb.cam.ac.uk/~ribo/30S/supplementary_info/ogle_TIBS_2003/ (Ogle et al. 2003).

acts with elongation factors, and the N-terminal domain of L11 was proposed to function as a switch, regulating factor entry to the ribosome (Wimberly et al. 1999; Simonson and Lake 2002). Mechanistically important is also the proximity of RF2 to helix 93 of the 23S rRNA (Rawat et al. 2003). Mutations in A2602 localized within this helix abolish RF-dependent hydrolysis of the ester bond in peptidyl-tRNA (Fig. 1; Polacek et al. 2003). Conformational changes upon ribosome binding induce large structural changes in RF2 (Klaholz et al. 2003; Rawat et al. 2003). To what degree termination factor binding impacts on ribosome structure is unclear. Besides movement in the L11 N-terminal domain upon RF2 binding, no major changes in ribosome structure have been observed by cryo-EM (Rawat et al. 2003). Superposition of a cryo-EM structure of the ribosome-RF2 complex with eRF1 suggests that class I release factors are likely to act by a conserved mechanism (Klaholz et al. 2003; Ma and Nussinov 2004).

Visualization of the bacterial RF3 on the ribosome by cryo-EM revealed that RF3 could be bound in two different conformational states associated with significant conformational changes within the ribosome (Klaholz et al. 2004). In state 1 ribosomes contain a tRNA in the P-site and RF3 is loosely associated. In state 2 the tRNA has moved to the E-site and RF3 is more tightly attached to the ribosomes (Klaholz et al. 2004). How eRF3 structurally influences eRF1 and the eukaryotic ribosome awaits future investigation.

Components of the translational apparatus and mutations thereof that affect translation termination

The overall fidelity of translation termination in the cell depends on how accurately eRF1 and eRF3 function. As outlined below, this can be affected by numerous components and conditions. However, two basic mechanisms underlie the majority of untimely translation termination events. Both increase the chance for a near-cognate aa-tRNA to bind to a stop codon in the mRNA. The first mechanism applies when insufficient amounts of functional translation termination factor are present in the cell. Examples include destabilizing mutations in eRF1 or eRF3, depletion of soluble eRF3 by [*PSI*$^+$] formation, mutations in eRF1 or eRF3 that corrupt binding to the ribosome, and possibly mutations in proteins interacting with eRF1 or eRF3. The second mechanism is related to mutations affecting the ability of the 30S subunit to perform the specific structural rearrangement, termed domain closure (Fig. 4). This group includes mutants in rRNA, ribosomal proteins of both subunits, and proteins loosely associated with ribosomes.

Mutants in ribosomal RNA that affect the fidelity of translation termination

Investigation of rRNA mutations in a eukaryotic organism requires special techniques, as rDNA genes are generally highly redundant. In yeast 100–200 tandemly repeated copies of rDNA genes are located at the *RDN* locus of chromosome XII. Several elegant systems to introduce mutations into rDNA genes have been developed and it is now possible to generate yeast strains expressing pure populations of ribosomes containing mutant rRNA (Liebman et al. 1995). Several of these rRNA mutations affect translational fidelity (Chernoff et al. 1994; Liebman et al. 1995; Velichutina et al. 2000, 2001).

Helix 44 of the 18S rRNA is the conserved core of the decoding region (Figs. 3, 5). At the same time, this long helix is involved in several of the contacts between the two ribosomal subunits, e.g. to the 5.8/25S rRNA, to Rpl23p, and to Rpl24p (Spahn et al. 2001). The yeast *rdn15* mutation (A1491G) creates a base pair analogous to that in helix 44 of prokaryotes (Fig. 5). This mutation compensates for conditional lethality caused by a GTPase mutant of eRF3 and also a mutant of eRF1. Based on these findings it was hypothesized that *rdn15* causes eRF1 to recognize stop codons more efficiently (compare also section on the GTPase activity of eRF3 and on aminoglycoside action; Velichutina et al. 2001).

Helix 27 of the 18S rRNA contacts helix 44 below the decoding region (Fig. 5; Carter et al. 2000) and is also involved in a bridge to the 5.8/25S rRNA of the large subunit of yeast (Spahn et al. 2001). In *E. coli* evidence suggests that helix 27 can perform a conformational switch, which has been implicated in the selection of tRNA at the A-site of the decoding region (Lodmell and Dahlberg 1997). Cryo-EM studies revealed that the switch induces large-scale structural changes, not only in the small but also in the large ribosomal subunit (Gabashvili et al. 1999). So far, it is not clear whether such a switch mechanism involving helix 27 is also operating in yeast (Velichutina et al. 2000). However, three yeast mutants in helix 27 that affect translational fidelity have been identified: *rdn4*, *rdn6*, and *rdn8* (Fig. 3). These mutants perform with increased translational fidelity. The double mutant *rdn4rdn6* displays synthetic effects in combination with mutations in eRF1, suggesting that helix 27 affects termination factor binding to the ribosome (Velichutina et al. 2000). In view of recent structural data it seems likely that mutations in helix 27, by affecting the interaction energy

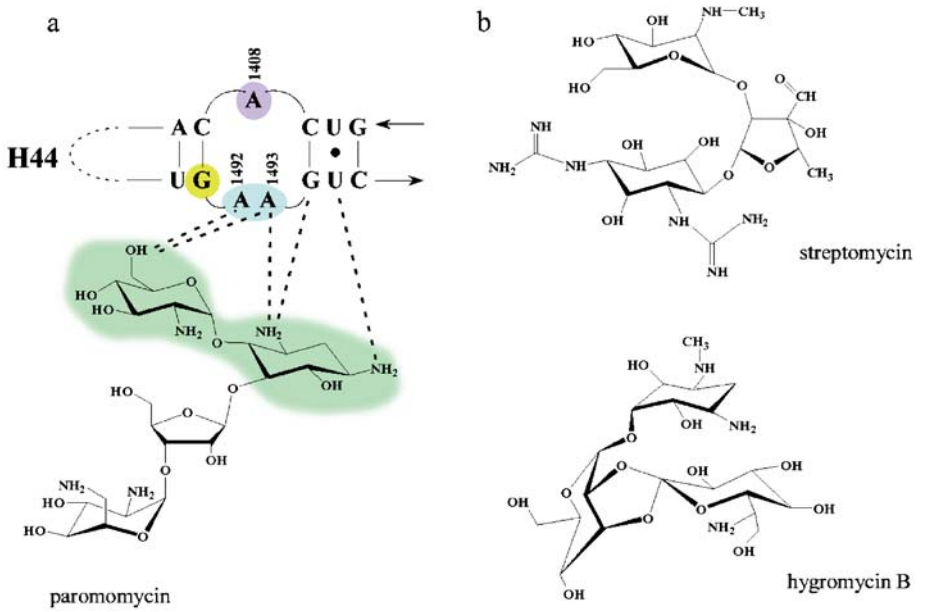

Fig. 5 Aminoglycosides interact with the rRNA of the decoding region. Aminoglycosides are oligosaccharides containing several ammonium groups. Different subclasses are distinguished on the basis of chemical structure and mechanism of action. **a** The paromomycin subclass is characterized by the presence of a 2-deoxystreptamine ring. Paromomycin interacts with helix 44 (*H44*) of the prokaryotic A-site. Important hydrogen bonds between the prokaryotic A-site and paromomycin are indicated by *dotted lines*. The part of paromomycin conserved in other A-site binding aminoglycosides is highlighted in *green*. A1492/1493 (*blue*) are displaced from the internal loop during domain closure (Fig. 4). Binding of paromomycin facilitates displacement of A1492/1493. G1491 (*yellow*) is involved in high affinity paromomycin binding. The nucleotide is not conserved in eukaryotes. A1408 (*violet*) is important for the effects exerted by aminoglycosides of the paromomycin subclass. Because the flipping of A1492/1493 is no longer induced by paromomycin after replacement of A1408 to G1408, the mutation confers paromomycin resistance to *E. coli*. **b** The aminoglycosides streptomycin and hygromycin B are non A-site binders (Vicens and Westhof 2003). For details, compare text.

at the interface to the small subunit shoulder, influence the ease of domain closure (Ogle et al. 2003).

In agreement with the view of an intimate cross talk between the two ribosomal subunits, mutations in the rRNA of the large subunit also influence translational fidelity (Fig. 3). Some evidence suggests that the sarcin–ricin loop, named after two toxins that target the same conserved loop region of the large subunit rRNA, is an important structural element in this process. Sarcin is a member of a fungal toxin family and specifically cleaves one phosphodiester bond in the large ribosomal subunit rRNA. Ricin belongs to a family of heterodimeric plant proteins that target the large ribosomal subunit RNA and cleave a single *N*-glycosidic bond (Spahn and Prescotta 1996). In both cases, elongation factor binding is disrupted and translation is inhibited. Mutations in the sarcin–ricin loop affect translational fidelity in prokaryotes (Tapprich and Dahlberg 1990; Melancon et al. 1992; Bilgin and Ehrenberg 1994; Bilgin et al. 1998) and in yeast the *rdn5* mutation in the sarcin–ricin loop also increases the rate of translational errors (Liu and Liebman 1996; Panopoulos et al. 2004). Additionally, mutations in the 5S rRNA can affect stop codon readthrough in yeast. A genetic screen identified both mutations that enhance and mutations that decrease translational fidelity. It has been speculated that 5S rRNA has an important role in transfer-

ring allosteric signals between the different functional centers of the ribosome (Smith et al. 2001).

Mutants in ribosomal proteins of the small subunit that affect translation termination

Small subunit proteins S4, S5, and S12 profoundly influence translational fidelity in *E. coli* and are referred to as proteins of the accuracy center (Alksne et al. 1993; Liebman et al. 1995). All three proteins localize in close proximity to the rRNA of the decoding region (Ogle et al. 2003). S4 and S5 have been extensively studied and a large number of mutants, termed *ram* (ribosomal ambiguity mutations) display reduced translational fidelity (Liebman et al. 1995; Kurland et al. 1996). *ram* ribosomes have higher affinity for aa-tRNA and lower affinity for peptidyl-tRNA (Karimi and Ehrenberg 1994, 1996). The crystal structure of the bacterial small subunit revealed that S4 and S5 contact each other and that the majority of *ram* mutations map to the interface of the two proteins (Carter et al. 2000). Later it was found that the conformational switch required for domain closure involves movement of S4 and S5 relative to each other. As a consequence, mutations that lower the stability of the S4/S5 interaction will reduce the cost of domain closure and thus increase the acceptance of near-cognate tRNA (compare above and Ogle et al. 2003). In agreement with this model, mutations in yeast Rps9p (homologous to S4) and Rps2p (homologous to S5) have been identified by their ability to enhance stop codon readthrough long before it was recognized that the corresponding genes encode for ribosomal proteins. Later it was shown that mutations in these proteins do indeed decrease the fidelity of translation (Fig. 4; Masurekar et al. 1981; Eustice et al. 1986; Liebman et al. 1995; Synetos et al. 1996).

In contrast to the *ram* mutations in S4/S5 mutations in protein S12, positioned at the opposite shoulder from the S4/S5 interface, increase translational fidelity (Ogle et al. 2003). Mutated sites in S12 contact helices 44 and 27 only after domain closure (Fig. 4). As a consequence these mutations destabilize the closed conformation and discourage incorporation of near-cognate amino acids (Ogle et al. 2003). Rps23p, the yeast S12 homolog, is also involved in the maintenance of translational fidelity (Alksne et al. 1993). A number of mutations in Rps23p lead to antisuppression in vivo. These mutations predominantly localize in the C-terminal part of Rps23p. Interestingly, mutations in the extended N-terminal tail of Rps23p lead to suppression (Anthony and Liebman 1995; Synetos et al. 1996; Wimberly et al. 2000) suggesting that alterations of this part of the protein may rather stabilize the closed conformation of the 30S subunit.

Mutants in a ribosomal tunnel protein and in ribosome-bound chaperones affect translational fidelity

L39e is a component of archaebacterial and eukaryotic ribosomes. The structure of the large ribosomal subunit of *H. marismortui* shows L39e in the neighborhood of the polypeptide tunnel exit, distant from the accuracy region of the ribosome. The tiny protein of extended structure partly enters the polypeptide exit tunnel (Nissen et al. 2000; Rospert 2004). The absence of yeast Rpl39p leads to translational errors at a rate comparable to that observed for mutants in proteins of the decoding region (Dresios et al. 2000). How Rpl39p exerts this strong effect on translational fidelity is not currently understood. It has been suggested that Rpl39p may allosterically transmit an effect along the polypeptide tunnel (Dresios et al. 2001). An interesting twist to this hypothesis comes from studies on the mechanism of

cotranslational translocation into the membrane of the ER. There is evidence that signals from deep within the ribosomal tunnel can be transmitted to the ER translocation machinery (Liao et al. 1997). Rpl39p is one of the ribosomal proteins and is thought to participate in signaling (Woolhead et al. 2004). From the perspective of the nascent polypeptide Rpl39p thus seems to communicate in both directions: backward to the decoding region of the small subunit and forward, beyond the tunnel exit to the ER translocon.

Communication between the polypeptide tunnel and the small ribosomal subunit is also required for the simultaneous and coordinated movement of mRNA and the nascent polypeptide. L22 and L4 are ribosomal proteins of the large subunit conserved in prokaryotes and eukaryotes. Like Rpl39p, these two proteins build part of the interior of the polypeptide tunnel (Ban et al. 2000; Nissen et al. 2000). The antibiotic erythromycin acts by physically blocking the tunnel, and thus it is not surprising that modifications in L22 or L4 can lead to erythromycin resistance. One type of mutation in L4 narrows the entrance of the tunnel in such a way that erythromycin binding is prevented (Gabashvili et al. 2001). In a genetic screen it was found that second-site suppressors in S12 and S5 (Saltzman and Apirion 1976) could overcome this type of L4 mutation. As discussed above, these two proteins are intimately linked to translational fidelity and affect domain closure of the small subunit (Fig. 4; Ogle et al. 2003). In combination with cryo-EM data on structural changes in L4-mutant ribosomes, the results suggest that conformational changes in the small subunit signal to the entrance of the polypeptide tunnel (Gabashvili et al. 2001).

Approximately 100 Å downstream of the PTC the tunnel ends and polypeptides exit to the cytosol. A number of ribosome-associated factors assist polypeptide passage and it is at this location where protein folding, translocation, and covalent modifications are initiated (Rospert et al. 2005). Among the midwives assisting the nascent polypeptide in yeast are the ribosome-associated Hsp70 homologs Ssb1/2p and Ssz1p, and the Hsp40 homolog zuotin. Ssz1p and zuotin form a dimeric complex termed RAC (*ribosome-associated complex*), which function together with Ssb1/2p in an unusual chaperone triad (Gautschi et al. 2001, 2002; Hundley et al. 2002; Rospert et al. 2005). Crosslinking studies revealed that Ssb1/2p, and most likely also RAC, localize close to the tunnel exit (Pfund et al. 1998; Gautschi et al. 2002, 2003). A prominent role of chaperones is to assist the folding of nascent polypeptides and RAC and Ssb1/2p are ideally positioned to mediate this process (Craig et al. 2003). It is thus unexpected that ribosomes lacking functional Ssb1/2p or RAC translate with decreased fidelity in vivo and in vitro (Rakwalska and Rospert 2004). How Ssb1/2p and RAC influence translation is not currently understood (compare also below). It may be speculated that they act as a functional extension of the tunnel and, like Rpl39p, participate in intersubunit communication. Δ*ssz1*/Δ*zuo1* and Δ*ssb1*/Δ*ssb2* strains are most severely affected in translation termination and only to a lesser extent in misincorporation (Rakwalska and Rospert 2004). Possibly, Ssb1/2p and RAC are involved in conformational changes of the ribosome that are specifically important for accurate translation termination (Fig. 1). However, other mechanisms may account for the termination defect. As we will see below, Ssb1/2p and RAC belong to a large group of ribosome-bound factors that affect translation termination. Although in most cases the mechanism is not understood direct interaction with eRF1/eRF3 seems to be a general theme. Ssb1/2p is connected to eRF3 by the unexplained observation that this chaperone affects the conversion of eRF3 to [*PSI*$^+$] (Chernoff et al. 1999; Kushnirov et al. 2000; Chacinska et al. 2001; Allen et al. 2005). Direct interaction between Sup35p and Ssb1/2p, however, has not been demonstrated so far.

Mutants in translation termination factors that affect the fidelity of translation termination

The effect of mutations in eRF1 and eRF3 on the efficiency of translation termination in yeast has been extensively studied. A variety of mutations in both translation termination factor subunits results in a nonsense suppression phenotype (Eustice et al. 1986; Song and Liebman 1989; All-Robyn et al. 1990; Wakem and Sherman 1990; Stansfield et al. 1995a, 1997; Bertram et al. 2000; Velichutina et al. 2001; Cosson et al. 2002; Bradley et al. 2003; Chabelskaya et al. 2004; Salas-Marco and Bedwell 2004).

Mutations in eRF3, which directly influence translation termination, localize to the essential C-domain (Fig. 2). One example of such a mutant is eRF3-R419G, which is thought to affect the GTPase activity of eRF3. eRF3-R419G causes increased stop codon readthrough and temperature sensitivity in yeast. Interestingly, the conditional phenotype can be rescued by the 18S rRNA mutants *rdn15* and *rdn4* (Velichutina et al. 2001). Mutations *rdn15*/*rdn4* may stabilize eRF1 on the ribosome long enough to provide sufficient time for mutant eRF3 to hydrolyze GTP and stimulate the termination activity of eRF1. Indeed, a recent study showed that decreased termination efficiency results when eRF1 activation becomes a less frequent event in the presence of eRF3 mutants with reduced GTPase activity (Salas-Marco and Bedwell 2004). Mutations in the non-essential N-domain of yeast eRF3 also affect translation termination, but by a very different mechanism. The N-terminal domain governs the conversion of eRF3 to its prion form [*PSI*$^+$] (Tuite 2000). In the context of translation termination, it is important to recall that formation of [*PSI*$^+$] depletes the cells of functional, i.e. soluble, eRF3. As eRF1 is unable to mediate polypeptide chain release in the absence of its partner subunit, [*PSI*$^+$] strains are defective in translation termination and display increased readthrough. Mutants with a less pronounced tendency to form [*PSI*$^+$] affect mostly Gln or Asn residues and cluster to the Gln/Asn-rich regions of eRF3 in its N-terminal portion (DePace et al. 1998). The [*PSI*$^+$] effect on translation termination may also involve eRF1 more directly, as it was found that eRF1 becomes incorporated into [*PSI*$^+$] aggregates (Paushkin et al. 1997).

As illustrated by the [*PSI*$^+$] phenomenon the relative abundance of the eRF1/eRF3 complexes is an important determinant for the efficiency of translation termination. Similar effects have been obtained in depletion studies and with eRF3 and eRF1 mutants that decrease their cellular concentration (Stansfield et al. 1996; Moskalenko et al. 2003; Chabelskaya et al. 2004; Salas-Marco and Bedwell 2004). Wild type yeast contains approximately 10- to 20-fold fewer termination factors compared to ribosomes (Didichenko et al. 1991; Stansfield et al. 1992). The threshold level of eRF1/eRF3 required to maintain viability is even lower. A 10-fold decrease reduces viability by only about 10% (Valouev et al. 2002) and a decrease in eRF3 of 99% does not affect viability of the cells significantly (Chabelskaya et al. 2004). As eRF3 is associated with polysomes or ribosomes, the mechanism of translation termination depends on efficient recycling (Didichenko et al. 1991 and compare below).

Proteins interacting with translation termination factors affect the fidelity of translation termination

Recent studies have shown that proteins interacting with the release factors can modulate the efficiency of stop codon readthrough. Physical and functional interaction with the translation termination factors was demonstrated for different components of the translational machinery.

The processes of nonsense-mediated mRNA decay and translational fidelity are intimately intertwined

Translation of abnormally truncated polypeptides from transcripts containing premature stop codons is minimized by mRNA degradation via the NMD pathway (Gonzalez et al. 2001; Wormington 2003; Baker and Parker 2004). Nonsense-containing transcripts differ from normal mRNAs and display accelerated deadenylation, loss of the poly(A) tail to inhibit decapping, and acceleration of the decapping rate. The close relation between NMD and translation termination is revealed by the observation that NMD can be prevented by suppressor tRNAs (Gozalbo and Hohmann 1990; Belgrader et al. 1993). Recently, it was noted that a ribosome toe print generated at a normal translation termination codon is distinct from the toe print of a ribosome terminating at a premature stop codon (Amrani et al. 2004; Baker and Parker 2004). How exactly a stop codon located within an *orf* is distinguished from a bona fide stop codon is still not fully understood and different models exist to explain this surprising finding.

Upf1p, Upf2p, and Upf3p are essential components of the NMD machinery in yeast. Recent results indicate that NMD is not restricted to degradation of transcripts containing premature stop codons, but plays a more general role in mRNA disposal (Lelivelt and Culbertson 1999; He and Jacobson 2001; Wang et al. 2001; He et al. 2003). One model suggests that during termination the NMD machinery is first assembled and then screens 3′ of the termination codon for specific sequences that target the mRNA for degradation (Czaplinski et al. 1999; Gonzalez et al. 2001). More recently, it was recognized that a specific spatial relationship among the stop codon, the structure shaped by mRNA, ribosome, and associated factors is required for proper termination (Amrani et al. 2004; Baker and Parker 2004).

Upf1p is the central component of the NMD machinery. The protein localizes to the cytosol and is mostly bound to polysomes (Atkin et al. 1995, 1997). At the N-terminus, it contains a cysteine- and histidine-rich region with similarity to the RING finger motif. The C-terminal domain places Upf1p into the RNA/DNA helicase superfamily and is responsible for nucleic acid-dependent helicase and ATPase activities (Altamura et al. 1992; Czaplinski et al. 1995). *UPF1* is not essential for viability; however, disruption of the gene results in stabilization of stop codon-containing transcripts and, quite unexpectedly, leads to a nonsense suppression phenotype. For example, cells harboring a nonsense mutation in the *leu2* gene are unable to grow on medium lacking leucine and the abundance of *leu2* mRNA is low. Deletion of *UPF1* from this strain restores the abundance of *leu2* mRNA to wild type level and allows growth on leucine-free medium. On the basis of these results it was suggested that the increased concentration of nonsense-containing mRNAs in a Δ*upf1* strain would fully account for the nonsense suppression phenotype (Leeds et al. 1991). However, later experiments demonstrated that the effect of Upf1p on mRNA stability and on nonsense suppression are separable functions: Some *upf1* alleles localized to the helicase domain inactivate NMD, but fail to suppress nonsense alleles, while certain mutations in the N-terminal region display normal NMD, but act as nonsense suppressors (Weng et al. 1996a,b). Also, overexpression of *UPF1* leads to a substantial decrease in nonsense suppression without any significant effect on mRNA levels (Maderazo et al. 2000). The combination of data is consistent with a model in that the amino-terminal, cysteine- and histidine-rich region of Upf1p is involved in modulating translation termination and affects translational fidelity. More recent results, however, suggest that the effect of the NMD machinery on stop codon readthrough might not be a general phenomenon (Harger and Dinman 2004).

Although the exact mechanism by which Upf1p influences translation termination remains unclear the function is connected to the well-documented physical interaction be-

tween Upf1p and eRF3 (Fig. 2; Czaplinski et al. 1998; Wang et al. 2001; Keeling et al. 2004; Kobayashi et al. 2004). As previously discussed, efficient recycling is a prerequisite for efficient translation termination due to the large excess of ribosomes over eRF1/eRF3. Moreover, ribosomes terminating prematurely are released much less efficiently compared with those encountering conventional termination signals (Amrani et al. 2004). As assembly of the NMD machinery on a transcript containing a premature stop codon has been suggested to involve translation termination factor release, recycling of the termination factors may become limiting in Δ*upf1* strains (Wang et al. 2001). Alternatively, Upf1p may catalytically influence release activity of the termination factors. The GTPase domain of eRF3 affects the ATPase activity of Upf1p (Czaplinski et al. 1998; Wang et al. 2001). GTPase mutants of eRF3 have been found to influence stop codon recognition in a termination signal-dependent manner (Salas-Marco and Bedwell 2004). In a similar fashion, readthrough in Δ*upf1* strains is influenced by the termination signal (Keeling et al. 2004). Possibly, Upf1p influences the GTPase activity of eRF3, which in turn influences the efficiency of eRF1-mediated polypeptide release (Salas-Marco and Bedwell 2004). A catalytic function of Upf1p in translation termination is consistent with the finding that there is only one molecule of Upf1p molecule per 200 ribosomes/20 eRF1/eRF3 (Didichenko et al. 1991; Maderazo et al. 2000).

Coupling between translation termination and translation initiation

Poly(A)-binding protein (PABP in higher eukaryotes, Pab1p in yeast) is a multifunctional protein. The common theme of PABP is binding to poly(A) tails of transcripts where the protein is thought to provide a scaffold for the binding of factors involved in polyadenylation, mRNA export, translation, and transcript turnover (Mangus et al. 2003). When bound to poly(A)-tails of cytosolic mRNA, PABP interacts with the initiation scaffold protein eIF4G. This interaction is thought to mediate formation of a closed mRNA loop structure and promote recruitment of the 40S ribosomal subunit (Tarun and Sachs 1996; Tarun et al. 1997; Coller et al. 1998; Wells et al. 1998; Gray et al. 2000; Mangus et al. 2003).

Initial evidence that PABP does not only interact with the initiation, but also the termination machinery came from higher eukaryotes. Later, it was also found in yeast that eRF3 interacts with Pab1p. Interaction occurs through the N-domain of eRF3 and the C-terminal domain of PABP/Pab1p is thought to be important for several steps of the translation process (Fig. 2; Hoshino et al. 1999a,b; Cosson et al. 2002). First, the interaction is a prerequisite for efficient recycling of ribosomes (Uchida et al. 2002). Second, the interaction is required for normal mRNA turnover as deletion or overexpression of the eRF3 N-domain results in prolongation of half-lives for all mRNAs and causes defects in poly(A) shortening (Hosoda et al. 2003; Amrani et al. 2004; Kobayashi et al. 2004). Third, the interaction enables Pab1p to act as an antisuppressor when overexpressed in strains in which translation termination is hampered for a variety of reasons (Cosson et al. 2002). An interesting genetic interaction also exists with Rpl39p, one of the ribosomal proteins involved in translational fidelity (compare above). Rpl39p was identified in a screen for mutants rescuing the otherwise lethal phenotype of Δ*pab1*. In the absence of functional Rpl39p, Pab1p-independent translation became possible (Sachs and Davis 1990). Whether the effects of Rpl39p and Pab1p on translational fidelity reflect the same functional interaction is unknown.

Translational fidelity and the cytoskeleton

The protein biosynthetic machinery in a living cell is highly organized and numerous studies have suggested a role of the actin cytoskeleton in translation (Lenk et al. 1977; Negrutskii et al. 1994; Kandl et al. 2002; Valouev et al. 2004, and references therein). Analysis of a large set of yeast actin mutants revealed that, quite unexpectedly, conditional growth defects were not related to reduced rates of translation. Instead, a subset of the actin mutants displayed increased stop codon readthrough, suggesting the role of actin in translational fidelity (Kandl et al. 2002). Additional evidence for a functional interaction between the cytoskeleton and specifically the termination machinery came from studies on Sla1p and Sla2p, two proteins involved in actin assembly. Sla1p interacts with the N-terminal domain of eRF3 and is thus a possible link between the translation termination machinery and the cytoskeleton (Fig. 2; Bailleul et al. 1999). Low levels of eRF3 not only increase stop codon readthrough but also induce defects in the actin cytoskeleton (Valouev et al. 2002). Sla2p was found to affect the status of the actin cytoskeleton and the stability of mRNA, which, as discussed above, is closely coupled to translation termination (Zuk et al. 1999). The physiological significance of the interactions between components of the translation apparatus and the cytoskeleton have not yet been established.

Proteins inducing readthrough when overexpressed

In yeast several proteins have been identified by their ability to induce nonsense suppression when overexpressed. The most intriguing readthrough inducer is Mtt1p, a protein homologous to Upf1p. Like Upf1p, Mtt1p is directly linked to the termination machinery and interacts with eRF1/eRF3 (Czaplinski et al. 2000). Itt1p, a protein with unknown molecular function, also interacts with the termination factors. In this case, the binding to eRF1 seems of specific importance since simultaneous overexpression of the two proteins abolishes the effect of Itt1p on readthrough (Urakov et al. 2001). A genetic screen aimed at the identification of factors that modulate translation termination revealed a number of additional candidates. Overexpression of Stu2p increases the rate of stop codon readthrough. Stu2p is a component of the spindle pole body, localizes to microtubules, and controls microtubule dynamics in vivo (Namy et al. 2002b). Whether there is direct interaction between Stu2p and the termination factors is not known. It may be speculated that Mtt1p, Itt1p, or Stu2p connect translation termination with other cellular processes like mRNA turnover pathways, recycling of terminating ribosomes or termination factors, and the spatial arrangement of the translation machinery.

Aminoglycoside antibiotics affect translation termination

The group of aminoglycoside antibiotics

Prokaryotes are sensitive towards aminoglycosides. In *E. coli* sublethal levels suppress auxotrophic mutations and increase the frequency of misincorporation obtained in vitro (Gorini and Kataja 1964; Davies et al. 1965). Yeast is normally quite resistant toward aminoglycosides, but mutant strains expressing translational suppressors display increased sensitivity in vivo and increased mistranslation frequencies in vitro (Palmer et al. 1979; Singh et al.

1979; Masurekar et al. 1981). These observations established early on that the binding of small molecules to the ribosome could affect translational fidelity. Evidently, the ribosome was involved in translation beyond its catalytic activity. Over the last few decades, aminoglycosides have been instrumental in the elucidation of the decoding mechanism.

At physiological pH, aminoglycosides are multiply charged, highly flexible compounds. Their positive charges are attracted to negatively charged RNA backbones and their flexible structure facilitates accommodation into binding pockets formed by loops of RNA helices or into ribozyme cores (Schroeder et al. 2000). Aminoglycosides are divided into several subclasses that differ in structure and biological action. The paromomycin group is characterized by a 2-deoxystreptamine ring (Fig. 5). Other commonly used members of the paromomycin subclass are gentamicin, G418, and kanamycin. Streptomycin and hygromycin B represent important members of two distinct subclasses (Fig. 5; Puglisi et al. 2000; Vicens and Westhof 2003). Aminoglycosides affect both the fidelity of the elongation and the fidelity of the translation termination process. The effect on the misincorporation of wrong amino acids has been studied in detail and is now understood at a molecular level (Ogle et al. 2003). Why higher concentrations of aminoglycosides kill cells, however, is only poorly understood (compare below).

Aminoglycoside action on prokaryotic ribosomes

The major target of aminoglycosides in living cells is the small subunit of the ribosome. Kinetic analysis revealed that paromomycin stabilizes binding of aa-tRNA at the A-site, irrespective of whether the codon–anticodon pair is cognate or near-cognate. In this way paromomycin accelerates GTP hydrolysis and peptide bond formation in the near-cognate complexes (Rodnina and Wintermeyer 2001a). On a structural level paromomycin affects domain closure (Fig. 4; Ogle et al. 2003). As previously discussed, one important consequence of domain closure is the displacement of A1492/A1493 from an internal loop in helix 44 (Fig. 5). The displacement is efficient only in the presence of cognate tRNA, since near-cognate tRNA does not trigger displacement. The trick of paromomycin and its structural relatives is to induce the flipping of A1492/A1493, even in the absence of cognate tRNA. As a consequence domain closure is greatly facilitated and translational fidelity is compromised (Fourmy et al. 1996, 1998a,b; Yoshizawa et al. 1998; Jerinic and Joseph 2000; Puglisi et al. 2000; Ogle et al. 2001, 2002).

Streptomycin, which binds to the same region of the 16S rRNA, acts by a different mechanism. This drug directly connects protein S12/helix 18 with helix 44/helix 27 of the decoding region (Fig. 4). Additional interactions between these two sites are thought to facilitate movements during domain closure (Carter et al. 2000). The model rationalizes how streptomycin affects restrictive S12 and error-inducing *ram* mutations in S4 and S5 (Piepersberg et al. 1979; Kurland et al. 1996). Streptomycin counteracts mutations in S12 that interfere with closure and aggravates mutations in S4/S5 that already facilitate domain closure (Ogle et al. 2003).

Stop codon readthrough is based on the competition between near-cognate tRNA and termination factor binding. It is therefore likely that termination efficiency in the presence of aminoglycosides suffers principally from similar structural changes. However, the effect of aminoglycosides on the competition between near-cognate tRNA and translation termination factor binding has not been studied in detail.

Aminoglycoside action on eukaryotic ribosomes

In general, eukaryotes are significantly more resistant to the killing effects of aminoglycosides than prokaryotes. Paromomycin, for example, binds to a yeast A-site oligonucleotide with approximately 25- to 50-fold lower affinity compared with the corresponding prokaryotic A-site oligonucleotide (Lynch and Puglisi 2001a). The reason lies in small differences in the rRNA sequence of the decoding region, just adjacent to A1492/A1493 in helix 44 (Fig. 5; Recht et al. 1999; Lynch and Puglisi 2001a,b). However, the eukaryotic ribosome is also susceptible to the misreading effects of aminoglycosides (Palmer et al. 1979).

The yeast mutant *rdn15*, in which A1491 of the 18S rRNA is exchanged for G1491, displays defects in translational fidelity (Figs 3, 5, and compare above). In addition, *rdn15* makes yeast cells sensitive to the killing effects of paromomycin. The finding suggests that the natural, high resistance of yeast ribosomes to paromomycin is related to the absence of this specific base pair (Velichutina et al. 2001). Consistent with this model, the eukaryotic organisms *Tetrahymena thermophila* and *Giardia lamblia* are unusually sensitive to aminoglycosides. Unlike yeast and higher eukaryotes they naturally contain the C1409–G1491 base pair (Recht et al. 1999). According to the model (Fig. 4), it may be speculated that the flipping of A1492/A1493 is facilitated by the creation of the C1409–G1491 base pair. Indeed, flipping of A1492/A1493 was not observed upon paromomycin binding to a eukaryotic A-site oligonucleotide (Lynch and Puglisi 2001a). Differences between the prokaryotic and eukaryotic decoding region is also revealed by the reciprocal situation. When A1408 was changed to G1408 in 16S rRNA to match the eukaryotic counterpart, *E. coli* turned resistant to aminoglycosides of the paromomycin subclass (Fig. 5; Recht et al. 1999).

In many cases, mutations in yeast rRNA helices and proteins affect aminoglycoside sensitivity just as the corresponding mutations in *E. coli*. As described for prokaryotic S4/S5 and S12, mutations in Rps2p (S5) and Rps9p (S4) confer paromomycin sensitivity, while most mutations in Rps23p (S12) increase the natural resistance of yeast (Masurekar et al. 1981; Eustice et al. 1986; Alksne et al. 1993; Liebman et al. 1995). Mutations in 18S rRNA can be more complex. *rdn2* (helix 18) and *rdn4* (helix 27) of 18S rRNA (Fig. 3) result in increased resistance toward some (paromomycin and G418), but decreased resistance in other (streptomycin) aminoglycosides (Chernoff et al. 1994). In the light of the different mechanisms of paromomycin and streptomycin action, *rdn2* and *rdn4* may affect the movement of S12/helix 18 relative to helix 44/helix 27 rather than the flipping of A1492/A1493 (Fig. 5). An interesting spin-off from the detailed studies on aminoglycoside action in yeast is that killing and translational fidelity are clearly separable functions (Wakem and Sherman 1990; Liu and Liebman 1996; Velichutina et al. 2000, 2001; Panopoulos et al. 2004). As an example, the mutant *rdn5* in the sarcin–ricin loop of the 25S rRNA causes phenotypic suppression and enhances misincorporation in vitro (Fig. 3). At the same time, yeast expressing *rdn5* is more resistant to paromomycin and hygromycin B (Liu and Liebman 1996; Panopoulos et al. 2004). Another example is the *rdn15*-mediated rescue of cell lethality caused by mutations in eRF1 and eRF3, which is not a result of decreased stop codon readthrough (Velichutina et al. 2001).

Aminoglycoside effects on mutants in translation termination factors and proteins interacting with the ribosome

Many mutants in yeast eRF3 and eRF1 display enhanced sensitivity toward aminoglycosides (Eustice et al. 1986; All-Robyn et al. 1990; Wakem and Sherman 1990; Stansfield

and Tuite 1994; Bertram et al. 2000; Cosson et al. 2002). A large group of aminoglycoside-sensitive mutants interact with the ribosome, or with the translation termination factors, or both. For example the effect of Pab1p on aminoglycoside sensitivity is clearly connected to eRF3: simultaneous overexpression of Pab1p and eRF3 leads to increased resistance toward paromomycin (Cosson et al. 2002). The sensitivity of Δ*sla1*, Δ*mtt1*, and possibly also actin mutants is most likely related to their interaction with eRF1/eRF3 or other components of the translation machinery (Bailleul et al. 1999; Czaplinski et al. 2000; Kandl et al. 2002). This group also includes mutants in components of the NMD machinery. Δ*upf3* and a strain expressing a point mutant of Upf1p (encoded by the *mof4-1* allele) are sensitive to paromomycin (Cui et al. 1996; Ruiz-Echevarria et al. 1998). Δ*upf1*, on the other hand, is not sensitive to the killing effect, but nonsense suppression is increased in the presence of the drug (Leeds et al. 1992; Lee et al. 1995; Cui et al. 1996). Strains lacking Upf1p thus represent another example of the dual effect of paromomycin on stop codon readthrough and cell viability.

Yeast strains lacking functional RAC or Ssb1/2p are sensitive toward aminoglycosides (Nelson et al. 1992; Yan et al. 1998; Gautschi et al. 2001). While these chaperones are among the most sensitive yeast mutants, the underlying mechanism of aminoglycoside sensitivity is not understood and different models have been suggested. In one model, the effect on translational fidelity is thought to be related to ribosome association (Rakwalska and Rospert 2004). This view is supported by the observation that aminoglycosides increase stop codon readthrough in the absence of ribosome-bound chaperones more severely than in the corresponding wild type strain. The effect of aminoglycosides was not only observed in vivo but also in vitro, suggesting that the absence of the chaperones primarily affected ribosome function (Rakwalska and Rospert 2004). Whether Rpl39p at the tunnel exit is functionally connected to RAC and Ssb1/2p is currently not clear; however, the absence of Rpl39p also confers aminoglycoside sensitivity (Dresios et al. 2000). According to another model, the sensitivity of Δ*ssb1*/Δ*ssb2* and Δ*zuo1* strains is rather indirect and related to a general increase in cation influx across the cytoplasmic membrane, possibly due to altered maturation of membrane proteins (Kim and Craig 2005; compare below).

Aminoglycoside effects on mutants without direct connection to the translation machinery

It has long been recognized that the aminoglycoside streptomycin causes membrane damage in *E. coli*. Although the drug may act on both the ribosome and the membrane, it is unlikely that single mutations in ribosomal proteins conferring streptomycin resistance could also directly alter the membrane (Fast et al. 1987; Kurland et al. 1996). Accordingly, membrane damage is viewed as an indirect effect of streptomycin resulting from its activity on ribosomes. As a mechanism it has been suggested that incorporation of misread proteins into the membrane results in the leakiness of small molecules (Davis et al. 1986; Davis 1987; Tok and Bi 2003). Consistent with this model, *ram* mutations or streptomycin treatment of *E. coli* leads to an overall increase in protein degradation (Goldberg 1972, 2003). In yeast the question of aminoglycoside-induced membrane damage has not been studied in detail. Due to the inherently low aminoglycoside affinity of eukaryotic ribosomes, this may not be a major concern for wild type yeast. However, a number of yeast mutants with defects in membrane integrity at the same time display increased sensitivity toward aminoglycosides. One type of mutants has been mapped to genes involved in all stages of the glycosylation process. Abnormal glycosylation is thought to increase permeability to aminoglycosides via defects in cell wall or plasma membrane components that either allow uptake or prevent

export of the drugs (Dean 1995). Another type of membrane permeability mutants affect the cation transporters encoded by *TRK1* and *TRK2*. Deletion of these genes results in increased cation sensitivity including sensitivity to hygromycin B and paromomycin. A similar phenotype results from the deletion of *HAL4* and *HAL5* encoding two homologous kinases implicated in the regulation of Trk1p/Trk2p (Mulet et al. 1999; Kim and Craig 2005). Glycosylation mutants, Δ*trk1*/Δ*trk2*, and Δ*hal4*/Δ*hal5* all increase aminoglycoside sensitivity by increasing the intracellular concentration of the drug. Such a mechanism may also apply for other paromomycin-sensitive mutants. For example, it has been suggested that the lack of the ribosome-bound chaperones Ssb1/2p or zuotin affects membrane integrity, possibly via folding defects in membrane components. This view is consistent with the observation that these strains are also sensitive to high concentrations of small cations, e.g. sodium. According to this model, cell death of Δ*ssb1*/Δ*ssb2* or Δ*zuo1* strains in the presence of aminoglycosides would be related to an increased intracellular aminoglycoside concentration and not to direct effects on ribosome function (Kim and Craig 2005). Another possibility, however, is that Δ*ssb1*/Δ*ssb2* or Δ*zuo1* primarily suffer from defects related to translational fidelity and loss of membrane integrity is a consequence of that. Δ*ssb1*/Δ*ssb2* or Δ*zuo1* strains would acquire defective membrane components by a mechanism similar to that suggested for *E. coli* during growth in the presence of aminoglycosides (Rakwalska and Rospert 2004, and compare above). A third possibility arises from recent results from genes with a naturally high tendency of stop codon readthrough (Namy et al. 2002a). One of the genes identified is *PDE2*, encoding a high-affinity cAMP phosphodiesterase, which is an important regulator of the cellular cAMP level (Namy et al. 2002a). Δ*pde2* strains suffer from defects in cell wall integrity believed to be caused by glycosylation defects, and interestingly, display hygromycin B sensitivity (Jones et al. 2003 and compare below). Rousset and coworkers found that stop codon readthrough in *PDE2* is a frequent event that results in a carboxy terminal extension of 20 amino acids on the protein level. The C-terminally extended version of Pde2p is destabilized and, as a consequence, the concentration of active Pde2p is reduced and the cellular cAMP level increased (Namy et al. 2002a).

In vivo roles and medical applications for stop codon readthrough

To some degree the efficiency of translation termination depends on which of the three stop codons is used to mark the end of an open reading frame. However, and even more so, readthrough depends on the 5′ and 3′ stop codon context (Bonetti et al. 1995; Namy et al. 2001 and references therein). A number of proteins are encoded by mRNA with a high natural tendency for stop codon readthrough (Namy et al. 2003; Williams et al. 2004). Little is known about the consequences of C-terminally extended protein versions on metabolism. First results suggest that high levels of stop codon readthrough might not just reflect some problematic cases, but also actively regulate cellular processes. One of the genes containing a highly leaky stop codon is *PDE2*, which was introduced above. If stop codon readthrough is a mechanism of regulation in the cell, there should be conditions that influence the efficiency of the process. Three factors have previously been associated with readthrough modulation: carbon source, temperature, and the presence of [*PSI*$^+$] (Namy et al. 2002a). The best evidence for a biologically important regulatory role on the level of stop codon readthrough exists for [*PSI*$^+$]. Depending on the strain background [*PSI*$^+$] produces a spectrum of phenotypes and it has been speculated that, via the level of stop codon readthrough, [*PSI*$^+$] might serve as an epigenetic modulator of cell function (Eaglestone et al. 1999; True and Lindquist 2000; True et al. 2004). There is evidence that one important target of

[*PSI*$^+$]-induced readthrough is *PDE2*. In a [*PSI*$^+$] background the cAMP level was increased compared to the isogenic [*psi*$^-$] strain, while [*PSI*$^+$] had no effect on the cAMP level in a Δ*pde2* background. The result suggests that the level of functional Pde2p might be directly connected to the frequency of readthrough (Namy et al. 2002a).

Viruses often exploit or subvert the host machinery for their own purposes. A recent example demonstrates that also here regulation of stop codon readthrough can be an important mechanism (Orlova et al. 2003). Murine leukemia viruses use a single mRNA to express both the structural Gag protein and the Gag-Pol fusion protein, which is processed to form the replication enzymes, including reverse transcriptase. The *gag* and *pol* genes are *in frame* and are separated by only a single stop codon. In about 5% of the translation events the stop codon is ignored and a glutamine is inserted. The event critically depends on an mRNA pseudoknot structure downstream of the stop codon (Goff 2004 and references therein). Goff and coworkers found that the efficiency of stop codon readthrough was dependent on the interaction between reverse transcriptase and eRF1. The interaction inhibited the release factor activity of eRF1, increased the level of readthrough, and subsequently the amount of Gag-Pol fusion protein generated (Orlova et al. 2003; Goff 2004).

As nonsense and frame shift mutations cause approximately 20–40% of the individual cases of more than 200 inherited diseases, they are of high medical relevance (Czaplinski et al. 2000). Recent reports have demonstrated that subinhibitory concentrations of aminoglycosides suppress translation termination and restore functional activity of genes harboring a nonsense mutation in vitro and in vivo (Barton-Davis et al. 1999; Keeling and Bedwell 2002; Schulz et al. 2002; Bidou et al. 2004; Howard et al. 2004; Lai et al. 2004; Sangkuhl et al. 2004). For example, 5–10% of disease causing G-protein-coupled receptor (GPCR) mutations represent nonsense mutations. One member of this GPCR family is non-peptide V2 vasopressin receptor (AVPR2) and patients who express non-functional AVPR2 suffer from X-linked nephrogenic diabetes insipidus (XNDI; Spiegel and Weinstein 2004). In XNDI patients, 18 different nonsense mutations in AVPR2 have been identified. When Schöneberg and coworkers investigated the effect of aminoglycosides on one of the AVPR2 stop codon mutations (E242X), they found that G418 not only efficiently increased stop codon readthrough, but also reduced the severity of XNDI in heterozygous female E242X mice (Yun et al. 2000; Schulz et al. 2002; Sangkuhl et al. 2004). Identification and characterization of the factors that regulate the efficiency of translation termination will thus not only be important for understanding the biology of the termination process, but also for the development of therapeutics treating a wide array of genetic disorders that arise as a consequence of nonsense mutations.

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Novel roles of aquaporins revealed by phenotype analysis of knockout mice

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Abstract The aquaporins (AQPs) are small integral membrane proteins that transport water and in some cases small solutes such as glycerol. Physiological roles of the ten or more mammalian AQPs have been proposed based on their expression in epithelial, endothelial and other tissues, their regulation, and in some cases the existence of humans with AQP mutation. Here, the role of AQPs in mammalian physiology is reviewed, based on phenotype analysis of transgenic mouse models of AQP deletion/mutation. Phenotype studies support the predicted roles of AQPs in kidney tubule and microvessel fluid transport for urinary concentrating function, and in fluid-secreting glandular epithelia. The phenotype studies have also shown unexpected roles of AQPs in brain and corneal swelling, in neural signal transduction, in regulation of intracranial and intraocular pressure, and in tumor angiogenesis and cell migration. The water/glycerol-transporting AQPs were found to play unexpected roles in skin hydration and in fat metabolism. However, many phenotype studies were negative, such as normal airway/lung and skeletal muscle function, despite AQP expression, indicating that tissue-specific AQP expression does not indicate physiological significance. The mouse phenotype data suggest that modulators of AQP expression/function may have such wide-ranging clinical applications as diuretics and in the treatment of brain swelling, glaucoma, epilepsy, obesity, and cancer.

Introduction

The aquaporins (AQPs) are a family of at least ten homologous water transporting proteins in mammals, with many more in plants and lower organisms. In mammals they are expressed in epithelial, endothelial, and other tissues, some of which are clearly involved in fluid trans-

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port, such as kidney tubules, while others are not, such as skin and fat cells. Structural studies of AQPs indicate a homotetrameric assembly in membranes in which each monomer contains six tilted helical segments that form a barrel surrounding a water pore (reviewed in Agre et al. 2002; Fujiyoshi et al. 2002). Functional measurements indicate that AQPs 1, 2, 4, 5, and 8 are primarily water-selective, whereas aquaporins 3, 7, 9, and 10 (the "aquaglyceroporins") also transport glycerol and possibly other small solutes. The importance of AQPs in mammalian physiology has been proposed based on the pattern and regulation of their tissue expression, and in some cases the existence of human mutations, such as nephrogenic diabetes insipidus caused by AQP2 mutation (Deen et al. 1994); however, the unavailability of aquaporin inhibitors suitable for use in vivo has precluded direct investigation of their function. Our laboratory has investigated the role of AQPs in mammalian physiology by analyzing the phenotype of transgenic mouse models of AQP mutation or deletion, which is the focus of this review. The phenotype analysis has been informative in defining the roles of AQPs and in establishing general paradigms about the situations in which AQPs are required. In some cases, the findings have clarified long-standing controversies about basic fluid transport mechanisms, as in the impairment of near-isosmolar fluid transport in kidney proximal tubule in AQP1 deficiency. In many cases the findings have pointed to previously unrecognized roles of AQPs, such as in neural signal transduction, skin hydration, fat metabolism, and cell migration. Yet in other cases, the findings have unexpectedly provided evidence against the significant physiological roles of AQPs, such as in the lung and airways, despite specific tissue expression and regulation data. In addition to providing new insights into basic physiological mechanisms, the ultimate significance of the mouse phenotype research will be in understanding mechanisms of human disease and in developing new therapies based on this understanding.

AQPs and the urinary concentrating mechanism

At least six AQPs are expressed in the kidney shown in Fig. 1a. AQP1 is expressed in apical and basolateral plasma membranes in proximal tubule and thin descending limb of Henle (TDLH), and in the microvascular endothelium of outer medullary descending vasa recta (OMDVR). AQP2 is expressed in collecting duct principal cells and undergoes vasopressin-regulated trafficking between an intracellular vesicular compartment and the cell apical plasma membrane. AQP3 and AQP4 are coexpressed at the basolateral membrane of collecting duct epithelial cells, with AQP3 prominently in proximal segments of the collecting duct and AQP4 in the inner medullary collecting duct. AQP6 is thought to be expressed in the intracellular vesicles in the intercalated cells of the collecting duct, and AQP7 is expressed in a small distal segment (S3 segment) of proximal tubule epithelium.

Renal function was assessed in a series of single and double knockout mice by measurement of fluid input-output and urinary concentrating ability. Given free access to food and water, AQP1 and AQP3 null mice were remarkably polyuric, consuming 2–3 (AQP1) to 7–10 (AQP3) times more fluid than wildtype mice, whereas the AQP4 null mice were not polyuric (Fig. 1b; Ma et al. 1997, 1998, 2000b). Polyuria was even more severe in AQP1/AQP3 double knockout mice (Yang et al. 2001b). Urinary osmolality was remarkably decreased in the AQP1 and AQP3 null mice (Fig. 1c). In response to a 36-h water deprivation, urine osmolality in AQP1 null mice did not change, whereas that in AQP3 null mice increased to a sub-maximal level. The AQP1 null mice manifested signs of severe de-

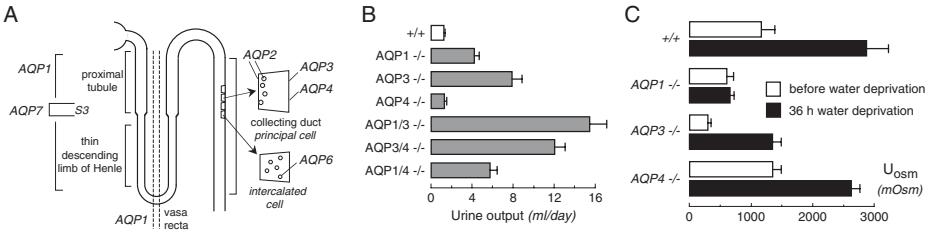

Fig. 1 Polyuria and impaired urinary concentrating function in aquaporin (*AQP*) null mice. **a** Location of AQPs in kidney tubules. **b** Daily urine output of mice of indicated genotype given free access to food and water. **c** Urine osmolality before and after a 36-h water deprivation in mice of indicated genotype. Data from Ma et al. (1997, 1998, 2000b)

hydration. AQP4 null mice had a small but significant reduction in maximal urine osmolality after a 36-h water deprivation.

The urinary concentrating defect in AQP1 null mice represents a combination of defective proximal tubule fluid absorption and defective countercurrent multiplication resulting in a relatively hypo-osmolar medullary interstitium. In isolated microperfused S2 segments of proximal tubule transepithelial osmotic water permeability (P_f) was decreased by nearly 5-fold in AQP1 knockout mice compared with wildtype mice (Schnermann et al. 1998), indicating that the major pathway for osmotically driven water transport in proximal tubule is transcellular and mediated by AQP1. Free-flow renal micropuncture in anesthetized mice showed an approximately 2-fold reduction in isosmolar fluid absorption (Schnermann et al. 1998), supporting the classical three-compartment model in which mild luminal hypotonicity drives osmotic water movement through highly water-permeable cell membranes. Indeed, AQP1 deletion caused a marked decrease in luminal fluid osmolality in end proximal tubule fluid (Vallon et al. 2000), indicating the active pumping of salt out of the tubule lumen without adequate water movement to dissipate the transepithelial osmotic gradient.

Defective countercurrent multiplication also contributes to the defective urinary concentrating ability in the AQP1 null mice. In the water-deprived AQP1 null mice, vasopressin stimulation of collecting duct water permeability, which should approximately equalize urine and medullary interstitial osmolalities, did not increase urine osmolality (Ma et al. 1998), indicating a relatively hypo-osmolar medullary interstitium. The TDLH and OMDVR play critical roles in generating a hypertonic medullary interstitium. As anticipated, AQP1 deletion resulted in a nearly 10-fold decreased transepithelial P_f in microperfused segments of TDLH (Chou et al. 1999), and an even more dramatic reduction in P_f in microperfused OMDVR (Pallone et al. 2000). These results indicate that AQP1 is the principal water channel in TDLH and OMDVR, and that osmotic equilibration along TDLH by water transport plays a key role in the renal countercurrent concentrating mechanism. Subsequent to these mouse studies, it was found that rare humans lacking AQP1 manifest a qualitatively similar urinary concentrating defect (King et al. 2001), though with different absolute osmolalities, as the mouse is able to concentrate its urine to >3000 mOsm, whereas maximal osmolality in humans rarely exceeds 1000 mOsm.

Collecting duct epithelial cells express AQP2, AQP3, and AQP4. The physiology of AQP2 is well-established from studies in humans with autosomal nephrogenic diabetes insipidus. Several AQP2 mutations causing recessive and dominant disease have been characterized. The AQP2 mutation T126M causes autosomal recessive disease by a mechanism involving retention of the mutant protein at the endoplasmic reticulum of mammalian cells. A mouse model of the human disease was created by targeted gene replacement so that the

mutant mice express the AQP2-T126M protein (Yang et al. 2001a). The AQP2-T126M protein was expressed mainly at the endoplasmic reticulum of collecting duct epithelial cells. However, the mice manifested severe polyuria and mortality in their first week of life. The data in Fig. 1b and c suggest a more important role of the basolateral membrane water channel AQP3 than AQP4 with respect to the urinary concentrating mechanism. Basolateral membrane P_f was reduced by >3-fold in microdissected cortical collecting duct from AQP3 null mice (Ma et al. 2000b), indicating that AQP3 is the main basolateral membrane water channel in this segment. Isolated tubule perfusion measurements in inner medullary collecting duct (IMCD) showed a 4-fold decrease in P_f in AQP4 null mice (Chou et al. 1998), indicating that AQP4 is responsible for the majority of basolateral membrane water movement in the IMCD. The remarkable urinary concentrating defect in AQP3 null mice and the very mild defect in AQP4 null mice support the conclusion that the amount of water absorbed in the cortical portion of the collecting duct greatly exceeds that absorbed in the medullary collecting duct. AQP8 was found by immunocytochemistry to also be expressed in kidney (Elkjaer et al. 2001), though urinary concentrating ability was unimpaired in AQP8 knockout mice (Yang et al. 2005), even in studies comparing AQP1 null mice with AQP1/AQP8 double knockout mice. Urinary studies of AQP6 and AQP7 knockout mice, both of which are now available, may be informative in defining their potential role in renal water, urea, and/or glycerol transport.

AQPs in active, near-isosmolar fluid secretion

As mentioned above, impaired fluid absorption in kidney proximal tubule in AQP1 deficiency indicates the need for high cell membrane water permeability for rapid, near-isosmolar fluid transport. The involvement of AQPs in fluid secretion by glands (salivary, submucosal, sweat, lacrimal), and by the choroid plexus and the ciliary body has been investigated using appropriate knockout mouse models. The general conclusion is that AQPs facilitate active fluid (secretion and absorption) when sufficiently rapid, in which case AQP deletion is associated with reduced volume and increased ion/solute content of secreted fluid. AQPs appear not to be needed when fluid secretion rate (per unit epithelial surface area) is low, as AQP-independent water permeability is high enough to support slow fluid secretion (or absorption).

AQP5-dependent fluid secretion by salivary glands

Saliva secreted by the salivary gland is the first fluid with which ingested food comes into contact. The interstitial-to-luminal transport of sodium and chloride across the acinar epithelium is the driving force for osmotic water flow. The salivary duct epithelium is believed to be relatively water-impermeable, where sodium and chloride are absorbed, and potassium and bicarbonate secreted to produce a hypotonic saliva. Upon stimulation the salivary gland can secrete saliva at high rates (up to 50 ml/min per 100 g tissue in humans), which relies on rapid water movement from serosa-to-mucosa across the capillary endothelium and acinar cells. AQP1 is expressed in the microvascular endothelial cells of the salivary gland, AQP5 in the apical membrane of acinar cells, and possibly AQP8 in the basolateral membrane. We found that pilocarpine-stimulated saliva production was reduced by more than 60% in AQP5 null mice, producing a viscous secretion (Fig. 2a; Ma et al. 1999). Compared with

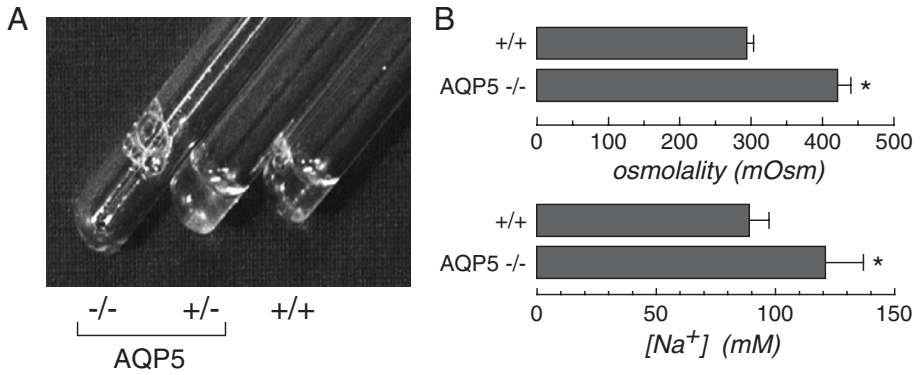

Fig. 2 Defective saliva secretion in AQP5 null mice. **a** Photograph of saliva collected over 5 min from mice of indicated genotype. Salivation was stimulated by pilocarpine. **b** Averaged (±SE) osmolality and sodium concentration of saliva collected from wildtype and AQP5 null mice. From Ma et al. (1999)

the saliva from wildtype mice, the saliva from AQP5 null mice was hypertonic and hypernatremic (Fig. 2b). Amylase and protein secretion, functions of salivary mucous cells, were not affected by AQP5 deletion. Saliva secretion was not impaired in AQP1 or AQP4 null mice. A subsequent study confirmed the defect in saliva secretion in AQP5 null mice and reported reduced water permeability, as expected, in acinar cells isolated from the null mice (Krane et al. 2001b). Recently, saliva secretion was measured in AQP8 knockout mice (Yang et al. 2005), but no impairment was found, nor was there a difference in saliva secretion in AQP8/AQP5 double knockout mice compared with AQP5 knockout mice.

AQP5-dependent fluid secretion by airway submucosal glands

Submucosal glands in mammalian airways secrete a mixture of water, ions, and macromolecules onto the airway surface. Glandular secretions are important in establishing airway surface liquid (ASL) composition and volume, and in antimicrobial defense mechanisms. Submucosal glands contain serous tubules, where active salt secretion into the gland lumen creates a small osmotic gradient driving water transport across a water permeable epithelium, as well as mucous cells and tubules, where viscous glycoproteins are secreted. AQP5 is expressed at the luminal membrane of serous epithelial cells. Novel methods were applied to measure the gland fluid secretion rate and composition in mice (Song and Verkman 2001). In mice breathing through a tracheotomy, total gland fluid output was measured from the dilution of a volume marker present in the fluid-filled nasopharynx and upper trachea. Pilocarpine-stimulated fluid secretion was reduced by >2-fold in AQP5 null mice compared with wildtype mice. Similar results were obtained when secreted fluid was collected in the oil-filled nasopharyngeal cavity, and by real-time video imaging of fluid droplets secreted from individual submucosal glands near the larynx. Analysis of secreted fluid showed a >2-fold increase in total protein in AQP5 null mice, suggesting intact protein secretion across a relatively water impermeable epithelial barrier. Submucosal gland morphology and density did not differ significantly in wild type versus AQP5 null mice. Therefore, as in salivary gland, the luminal membrane of submucosal gland epithelial cells is the rate-limiting barrier to water movement. It was proposed that modulation of gland AQP5 expression or

function might provide an approach to treating hyperviscous gland secretions in cystic fibrosis, and excessive fluid secretions in infectious or allergic bronchitis/rhinitis.

AQP1-dependent CSF secretion by choroid plexus

Aquaporin-1 is expressed strongly at the ventricular-facing surface of choroid plexus epithelium. To investigate its role in cerebrospinal fluid (CSF) secretion novel methods were developed to compare water permeability in isolated choroid plexus of wildtype versus AQP1 null mice, as well as intracranial pressure (ICP), and CSF production and absorption (Oshio et al. 2005). Osmotically induced water transport was rapid in choroid plexus from wildtype mice and reduced by 5-fold by AQP1 deletion, confirming AQP1 is the major water pathway in choroid plexus. AQP1 deletion did not affect choroid plexus size or structure. By stereotaxic puncture of the lateral ventricle with a microneedle, ICP was 9.5±1.4 cm H_2O in wildtype mice and 4.2±0.4 cm H_2O in AQP1 null mice. CSF production, as measured by a dye dilution method involving fluid collections using a second microneedle introduced into the cisterna magna, was 0.37±0.04 μl/min in wildtype mice, and reduced ~25% in AQP1 null mice. However, pressure-dependent CSF outflow, measured from steady-state ICP at different ventricular infusion rates, was not affected by AQP1 deletion. The potential clinical relevance of the reduced ICP in AQP1 null mice was demonstrated in a model of focal brain injury, where AQP1 null mice had remarkably reduced ICP and improved survival compared with wildtype mice. AQP1 thus plays a role in CSF secretion and ICP regulation. A similar conclusion was found for AQP-dependent fluid secretion by the ciliary epithelium in the eye and regulation of intraocular pressure (Zhang et al. 2002, and see below).

AQP-independent fluid secretion

As in salivary and submucosal glands, AQP5 is expressed at the apical membrane in acinar epithelial cells in the lacrimal gland and the sweat gland. However, secretion of tear fluid (Moore et al. 2000) and sweat (Song et al. 2002) were not significantly reduced in AQP5-deficient mice. Together with data from lung showing unimpaired alveolar fluid absorption in AQP1/AQP5 deficiency (see below), it was concluded that AQPs are not required for active fluid absorption/secretion when fluid transport rates are low.

AQPs in the lung and airways

The lung and airways are potentially important sites of fluid movement in airway hydration, reabsorption of alveolar fluid in the neonatal period, and formation/resolution of pulmonary edema resulting from heart failure or lung injury. Figure 3a depicts the barriers to water movement between the airspace and capillary compartments, which consist of an epithelium, interstitium, and endothelium. The alveolar epithelium is composed mainly of type I cells, which express AQP5 at their apical membrane. The microvascular endothelium throughout the lung/airways expresses AQP1. AQP4 is expressed at the basolateral membrane of surface epithelial cells in large and small airways, and AQP3 is expressed in airway epithelial cells in nasopharynx and trachea. The airways also contain submucosal glands that express AQP5 as described above.

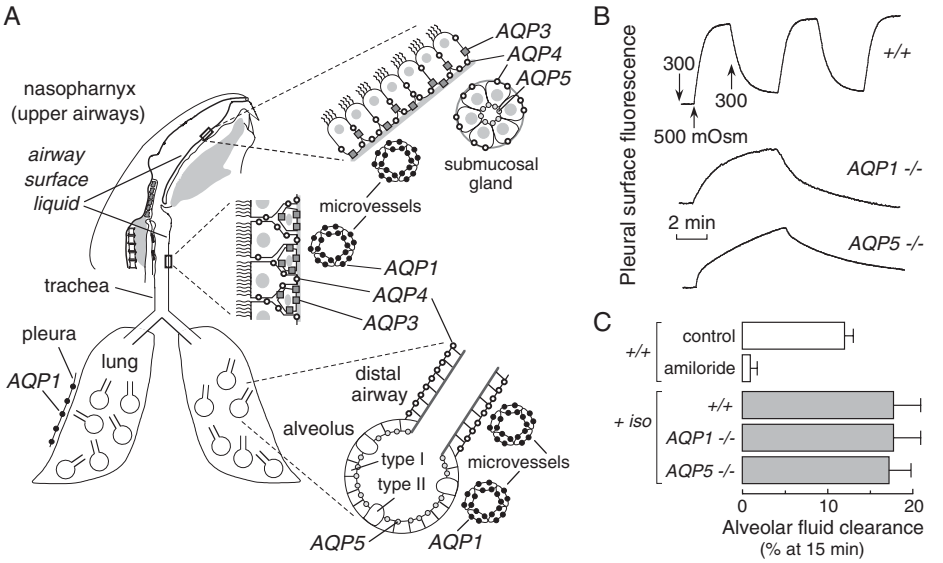

Fig. 3 Aquaporin deletion reduces osmotic water permeability in lung, but does not impair active fluid absorption. **a** AQP expression in epithelia and endothelia in nasopharyngeal cavity, upper and lower airways, and alveoli. **b** Osmotically driven water transport across the airspace/capillary barrier in perfused lungs from wildtype and indicated AQP null mice. Note the remarkable slowing of osmotic equilibration in mice lacking AQP1 or AQP5. **c** Alveolar fluid clearance measured from the increased concentration of a volume marker 15 min after instillation of isosmolar fluid at 37°C. Where indicated, fluid absorption was inhibited by amiloride or stimulated by isoproterenol. From Bai et al. (1999) and Ma et al. (2000a)

Peripheral lung

Major fluid transport-related functions in the peripheral lung include alveolar fluid clearance, gas exchange, and adaptation to acute and subacute lung injury. The principal finding of a series of studies on AQP knockout mice was that although AQPs provide a major route for osmotically-driven water transport among the airspace, interstitial, and capillary compartments, they are not required for physiologically important lung functions (Bai et al. 1999; Ma et al. 2000a; Song et al. 2000b). Figure 3b shows an approximately 10-fold reduced osmotic water permeability in mice lacking AQP1 or AQP5 compared with wildtype mice. Here, osmotic water transport between the airspace and capillary compartments was measured in isolated perfused lungs by a pleural surface fluorescence method. However, despite the much reduced water permeability in AQP deficiency, active alveolar fluid absorption was not impaired (Fig. 3c). Also, the rapid reabsorption of fluid from the airspace just after birth was not impaired by AQP deletion, nor was the response of the adult lung in models of lung injury, including acid-induced epithelial injury, thiourea-induced endothelial injury, and hyperoxic subacute lung injury (Song et al. 2000a). As discussed above with regard to tear secretion by the lacrimal gland and sweat secretion by the sweat gland, the remarkably slower rate of alveolar fluid absorption compared with proximal tubule fluid absorption or saliva secretion probably accounts for the lack of effect of AQP1 and AQP5 deletion on alveolar fluid clearance. Also, lung carbon dioxide transport, which was proposed to involve AQP1, was not impaired in AQP1 null mice as demonstrated in isolated perfused lung experiments (Fang et al. 2002), as well as in measurements of carbon dioxide exchange in vivo (Yang et al. 2000a).

Airways

Major fluid-transport related functions of the airways include humidification of inspired air, regulation of ASL volume and tonicity, isosmolar fluid absorption in upper airways, and glandular fluid secretion. Methods were developed to measure each of these functions quantitatively in mice. For example, airway humidification was measured from the water content of exhaled gas in mice that were ventilated with dry air (Song et al. 2001). Airway surface liquid thickness and ionic composition were measured using confocal microscopy and ratio imaging after staining with fluorescent indicators (Jayaraman et al. 2001). The principal conclusion from measurements on wildtype versus AQP3/AQP4 single and double knockout mice (as well as AQP1/AQP5 double knockout mice) was that AQPs are of at most minimal importance in humidification of upper and lower airways, airway surface liquid hydration, and fluid absorption in upper airways (Song et al. 2001). In a report from the Menon group (Krane et al. 2001a), altered airway reactivity in response to bronchoconstricting agents was found in AQP5 null mice. A mechanism was not established for the small differences observed, but may be related to indirect effects of AQP5 deletion on agonist-induced fluid secretion from submucosal glands as described above.

Pleura

Aquaporin-1 is also expressed in microvascular endothelia and surface mesangial cells in the pleura. Deletion of AQP1 resulted in a substantial reduction in osmotic water permeability of the pleural surface; however, physiologically important pleural functions, including the formation and resolution of pleural effusions, were not affected by AQP1 deletion (Song et al. 2000c). Similar findings were reported for AQP1 deletion in peritoneal microvessels (Yang et al. 1999). Although AQP1 deletion reduced osmotically driven water transport into the peritoneal cavity, isosmolar fluid accumulation and absorption were not affected. Thus, the aquaporins do not appear to be physiologically important in peripheral lung and pleura despite their major role in airspace–capillary osmosis.

Thus, despite the high water permeability in lung/airway epithelia, and the specific expression pattern and regulation of lung AQPs, the data in AQP-deficient mice do not support a physiologically important role of AQPs in lung/airways. The negative phenotype cannot be explained by upregulation of other AQPs since measured water permeabilities were very low in the AQP knockout mice. It remains to be determined whether AQPs in lung/airways will be shown to be important for physiological stresses not yet tested, though the available data strongly argue against an important role of AQPs in the major fluid transport-related functions in lung/airways.

AQPs in brain swelling and neural signal transduction

Work from several laboratories has shown AQP4 expression in the brain and spinal cord at putative sites of fluid transport at blood–brain and brain–CSF interfaces. AQP4 is expressed in the dense astrocyte cell processes that form the glia limitans, which lines the CSF-bathed pial and ependymal surfaces in the subarachnoid space and the ventricles. AQP4 expression is highly polarized to astrocytic foot processes in contact with blood vessels (Nielsen et al. 1997). Evidence that AQP4 provides the major route for water transport across astrocyte cell

membranes came from osmotic water permeability measurements showing 7-fold reduced water transport in astrocytes cultured from AQP4 null mice compared with wildtype mice (Solenov et al. 2004). Also, accumulation of brain water was found to be greatly slowed in AQP4 null mice in response to serum hypo-osmolality, as monitored continuously in vivo by a non-invasive near-infrared optical method (Thiagarajah et al. 2005), or by brain wet-to-dry weight ratios measured at different times after water intoxication (Papadopoulos et al. 2004).

AQP4 is the orthogonal array protein

Aquaporin-4 has been identified as a structural component of membrane "orthogonal arrays of proteins" (OAPs), which are characteristic cobblestone-like intramembrane particles in astrocytes seen by freeze-fracture electron microscopy (FFEM). From the observation that AQP4 was present in cell types in which OAPs had been identified, it was first proposed that AQP4 is a component of OAPs (Frigeri et al. 1995). Support for this hypothesis came from freeze-fracture studies showing OAPs in stably transfected Chinese hamster ovary (CHO) cells expressing functional AQP4 (Yang et al. 1996). Direct evidence that AQP4 is a component of OAPs in vivo came from FFEM studies of brain, kidney and skeletal muscle showing an absence of OAPs in AQP4 null mice (Verbavatz et al. 1997). Subsequent anti-AQP4 antibody labeling of OAPs in brain tissue confirmed the immunoreactivity of AQPs in OAPs (Rash et al. 1998). The functional significance of AQP4 assembly in OAPs remains unclear. One proposal is that multiple AQP4 pores in an OAP may facilitate siphoning, whereby the per-channel water permeability of the AQP4 macro-assembly is greater than that of independently functioning AQP4 tetramers (Yang et al. 1996).

AQP4 deletion improves outcome in cytotoxic edema

The expression of AQP4 at the blood–brain and brain–CSF interfaces suggested possible involvement in the brain water accumulation in cytotoxic (cellular) brain edema. Initial studies of cytotoxic brain edema were carried out using water intoxication and cerebral ischemia models (Manley et al. 2000). It was reasoned that slowed brain water accumulation in AQP4 deficiency should reduce brain swelling and improve clinical outcome in these models. In water intoxication produced by intraperitoneal injection of distilled water, which reduced serum sodium concentration to approximately 105 mM, mice lacking AQP4 had improved outcome as assessed by clinical examination and by survival (Fig. 4a). Reduced brain water accumulation in brain tissue of AQP4 null mice following water intoxication was confirmed using a densitometric method. By transmission electron microscopy the wildtype mice had much greater and widespread pericapillary astrocytic foot process swelling than the AQP4 null mice. A subsequent similar study was carried out on dystrophin null (*mdx*) transgenic mice, which manifest secondary reduced AQP4 expression in astrocyte foot processes (Vajda et al. 2002). Following water intoxication, wildtype mice began to deteriorate neurologically after 30 min, whereas the dystrophin null mice deteriorated after >50 min. However, in contrast to our study demonstrating a survival benefit of AQP4 deletion, all dystrophin null mice died. The differences in survival between the dystrophin and AQP4 null mice may be in part related to baseline morphological alterations found in the dystrophin null mice such as increased blood–brain barrier permeability (Nico et al. 2003), which may enhance

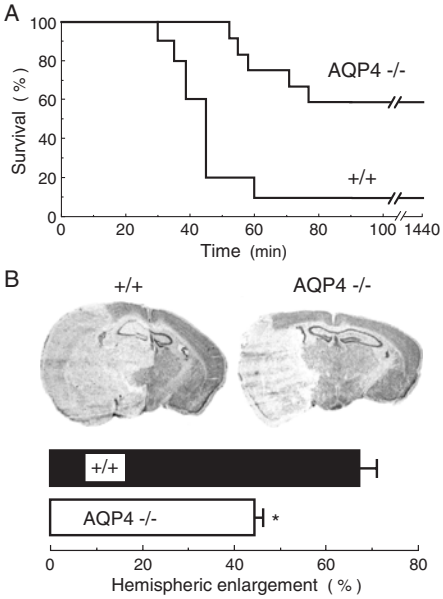

Fig. 4 Aquaporin-4 deletion reduces brain swelling in cytotoxic edema. **a** Survival of mice after intraperitoneal injection of distilled water (20% body weight) containing desmopressin (DDAVP). **b** Reduced brain edema in AQP4 null mice following ischemic stroke. *Top*: representative low magnification image of cresyl violet-stained brain at 24 h after permanent middle cerebral artery (MCA) occlusion. Note the increased swelling and hemispheric enlargement in the wildtype mouse brain section. *Bottom*: hemispheric enlargement, determined by quantitative image analysis, was significantly lower in the AQP4 null mice. From Manley et al. (2000)

the transport of water from the intravascular space to the brain, thereby increasing cerebral edema and worsening outcome.

The protective effect of AQP4 deletion was also tested in a clinically relevant model of ischemic stroke, produced primarily in cytotoxic edema (Manley et al. 2000). Following permanent middle cerebral artery occlusion wildtype mice had a higher mortality rate and significantly greater neurological deficit after 24 h compared with AQP4 null mice. Histological examination 24 h after arterial occlusion showed significantly greater hemispheric enlargement and midline shift in wildtype than in AQP4 null mice (Fig. 4b, top). Infarct volume was also reduced in the AQP4 null mice (Fig. 4b, bottom). A similar protective effect was reported subsequently for α-syntrophin null mice following transient cerebral ischemia (Amiry-Moghaddam et al. 2003a).

We recently investigated the role of AQP4 in brain edema and clinical outcome in a bacterial meningitis model of brain swelling (Papadopoulos and Verkman 2005). *Streptococcus pneumoniae* was injected into CSF in wildtype and AQP4 null mice. CSF bacterial and white blood cell counts were similar in both groups of mice. However, AQP4-deficient mice had remarkably lower ICP (9±1 versus 25±5 cm H_2O) and brain water accumulation (2±1 versus 9±1 μl) after 30 h, and improved survival (80 versus 0% survival) after 60 h, though comparable CSF bacterial and white cell counts. Meningitis produced marked astrocyte foot process swelling in wildtype but not AQP4 null mice, and slowed diffusion of an inert macromolecule in the extracellular space of the brain, confirming a primarily cytotoxic brain edema mechanism. AQP4 protein was strongly upregulated in meningitis, resulting in an approximately 5-fold higher water permeability across the blood-brain bar-

rier compared with non-infected wildtype mice. Mathematical modeling using measured blood–brain barrier water permeability and CSF dynamics accurately simulated the elevated ICP and brain water produced by meningitis and predicted a beneficial effect of prevention of AQP4 upregulation. The data suggested that inhibition of AQP4 function or upregulation may dramatically improve clinical outcome in bacterial meningitis.

AQP4 deletion worsens outcome in vasogenic edema

As expected for all AQPs, AQP4 permits bidirectional water transport, so that AQP4 should not only provide the major pathway for water entry into the brain, but also for water exit. Several models of vasogenic edema were used to test whether AQP4 is involved in the removal of excess brain water (Papadopoulos et al. 2004, 2005). After continuous intracerebral fluid infusion, AQP4 null mice developed significantly higher ICP (Fig. 5a) and brain water content compared with wildtype mice. AQP4 deficient mice also had a worse clinical outcome, higher ICP, and greater brain water content than wildtype mice in a freeze-injury model of vasogenic brain edema. Brain tumor was also studied as a clinically relevant model of vasogenic edema. Melanoma cells were injected into the right striatum as illustrated (Fig. 5b, top). The injected cells produced comparably growing, well-demarcated dark tumors in the wildtype and AQP4 null mice (Fig. 5b, bottom). However, the AQP4 null mice had a significantly worse neurological score at days 6–8, as well as higher ICP (Fig. 5c). In recent studies (Bloch et al. 2005), higher ICP and brain water were found in an intraparenchymal bacterial abscess model of vasogenic edema, providing further support for the clinical relevance of AQP4-dependent clearance of excess brain water.

It is not yet clear exactly how AQP4 worsens clinical outcome in vasogenic brain edema. Clearance of excess brain water involves bulk flow of fluid through the extracellular space and glia limitans into the ventricles and subarachnoid space. The AQP4-rich astrocyte processes of the glia limitans interna and glia limitans externa form a dense mesh at the brain–CSF boundaries. Ultrastructural studies show that these long astrocytic processes are separated by narrow ($<$20 nm) intercellular clefts and interconnected by gap junctions. The long diffusion path presents a significant permeability barrier to the extracellular flow of water and solutes. The impaired clearance of brain edema fluid in AQP4 deficiency in models of vasogenic brain edema suggests that AQP4 provides a low resistance route, which allows edema fluid to move across the astrocyte cell membranes of the glia limitans into the CSF. The enhanced elimination of excess water through this transcellular pathway would also accelerate the removal of associated solutes by creating a solute concentration gradient between brain parenchyma and CSF. Experimental evidence will be needed to test this hypothesis.

AQP4 and neural signal transduction

The first evidence for a role of AQP4 in neural signal transduction came from studies of hearing in AQP4 null mice (Li and Verkman 2001). AQP4 was immunolocalized to supporting epithelial cells (Hensen's and Claudius cells) in the organ of Corti, which are adjacent to electrically excitable hair cells. In 4- to 5-week-old mice from a CD1 genetic background, auditory brainstem response (ABR) thresholds in response to a click stimulus were increased by $>$20 db in AQP4 null mice compared with wildtype mice. In a C57/bl6 background, nearly all AQP4 null mice were deaf whereas ABRs could be elicited in wild-

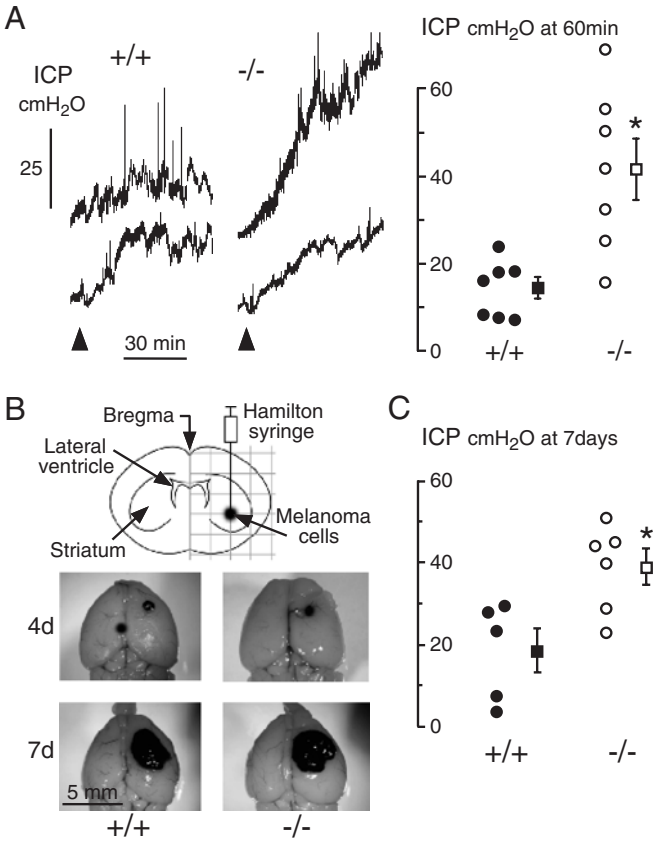

Fig. 5 Aquaporin-4 deletion increases brain swelling in vasogenic edema. **a** Increased ICP in AQP4-null mice in response to intraparenchymal fluid infusion. *Left*: representative ICP traces from two wildtype and AQP4 null mice. *Right*: increased ICP at 60 min in response to isotonic fluid infusion (0.5 μl/min). Data shown for individual mice and mean±SE. **b** Increased ICP and in AQP4 null mice with melanoma brain tumor. *Top*: site of injection of melanoma cells. *Bottom*: tumor size at 4 and 7 days after implantation showing similar-sized tumors in wildtype and AQP4 null mice. **c** ICP measured 7 days after tumor implantation. From Papadopoulos et al. (2004)

type mice. Light microscopy showed no differences in inner ear morphology of wildtype versus AQP4 null mice. Similar hearing impairment in AQP4 deficiency was reported subsequently by Mhatre et al. (2002). The exact mechanism by which AQP4 deletion impairs hearing remains unclear. One possibility is that AQP4 facilitates rapid osmotic equilibration in epithelial cells in the organ of Corti, which are subject to large K^+ fluxes during mechanoelectric signal transduction.

Epilepsy is an example of excessive neural signaling in the central nervous system. Relative cellular and extracellular space (ECS) volume has been demonstrated to play an important role in the propensity for epileptic seizures. For example, reducing ECS volume by exposure to hypotonic medium produces hyperexcitability and enhanced epileptiform activity, whereas hyperosmolar medium reduces excitability. The hypothesis that AQP4-dependent water transport in astrocytes might modulate intrinsic brain excitability was tested by seizure susceptibility in response to the $GABA_A$ antagonist convulsant pentylenetetrazol

(PTZ; Binder et al. 2004a). At 40 mg/kg PTZ all wildtype mice exhibited seizure activity, whereas only one out of seven AQP4 null mice showed seizure activity. At a higher PTZ dose (50 mg/kg), both groups exhibited seizure activity; however, the latency to generalized (tonic-clonic) seizures was significantly reduced in wildtype mice. In recent experiments, in vivo EEG characterization of seizures induced by electrical stimulation in the hippocampus indicated greater seizure threshold and remarkably longer seizure duration in AQP4 null mice compared with wildtype mice (Binder et al., unpublished data). Although these studies suggest that AQP4 modulates brain excitability, as well as the propagation and termination of seizure activity, they do not establish a mechanism for the phenotype. Since seizure propensity is sensitive to ECS volume, AQP4 deletion may alter ECS volume or composition at baseline and/or following neural activity. A larger ECS volume fraction prior to seizure-inducing stimuli and/or a blunted reduction in ECS volume during neural activity via abrogation of water influx through glial AQP4 would be expected to limit excitability and synchrony. Indeed, a significantly greater ECS volume was found in AQP4 null mice at baseline using a cortical surface photobleaching method to quantify the diffusion of extracellular markers in the ECS (Binder et al. 2004b), which probably provides part of the explanation for the altered seizure phenotype in AQP4 deficiency.

In prior work, Amiry-Moghaddam et al. (2003b) studied hippocampal slices from α-syntrophin-deficient mice and found a deficit in extracellular K^+ clearance following evoked neuronal activity. In addition, using a hyperthermia model of seizure induction, they found that a greater fraction of the α-syntrophin-deficient mice had more severe seizures than wildtype mice. These data are consistent with the idea that AQP4 and its molecular partners (e.g. Kir4.1, α-syntrophin, dystrophin) together comprise a multifunctional "unit" responsible for clearance of K^+ and/or H_2O following neural activity. However, the existence of a functionally important macromolecular complex involving AQP4 remains speculative.

AQPs and eye physiology

The eye expresses several AQPs at putative sites of fluid transport (Fig. 6a). The expression of MIP (major intrinsic protein, also referred to as AQP0) in lens fiber has been known for many years. Mutations in AQP0 in humans are associated with congenital cataracts (Berry et al. 2000). AQP1 is expressed in corneal endothelia, and at sites of aqueous fluid production (ciliary epithelium) and outflow (trabecular meshwork). AQP3 is expressed in the conjunctival epithelium. AQP4 is expressed in Müller cells in the retina, which support the electrically excitable hair cells, as well as in the optic nerve. AQP4 is also expressed with AQP1 in non-pigmented ciliary epithelium. AQP5 is expressed in corneal epithelia and lacrimal gland. The expression pattern of AQPs provides indirect evidence for their involvement in intraocular pressure (IOP) regulation (AQP1 and AQP4), corneal and lens transparency (AQP0, AQP1, and AQP5), visual signal transduction (AQP4), tear film homeostasis (AQP3 and AQP5), conjunctival barrier function (AQP3), and tear formation by the lacrimal glands (AQP5).

Role of AQP1 in corneal swelling

Maintenance of corneal transparency requires precise regulation of stromal water content. AQP1 is expressed in corneal endothelial cells and AQP5 in epithelial cells. Corneal thickness, water permeability, and the response to experimental swelling was studied in wildtype

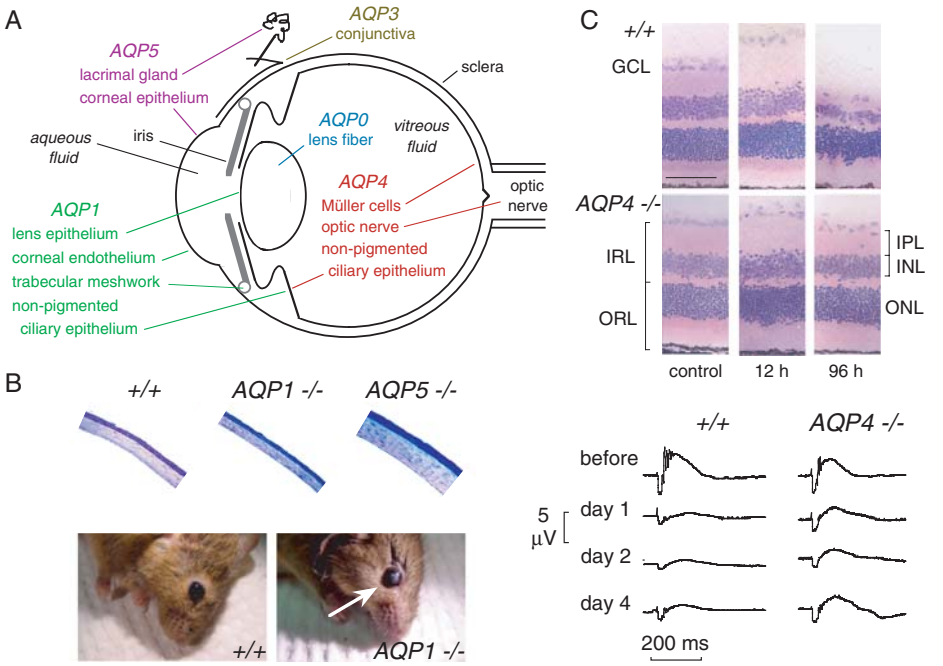

Fig. 6 Ocular phenotype in AQP-deficient mice. **a** Sites of AQP expression in the eye. **b** *Top*: stained plastic sections of corneas of mice of indicated genotype showing epithelium (upper surface), stroma, and endothelium. *Bottom*: photographs of wildtype and AQP1 null mice 40 min after a 10-min exposure of the corneal surface to distilled water showing corneal opacification in the AQP1 null mouse (*white arrow*). **c** *Top*: retinal morphology in an ischemia-reperfusion model of retinal injury. Hematoxylin and eosin-stained retinal sections before and 12 and 96 h after 60-min retinal ischemia in wildtype and AQP4 null mice. Note retinal swelling after 12 h and degeneration after 96 h. *Bottom*: functional analysis by electroretinography. Representative electroretinograms before and 1, 2, and 4 days after 45-min retinal ischemia in wildtype and AQP4 null mice. From Thiagarajah and Verkman (2002) and Da and Verkman (2004)

mice versus mice lacking AQP1 or AQP5 (Thiagarajah and Verkman 2002). Compared with wildtype mice, which have a corneal thickness of 123 μm, corneal thickness was reduced in AQP1 null mice (101 μm) and increased in AQP5 null mice (144 μm). Thickness measurements were made in fixed eyes (Fig. 6b, top), as well as by brightfield scanning confocal microscopy in vivo. After exposure of the external corneal surface to hypotonic saline (100 mOsm), the rate of corneal swelling (5 μm/min) was reduced approximately 2-fold by AQP5 deletion. After exposure of the corneal endothelial surface to hypotonic saline by anterior chamber perfusion, the rate of corneal swelling (7 μm/min) was reduced ~4-fold by AQP1 deletion. Although baseline corneal transparency was not impaired by AQP1 deletion, the recovery of corneal transparency and thickness after hypotonic swelling (10-min exposure of corneal surface to distilled water) was remarkably delayed in AQP1 null mice, with approximately 75% recovery at 7 min in wildtype mice compared with 5% recovery in AQP1 null mice (Fig. 6b, bottom). The impaired recovery of corneal transparency in AQP1 null mice provides evidence for the involvement of AQP1 in active extrusion of fluid from the corneal stroma across the corneal endothelium. A recent study of primary cultures of corneal endothelial cells showed reduced water permeability and impaired cell volume regulation in AQP1 deficiency (Kuang et al. 2004). The exact mechanism by which AQP1 appears to play

a major role in corneal volume homeostasis remains to be determined, as does the possible clinical benefit of increasing endothelial AQP1 expression to reduce corneal edema.

AQPs and water transport at the ocular surface

The cornea and conjunctiva are semipermeable barriers at the ocular surface that are involved in regulation of tear film volume and composition. Fluorescence methods were developed recently to measure the cell and tissue water permeabilities of the cornea and conjunctiva in vivo (Levin and Verkman 2004). Water permeability in calcein-stained surface epithelial cells was measured from the kinetics of fluorescence quenching in response to rapid (<0.2 s) changes in extraocular fluid osmolarity. Osmotic equilibration occurred with an exponential time constant of 1.3±0.2 s (water permeability P_f 0.045 cm/s) in the corneal epithelial cells of wildtype mice, slowing 2.1±0.4-fold in AQP5-deficient mice; the time constant was 2.4±0.1 s in conjunctiva (P_f 0.025 cm/s), slowing 3.6±0.7-fold in AQP3-deficient mice. P_f across the intact cornea and conjunctiva, the relevant parameters describing water movement into the hyperosmolar tear film in vivo, was determined by a dye dilution method from the fluorescence of Texas Red-dextran in an anisosmolar solution in a microchamber at the ocular surface. P_f of whole cornea was 0.0017 cm/s, and reduced by greater than 5-fold in AQP5-deficient mice. P_f in AQP5 null mice was restored to 0.0015 cm/s after epithelial removal. P_f of whole conjunctiva was 0.0011 cm/s, and not sensitive to AQP3 deletion. The permeability data were incorporated into a mathematical model of tear film osmolarity, providing insights into the pathophysiology of dry eye disorders and therapies directed at increasing fluid secretion across ocular surface tissues.

AQPs and intraocular pressure regulation

The principal determinants of IOP are the rate of aqueous fluid production by the ciliary epithelium and the rate of fluid drainage (outflow) in the canal of Schlemm. Aqueous fluid production involves passive, near-isosmolar fluid secretion driven by active salt transport across the ciliary epithelium. Ion and solute transporters have been identified on pigmented and non-pigmented layers of the ciliary epithelium that probably facilitate active solute secretion. Aqueous fluid drainage is believed to involve pressure-driven bulk fluid flow in the canal of Schlemm, as well as fluid movement through the sclera by seepage across the ciliary muscle and supraciliary space.

AQP1 and AQP4 are expressed in non-pigmented ciliary epithelium, and AQP1 in trabecular meshwork endothelium. Using a modified microneedle method to measure IOP, a small though significant reduction in IOP was found in mice lacking AQP1 and/or AQP4 compared with wildtype mice in both outbred (CD1) and inbred (C57/bl6) mouse genetic backgrounds (Zhang et al. 2002). Whether larger differences in IOP will be found with AQP deficiency in models of glaucoma remains to be determined. Methods were also developed to measure aqueous fluid production and influx in mice in order to resolve whether the reduced steady-state IOP in AQP deficiency is due to reduced aqueous fluid production and/or altered outflow. Aqueous fluid outflow in wildtype and AQP1-deficient mice was approximately 0.35 μl/h per mmHg. The lack of effect of AQP1 deletion on aqueous fluid outflow is consistent with a bulk fluid flow mechanism that is predicted not to involve water-selective AQPs. Aqueous fluid production was measured by confocal microscopy from the disappearance of fluorescein from the aqueous fluid space following pulsed iontophoretic in-

troduction. The aqueous fluid production rate was 3.6 μl/h and slowed significantly in AQP1 deficiency. Thus, the decreased IOP in AQP1 null mice probably results from reduced aqueous fluid production, which is probably a consequence of impaired isosmolar fluid secretion across the ciliary epithelium.

AQP4 and retinal function and response to injury

AQP4 is expressed in Müller cells in the retina, which are similar to astroglial cells in the central nervous system where AQP4 deletion protects against cytotoxic brain edema after cerebral ischemia as discussed above. The possibility was tested that AQP4 deletion in mice protects the retina in a transient ischemia-reperfusion model (Da and Verkman 2004). Retinal function and morphology were assessed in wildtype and AQP4-deficient mice after ischemic damage produced by 45–60 min elevation of IOP to 120 mmHg. Retinal structure and cell number were preserved in AQP4 null mice, particularly in inner nuclear and plexiform layers of retina where Müller cells are concentrated (Fig. 6c). For example, 4 days after ischemia inner retinal thickness was thinned by 43% in wild-type mice versus 11% in AQP4 null mice. Retinal function and cell survival were also significantly improved in AQP4-deficient mice. By electroretinography, b-wave amplitude was reduced by 75–83% 1–4 days after ischemia in wildtype mice versus 48–51% in AQP4 null CD1 mice; reductions were 53–72% versus <34% in C57/bl6 mice (Fig. 6d). As reported previously (Li et al. 2002), there were small but significant abnormalities in baseline electroretinograms of AQP4 null mice, consistent with the role of AQP4 in neural signal transduction as discussed above. Several possible mechanisms for retinal protection in AQP4 deficiency were investigated and ruled out, including ECS expansion, reduced early swelling, and altered Kir4.1 K^+ channel expression. The results showed that AQP4 deletion in mice is neuroprotective in a transient ischemia model of retinal injury, suggesting the possible use of AQP4 inhibitors in retinal vascular occlusive and ischemic diseases. However, the precise mechanism(s) by which AQP4 deletion is/are neuroprotective remain(s) to be established.

Physiological role of glycerol transport by the aquaglyceroporins

AQP3 and skin function

The most superficial layer of skin is the stratum corneum (SC), which consists of terminally differentiated keratinocytes (corneocytes) that originate from actively proliferating keratinocytes in lower epidermis (basale, spinosum, and granulosum cells), and contain a lamellar lipid layer secreted from lamellar bodies (Fig. 7a). Hydration of the SC is an important determinant of skin appearance and physical properties, and depends on a number of factors including the external humidity, and its structure, lipid/protein composition, barrier properties, and concentration of water-retaining osmolytes (natural moisturizing factors, NMFs) including free amino acids, ions, and other small solutes.

Phenotype analysis of AQP3-deficient mice has provided compelling evidence for a role of AQP3 in epidermal biology. AQP3 is expressed strongly in the basal layer of kerinocytes in mammalian skin (Fig. 7b, left). AQP3 null mice were generated and studied in a hairless genetic background to facilitate skin studies (Ma et al. 2002). Figure 7b (right) shows reduced SC hydration in AQP3 null mice as measured by high frequency skin conductance,

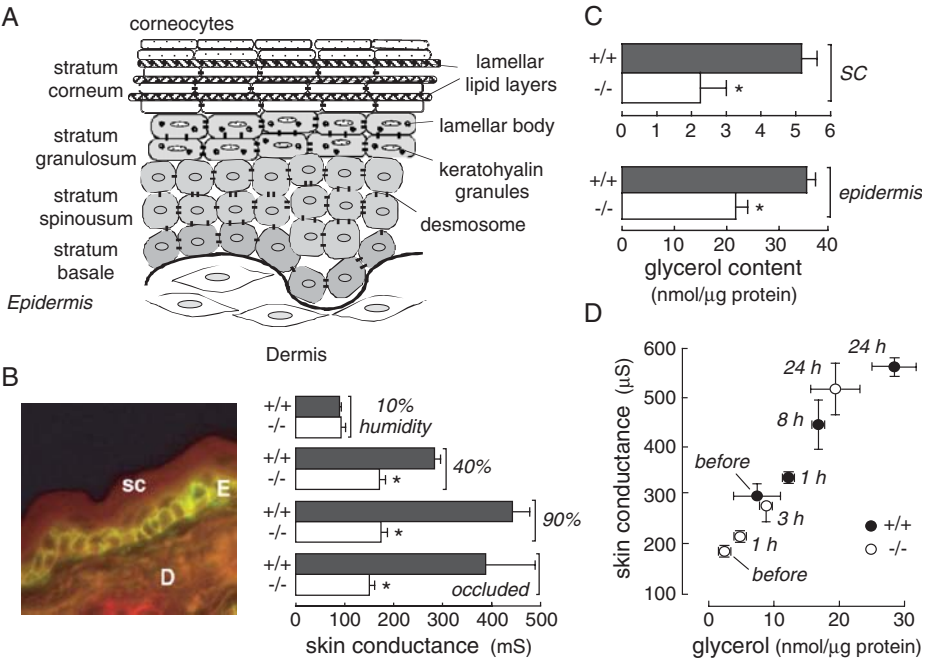

Fig. 7 Reduced skin hydration in AQP3 deficiency. **a** Schematic showing stratum corneum and epidermal layers. **b** *Left*: immunofluorescence showing AQP3 in mouse epidermal cells. *E* epidermis, *D* dermis, *SC* stratum corneum. *Right*: high-frequency superficial skin surface conductance in dorsal skin of hairless wild-type and AQP3 null mice (SE, 20 mice/group). Skin conductance measured after 24-h exposure to relative humidity of 10, 40, or 90%; *occluded* indicates a plastic occlusion dressing that prevents evaporative water loss (SE, 5 mice/group). **c** Glycerol content measured in SC and epidermis. **d** Correlation between SC glycerol content and skin conductance for wildtype (*filled circles*) and AQP3 null (*open circles*) mice in a 90% humidity atmosphere for the indicated times. Mice were given glycerol orally ad libitum as their only fluid source (10% for wildtype mice, 2% for AQP3 null mice). From Hara et al. (2002), Ma et al. (2002) and Hara and Verkman (2003)

which is a linear index of SC water content. An interesting observation was that exposure of mice to high humidity or occlusion increased SC hydration in wildtype but not AQP3 null mice. Skin conductance of the wildtype and AQP3 null mice became similar after exposure to 10% humidity. These results indicate that water transport through AQP3 is not a rate-limiting factor in transepidermal water loss. If reduced SC hydration were related to a balance between evaporative water loss from the SC and water replacement through AQP3-containing basal keratinocytes, then preventing water loss by high humidity or occlusion should have corrected the defect in SC hydration in the AQP3 null mice. Additional skin phenotype analysis indicated delayed barrier recovery after SC removal by tape-stripping in AQP3 null mice, as well as decreased skin elasticity, and delayed wound healing (Hara et al. 2002).

A series of experiments was performed to investigate the mechanism by which AQP3 deficiency produces the pleiotropic defects in skin function. Initially, functional measurements of water and glycerol transport were carried out to verify AQP3 functionality in epidermis (Ma et al. 2002). A systematic analysis of the ultrastructure and composition of the epidermis and stratum corneum in AQP3-deficient mice revealed reduced glycerol content in SC and epidermis (Fig. 7c), with normal glycerol in dermis and serum, suggesting reduced

glycerol transport from blood into the epidermis in AQP3 deficiency through the basal keratinocytes. No significant differences in wildtype versus AQP3-deficient mice were found in SC structure, cell turnover, lipid profile, protein content, and the concentrations of amino acids, ions, and other NMFs (Hara et al. 2002).

From these observations it was postulated that reduced epidermal and SC glycerol content was responsible for the abnormal skin phenotype in AQP3 null mice. Because glycerol is an NMF, reduced SC glycerol is predicted to reduce SC hydration and skin elasticity; because of the biosynthetic role of glycerol in the epidermis, reduced epidermal glycerol is predicted to delay barrier recovery function and wound healing. In support of this hypothesis, it was found that glycerol replacement by topical or systemic routes corrected each of the phenotype abnormalities in AQP3 null mice (Hara and Verkman 2003). SC hydration was increased in wildtype and AQP3 null mice placed in a 40% humidity atmosphere after oral glycerol administration. SC glycerol content and water content, as assessed by skin conductance, correlated well for mice placed in a 90% humidity atmosphere and given oral glycerol (Fig. 7d). Furthermore, glycerol transport from blood into the epidermis and SC was found to be reduced in AQP3 deficiency, suggesting impaired glycerol transport into the epidermis and SC through the relatively glycerol impermeable basal keratinocyte layer resulting in reduced steady-state epidermal and SC glycerol content. These findings indicated an important role of AQP3 and glycerol in epidermal function, providing a rational scientific basis for the long-standing practice of including glycerol in cosmetic and skin medicinal preparations.

AQP7 and adipocyte metabolism

The aquaglyceroporin AQP7 was cloned initially from human adipose tissue (originally named AQPap; Kuriyama et al. 1997), and shown in heterologous expression systems to function as a water/glycerol transporter. In a series of studies from the same group and others, AQP7 expression in adipocytes was found to be sensitive to a number of metabolic stresses, including fasting/refeeding, insulin deficiency, and steroids and adrenergic agonists, providing indirect evidence for a role of AQP7 in adipocyte function.

We recently discovered a remarkable phenotype in AQP7 null mice (Hara-Chikuma et al. 2005). Although wildtype and AQP7 mice grew at similar rates as assessed by mouse weight, the AQP7 null mice had a remarkably greater fat mass compared with wildtype mice as seen grossly (Fig. 8a, top). Indeed, fat mass from multiple sites was significantly elevated in both male and female AQP7 null mice at age 16 weeks. Epididymal fat mass was comparable in wildtype and AQP7 null mice until age 4 weeks, but became different as the mice aged (Fig. 8b). Histologically, adipocytes at 16 weeks were remarkably larger in AQP7 null mice than in wildtype mice (Fig. 8a, bottom), suggesting that the greater fat mass in the AQP7 null mice is a consequence of adipocyte hypertrophy. Adipocyte size was similar in young wildtype and AQP7-deficient mice.

The concentrations of glycerol and triglycerides in serum were unaffected by AQP7 deletion, though adipocyte glycerol and triglyceride concentrations were significantly elevated in the AQP7 null mice (Fig. 8c). To investigate the mechanism for the progressive adipocyte hypertrophy in AQP7 deficiency, measurements were made of adipocyte plasma membrane glycerol permeability, glycerol release, lipolysis and lipogenesis. Plasma membrane glycerol permeability was measured from the initial uptake of ^{14}C-glycerol into isolated adipocytes from the younger wildtype and AQP7 null mice, where adipocyte size is comparable. ^{14}C-glycerol uptake was reduced by ~3-fold in AQP7 null mice compared with

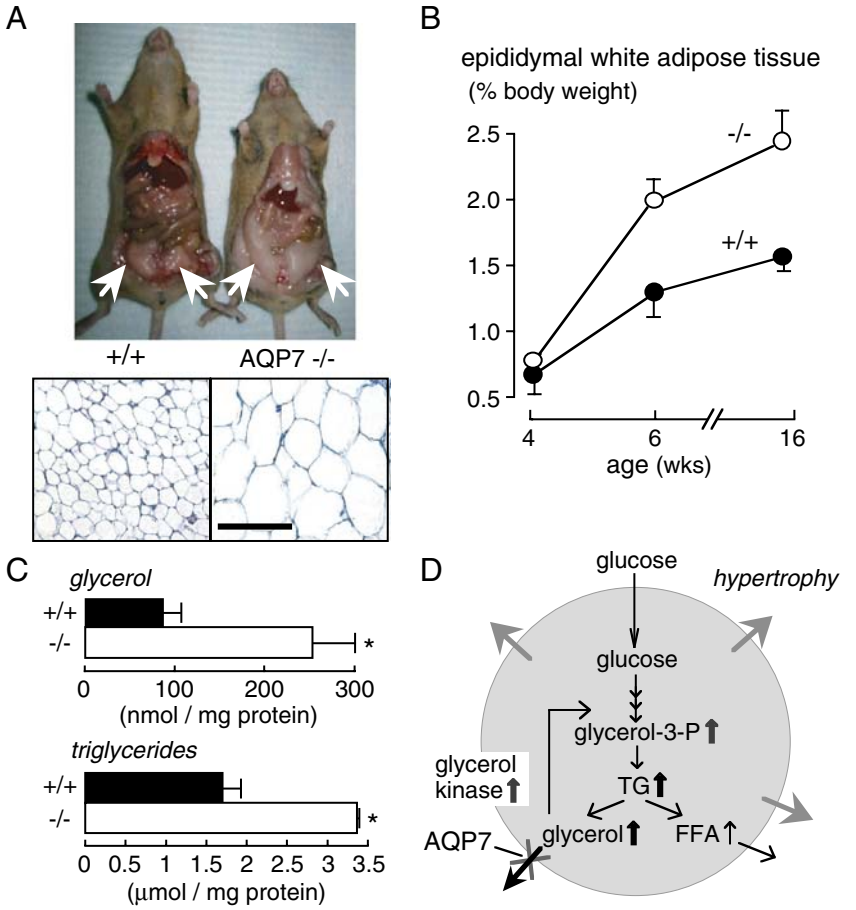

Fig. 8 Progressive fat accumulation and adipocyte hypertrophy in AQP7 deficiency. **a** *Top*: photographs showing increased gonadal fat in AQP7 null mice at age 16 weeks (*white arrows*). *Bottom*: histology of gonadal fat (stained with hematoxylin and eosin). Bar, 100 μm. **b** Age-dependent epididymal fat mass (SE, 6 mice). **c** Glycerol and triglyceride content in adipocytes from mice of age 16 weeks. **d** Proposed mechanism for adipocyte hypertrophy in AQP7 deficiency. From Hara-Chikuma et al. (2005)

wildtype mice. Glycerol release from minced fat tissue was determined by assay of glycerol in the physiological bathing solution 1 and 3 h after incubation at 37°C. Glycerol release was significantly reduced in the AQP7 null mice, though release data do not provide direct information about adipocyte lipolysis because glycerol release depends on several factors, including the rate of lipolysis, cellular glycerol concentration, and plasma membrane glycerol permeability.

Lipolysis, as measured by free fatty acid release from isolated adipocytes and the activity of hormone-sensitive lipase (a key regulator of lipolysis), was found to be similar in wildtype and AQP7-deficient mice. Similarly, lipogenesis was similar in the wildtype and knockout mice, as assayed from the incorporation of ^{14}C-glucose into triglycerides. Also, measurements of PPARγ and C/EBPα transcript and protein expression indicated that upregulation of these adipocyte transcription factors could not account for the progressive adipocyte hypertrophy in AQP7-deficient mice.

Figure 8d shows a proposed mechanism for progressive triglyceride (TG) accumulation in AQP7-deficient adipocytes. Reduced plasma membrane glycerol permeability in AQP7 deficiency results in an increase in steady-state glycerol concentration in adipocyte cytoplasm. Increased adipocyte glycerol concentration would then increase glycerol 3-phosphate and hence TG biosynthesis. These results thus focus attention on adipocyte glycerol permeability as a novel regulator of adipocyte size and whole body fat mass. Modulation of adipocyte AQP7 expression and/or function may thus alter fat mass, providing a rational basis for investigation of AQP7 upregulation as therapy in some forms of obesity.

Another study of AQP7 knockout mice (Maeda et al. 2004) reported a mild phenotype of reduced serum glycerol concentration in AQP7-deficient young male mice after adrenergic stimuli and prolonged fasting. The latter study did not report data on adipocyte morphology or fat mass, but looked only at relatively young mice focusing on basal and stimulated release of glycerol into the blood. However, the physiological relevance of altered serum glycerol concentration in response to metabolic stress is unclear.

AQPs in angiogenesis and cell migration

Based on evidence of strong AQP1 protein expression in tumor microvessels (Endo et al. 1999; Vacca et al. 2001), the possible role of AQP1 in tumor angiogenesis was investigated (Saadoun et al. 2005). Melanoma cells were implanted subcutaneously in wildtype and AQP1 null mice. Remarkably, tumor growth was greatly slowed in the AQP1-deficient mice and associated with enhanced survival (Fig. 9a). In control studies, tumor growth was not affected in mice lacking AQP3, a water channel that impairs urinary concentrating ability to an even greater extent than AQP1. Slow growth and improved survival of subcutaneous melanoma was also found in syngeneic mice (C57/BL6) lacking AQP1, and of a brain tumor assessed histologically 7 days after intracranial melanoma cell implantation. A consistent histological finding in tumors of AQP1 null mice was a much lower density of microvessels and the presence of islands of viable tumor cells surrounded by necrotic tissue (Fig. 9b). All vessels in tumors of wildtype mice immunostained strongly for AQP1, with no staining seen in tumor cells or vessels of AQP1 null mice.

The mechanism of the apparent reduction in angiogenesis in AQP1 deficiency was studied using primary cultures of aortic endothelia from wildtype and AQP1 null mice. Intrinsic endothelial cell functions required for angiogenesis were measured, such as proliferation, adhesion, and migration. Cultured endothelial cells from wildtype and AQP1 null mice had similar appearance, size, and growth/proliferation, with $>90\%$ of cells from wildtype mice staining positive for AQP1 and having high water permeability. As shown in Fig. 9c, cell adhesion, measured from the number of cells adhering to a gelatin support within 4 h of plating, was not significantly altered by AQP1 expression. However, cell migration toward fetal bovine serum, a potent chemotactic stimulus, quantified using a modified Boyden chamber, was significantly impaired in AQP1-deficient endothelial cells. Even larger differences were found in a cell "invasion" assay in which cells migrated through a Matrigel layer followed by the porous filter.

Cell migration involves transient formation of membrane protrusions (lamellipodia, cell membrane ruffles) at the leading edge of the cell that are thought to require rapid local changes in ion fluxes and cell volume, probably accompanied by rapid transmembrane water movement (Condeelis 1993; Lauffenburger and Horwitz 1996). If transmembrane water movement is a fundamental determinant of cell motility, then a different water channel

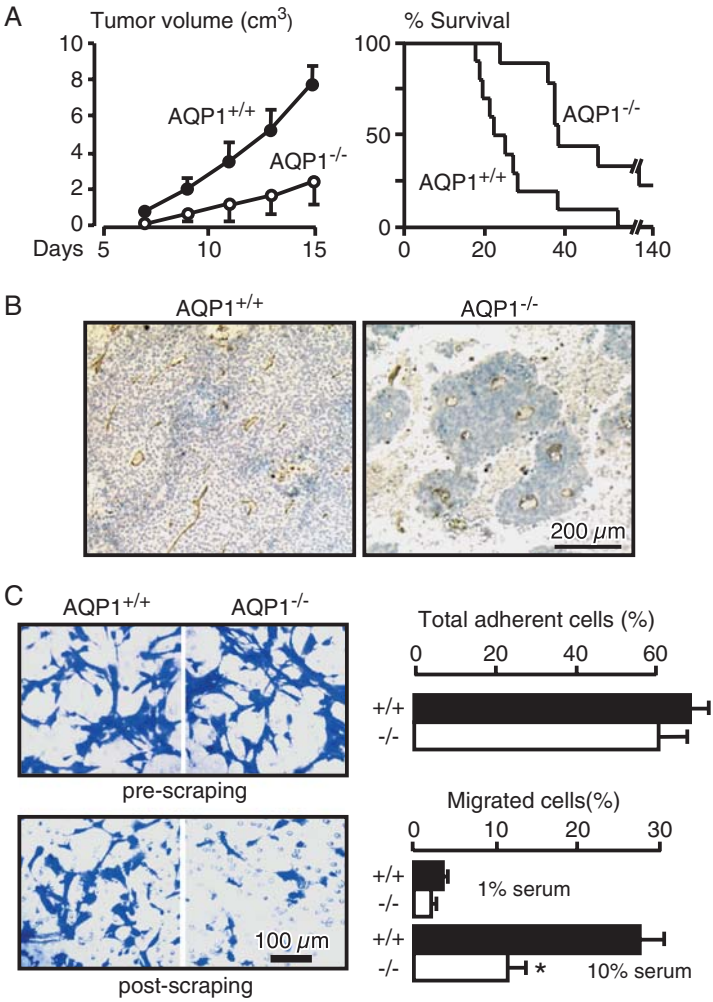

Fig. 9 Impaired tumor angiogenesis and endothelial cell migration in AQP1 deficiency. **a** Reduced tumor growth in AQP1 null mice. Growth (*left*) and survival (*right*) of subcutaneous melanoma in wildtype versus AQP1 null mice. **b** Tumor stained with isolectin-B4 (*brown*). Note islands of tumor surrounded by necrotic tissue in AQP1-deficient mice. **c** Impaired migration of endothelial cells lacking AQP1. *Left*: adhesion and migration of endothelial cells quantified by counting cells in Boyden chamber assay before and after scraping. *Right*: summary of percentages of adherent and migrated cells. Where indicated, cells migrated from 1 to 10% serum medium. From Saadoun et al. (2005)

would also accelerate cell migration, and water permeability-dependent migration might be a general phenomenon seen in many cell types. To test this hypothesis, wound healing and cell migration were studied in non-endothelial CHO cells and Fischer rat thyroid (FRT) epithelial cells, after stable transfection with control plasmid, or plasmids encoding AQP1 or AQP4. Plasma membrane expression of AQP1 or AQP4 was confirmed in the transfected cells. Although cell growth and adhesion were not affected by AQP1 or AQP4 expression, cell migration through a porous filter was remarkably enhanced in both cell types when either AQP was expressed. Wound closure experiments supported the conclusion of increased

migration in the AQP-expressing cells. An interesting observation made in many cells was the polarized expression of AQP1 at the leading edge of the cell membrane, which has also been found for several transporters involved in migration, including Na^+/H^+ and Cl^-/HCO_3^- exchangers, and the Na^+/HCO_3^- cotransporter. In rapid time-lapse video microscopy, it was found that AQP1 expression produced more protrusions and a shorter mean residence time of protrusions, suggesting that AQPs accelerate cell migration by facilitating the rapid turnover of cell membrane protrusions at the leading edge.

From these data we proposed that actin cleavage and ion uptake at the tip of a lamellipodium creates local osmotic gradients that drive the influx of water across the cell membrane. Water entry then increases local hydrostatic pressure to cause cell membrane protrusion, which may create space for actin polymerization. AQPs in the region of membrane protrusions thus enhance water entry and thereby the dynamics of cell membrane protrusions and cell motility. Further biophysical studies are needed to test this proposed mechanism. Studies are also needed to test whether AQP-dependent cell migration is a general phenomenon in other biological processes besides angiogenesis, such as tumor spread, wound healing, and organ regeneration.

Summary and perspective

The phenotype data on AQP-deficient mice suggest that AQP-facilitated water permeability is important: *a*. When water movement is driven across a barrier by a continuous osmotic gradient (as in the kidney collecting duct *b*. For active, near-isosmolar fluid absorption/secretion (as in the kidney proximal tubule and salivary gland *c*. For neural signal transduction (as in the brain and inner ear) *d*. For cell migration (as in tumor angiogenesis) Aquaglyceroporin-facilitated glyercol transport has been shown to be important in skin hydration (AQP3) and adipocyte metabolism (AQP7). It is likely that other new roles of AQPs will be discovered. Another general conclusion from the phenotype studies is that the tissue expression of an aquaporin does not ensure its physiological significance, indicating that evaluation of tissue AQP function should be done on a case-by-case basis. For example, intestinal fluid absorption and secretion were not affected by AQP4 or AQP8 deletion (Wang et al. 2000b; Yang et al. 2005), hepatobiliary function was not impaired by AQP1 or AQP8 deletion (Ma et al. 2001; Yang et al. 2005), lacrimal and sweat gland secretion were not impaired by AQP5 deletion (Moore et al. 2000; Song et al. 2002), skeletal muscle function was not impaired by AQP4 deletion (Yang et al. 2000b), and gastric acid/fluid secretion were not impaired by AQP4 deletion (Wang et al. 2000a).

Much work remains to be done in following up the mouse phenotype observations made thus far, in discovering new roles of AQPs in mammalian physiology, and in developing clinical therapies because of the new insights coming from the mouse phenotype analyses. Small-molecule modulators of AQP expression/function could have clinical applications in the therapy of congestive heart failure and hypertension (AQP1 and AQP2 inhibitors), of cytotoxic and vasogenic types of brain swelling (AQP4 modulators), of obesity (AQP7 upregulators), of tumor angiogenesis (AQP1 inhibitors), and others. Additional data are needed to establish a firm mechanistic basis for AQP involvement in neural signal transduction and the clearance of excess brain water in vasogenic edema, and in the precise role of the aquaglyceroporins in epidermal and adipocyte metabolism. Finally, the proposed new role for AQPs in cell motility mandates examination of the possible role of AQPs in tumor metastasis, wound healing, leukocyte chemotaxis, and organ regeneration.

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Activation of heterotrimeric G-proteins independent of a G-protein coupled receptor and the implications for signal processing

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Abstract Heterotrimeric G-proteins are key transducers for signal transfer from outside the cell, mediating signals emanating from cell-surface G-protein coupled receptors (GPCR). Many, if not all, subtypes of heterotrimeric G-proteins are also regulated by accessory proteins that influence guanine nucleotide binding, guanosine triphosphate (GTP) hydrolysis, or subunit interactions. One subgroup of such accessory proteins (activators of G-protein signaling; AGS proteins) refer to a functionally defined group of proteins that activate selected G-protein signaling systems in the absence of classical G-protein coupled receptors. AGS and related proteins provide unexpected insights into the regulation of the G-protein activation–deactivation cycle. Different AGS proteins function as guanine nucleotide exchange factors or guanine nucleotide dissociation inhibitors and may also influence subunit interactions by interaction with G$\beta\gamma$. These proteins play important roles in the generation or positioning of signaling complexes and of the regulation of GPCR signaling, and as alternative binding partners for G-protein subunits. Perhaps of even broader impact is the discovery that AGS proteins provide a foundation for the concept that heterotrimeric G-protein subunits are processing signals within the cell involving intrinsic cues that do not involve the classical signal input from a cell surface GPCR.

Abbreviations *AGS:* Activator of G-protein signaling · *ER:* Endoplasmic reticulum · *ERK:* Extracellular signal-regulated kinase · *GAP:* GTPase-activating protein · *GDI:* Guanine nucleotide dissociation inhibitor · *GEF:* Guanine nucleotide exchange factor · *GPCR:* G-protein coupled receptor · *GPR:* G-protein regulatory · *GPSM:* G-protein signaling

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modulator · *GTPγS:* Guanosine 5′-3-*O*-(thio)triphosphate · *MHC:* Major histocompatibility complex · *NMDA:* N-methyl-D-aspartate · *nNOS:* Neuronal nitric oxide synthase · *PDZ:* PSD95/DLG/ZO-1 domain · *PI3K:* Phosphatidylinositol-3 kinase · *PLC:* Phospholipase C · *PTB:* Phosphotyrosine binding · *RBD:* Ras binding domain · *RGS:* Regulator of G-protein signaling · *TPR:* Tetratricopeptide repeat

Introduction

G-protein coupled receptor signaling represents one of the most conserved and ubiquitous signaling systems in eukaryotes for transferring information across the plasma membrane to the intracellular environment. In the canonical G-protein coupled receptor (GPCR) signaling pathway, the binding of a ligand to a cell surface receptor initiates conformational changes in receptor subdomains that transfer this signal to a heterotrimeric G-protein on the inner face of the membrane. This ligand-induced signal triggers the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the Gα subunit, followed by altered subunit interactions and activation of Gα-GTP- and Gβγ-specific downstream signaling pathways. Upon GTP hydrolysis, the heterotrimer can reform and be activated again by the receptor. Tight regulation of each of these discrete events within the cell ensures appropriate integration of cellular responses, and dysfunctional regulation contributes to pathophysiological situations in various tissues.

Regulation of these events extends beyond selective interactions of receptors, G-protein, and effectors, and includes subgroups of accessory proteins or signal regulators that directly influence the activation state of G-proteins. In addition to their influence on receptor-mediated signaling events, such proteins provide alternative modes of input to G-protein signaling pathways independent of GPCR function. One such subgroup of signal regulators was identified functionally based upon its ability to directly activate heterotrimeric G-proteins. Following a brief background, this article will focus on this newly-discovered subgroup, the nonreceptor activators of G-protein signaling (AGS) 1–8.

During the initial stages of the characterization of G-proteins, several observations suggested that their activation state could be regulated independently of a GPCR. Short cationic peptides, such as the family of wasp venom mastoparans, the kinin family, and peptide derivatives of Rab3 effector domains, directly activated heterotrimeric Gαi/Gαo proteins, and this was blocked by pertussis toxin treatment, which ADP-ribosylates Gαi/Gαo and prevents G-protein activation by a GPCR (Repke and Bienert 1987; Bueb et al. 1990; Higashijima et al. 1990; Mousli et al. 1990; Tomita et al. 1991; Kavelaars et al. 1993; Law et al. 1993; Ross and Higashijima 1994; Rupnik et al. 1997). Their mechanism of action is through facilitating GTP/GDP exchange on the Gα subunit without altering basal GTP hydrolysis rates (Higashijima et al. 1990), and thus these entities behave as guanine nucleotide exchange factors. Several small molecule ligands (2-substituted histamines, β-carbolines, suramin and derivatives, morphine derivatives) also directly alter nucleotide binding and/or hydrolysis by Gα (Seifert et al. 1994; Burde et al. 1996; Detert et al. 1996; Klinker et al. 1997; Klinker and Seifert 1997; Leschke et al. 1997). The bioactivity of such peptides and small molecules may involve cationic amphiphilic-like structures, and both charge distribution and hydrophobicity are important for Gα activator function. This may be similar to mechanisms of G-protein activation via juxtamembrane amphipathic helices in the third intracellular loop of a GPCR (Strader et al. 1989; Burstein et al. 1998; Franzoni et al. 1999; however, see also Voss et al. 1993). Additional activity in this area of research led to the

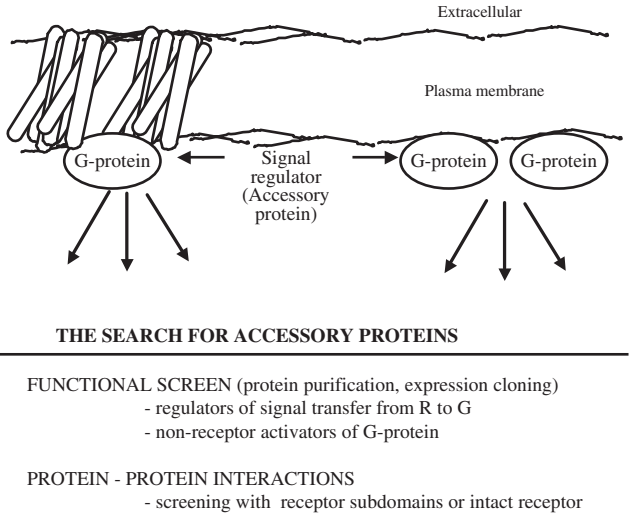

Fig. 1 Signal regulators for heterotrimeric G-proteins. A signal regulator or accessory protein was postulated to influence the transfer of signal from receptor to G-protein or to directly regulate the activation state or localization of G-proteins. Various strategies were initiated to identify such accessory proteins. (*G* G-protein, *R* G-protein coupled receptor)

identification of small molecule ligands and factors that actually behave as G-protein antagonists (Freissmuth et al. 1999; Nanoff et al. 2002; Takasaki et al. 2004).

The search for intracellular proteins other than a cell surface receptor that regulated G-protein function was initiated in part following observations of variations in the efficiency and/or specificity of receptor-heterotrimeric G-protein coupling in different cell backgrounds, as well as differences between the kinetic properties of the G-protein activation/deactivation cycle for selected signaling pathways within the cell and those observed with purified proteins (Duzic and Lanier 1992; Herzog et al. 1992; Perez et al. 1993; Sato et al. 1995, 1996 ; Marjamaki et al. 1997 and references therein). Additional studies demonstrating the localization of heterotrimeric G-protein subunits in intracellular compartments, suggesting a role in controlling such basic cellular functions as vesicular trafficking and exocytosis, lent further momentum to this search (Toutant et al. 1987; Ngsee et al. 1990; Stow et al. 1991; Aronin and DiFiglia 1992; Bomsel and Mostov 1992; Ahnert-Hilger et al. 1994; Helms 1995; Maier et al. 1995; Denker et al. 1996; Nurnberg and Ahnert-Hilger 1996; Ogier-Denis et al. 1996; Crouch and Simson 1997; Pinxteren et al. 1998; Martin et al. 1999; Crouch et al. 2000; Yamaguchi et al. 2000; Chatterjee and Fisher 2000a,2000b; Blackmer et al. 2001; Blumer et al. 2002). From this body of work, it was hypothesized that there were cell-specific accessory proteins or signal regulators that influence the transfer of signal from receptor to G-protein or that regulate the activation state and/or subcellular localization of heterotrimeric G-proteins (Fig. 1). Various experimental strategies were used to identify such proteins, including protein interaction screens, genetic screens, protein purification, and expression cloning systems.

One major class of such accessory proteins discovered during the 1990s, regulators of G-protein signaling or RGS proteins, augment the GTPase activity of Gα subunits, and subsequent studies indicate that some members of this group may also function as a scaffold and/or G-protein effector (Dohlman and Thorner 1997; Berman and Gilman 1998; DeVries and Farquhar 1999; Ross and Wilkie 2000; Hollinger and Hepler 2002). Several

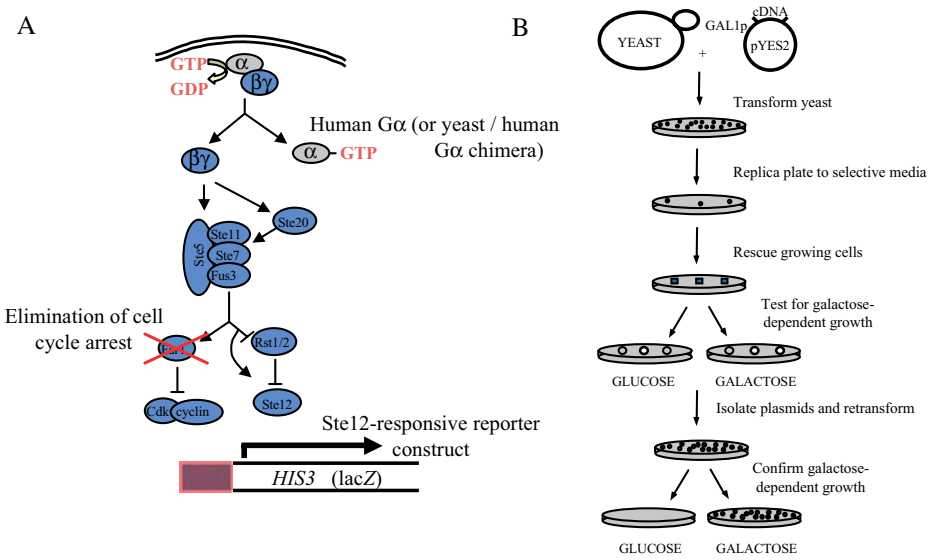

Fig. 2 Yeast-based functional screen for receptor-independent activators of G-protein signaling. The yeast strain indicated in **A** was generated as described previously (Cismowski et al. 1999, 2002) and transformed with mammalian cDNA libraries in the galactose-inducible yeast expression vector pYES2. The strategy for identifying mammalian cDNAs that promoted growth (i.e., activated G-protein signaling pathway) is indicated in **B**. (*GAL1p* GAL1 promoter)

biochemical studies suggested an additional class of proteins that actually influenced nucleotide exchange on Gα in the absence of a receptor. Such proteins include the growth-associated protein (GAP-43) and the NG-G-protein activator (Strittmatter et al. 1991; Sato et al. 1996). GAP-43, which is enriched in the neuronal growth cone, increases guanosine 5′-3-*O*-(thio)triphosphate (GTPγS) binding to purified Gαo (Strittmatter et al. 1991) and is suggested to be involved in neuronal guidance or growth cone stability (Lankford et al. 1990). Differences in α2-adrenergic receptor signaling efficiency in response to agonists in PC-12 cells versus NIH-3T3 cells led to the biochemical characterization of a G-protein activator from N108-15 cells that increased GTPγS binding to heterotrimeric Gαi and Gαo (Sato et al. 1996). Although not yet fully identified, the activation of G-protein by the NG-G-protein activator was insensitive to pertussis toxin pretreatment of purified brain G-protein, which distinguished it from a GPCR in terms of mechanism of action (Ribas et al. 2002). Additional entities were recently identified as influencing G-protein nucleotide binding and/or hydrolysis, including phosphatidylethanolamine binding protein, caveolins, presenilin, and Ric-8 (Sato et al. 1995, 1996 and references therein; Smine et al. 1998; Kroslak et al. 2001; Blumer and Lanier 2003; Tall et al. 2003)

As part of a broad strategy to identify postreceptor signal regulators, we developed functional screening methodologies in the yeast *Saccharomyces cerevisiae*, taking advantage of the yeast's endogenous mating pathway which utilizes heterotrimeric G-proteins (Fig. 2; Klein et al. 1998; Cismowski et al. 1999; Takesono et al. 1999; Cismowski et al. 2002; Cao et al. 2004).

This pathway, which is driven by the presence of free Gβγ, was modified to allow for screening of mammalian cDNA expression libraries to isolate those cDNAs that activated transcription of a pheromone-responsive promoter downstream of activated G-protein. Modifications included eliminating the pheromone receptor, replacing the endogenous yeast Gα

	Activators of G-protein Signaling
GEF	AGS1 - (RASD1, DexRas1)- ras-related protein
GDI	AGS3 (GPSM1) - four GPR motifs (partial cDNA) AGS4 - (GPSM3, G18.1b) - three GPR motifs AGS5 - (GPSM2, LGN) - four GPR motifs (partial cDNA) AGS6 - (RGS12) - one GPR motif (partial cDNA)
OTHER	AGS2 - (tctex-1) - light chain of cytoplasmic dynein AGS7 - uncharacterized mRNA AGS8 - uncharacterized mRNA

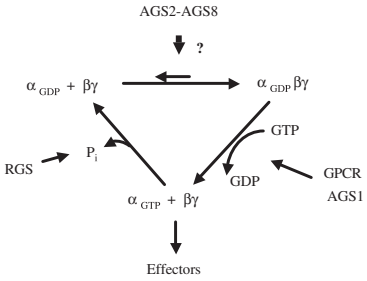

Fig. 3 **A** AGS proteins isolated in yeast-based functional screen; G-protein signaling modulator (GPSM) as named by the Human Genome Nomenclature Committee. The major sites of action of AGS proteins in the context of the G-protein activation–deactivation cycle are indicated **B** on the right. (*GPR* G-protein regulatory, *GEF* guanine nucleotide exchange factor, *GDI* guanine nucleotide dissociation inhibitor, *GPCR* G-protein coupled receptor)

with different mammalian Gα sequences (e.g., Gαs, Gαi2, Gαi3, Gα16) that efficiently couple to the yeast Gβγ, and introducing a reporter construct allowing for selection of active cDNAs via a simple growth assay. Mammalian Gα proteins were introduced in an attempt to bias screens of mammalian cDNAs toward the mammalian signaling component. Epistasis tests using other similarly modified yeast strains identified those cDNAs whose expressed proteins functioned at the heterotrimeric G-protein itself (Cismowski et al. 1999; Takesono et al. 1999; Cao et al. 2004). Screening of mammalian cDNA libraries derived from both normal and diseased tissues identified several activators of G-protein signaling, or AGS proteins, which fall into three broad classes: direct Gα activators, guanine nucleotide dissociation inhibitors (GDIs), and Gβγ-binding proteins (Fig. 3). With the exception of the group of guanine nucleotide dissociation inhibitors, the isolated AGS proteins did not share common structural motifs, but instead were functionally defined. Further experiments suggest that these classes of AGS proteins provide distinct and unexpected input into heterotrimeric G-protein signaling pathways.

AGS1-A Ras-related GEF

Full-length AGS1 (RASD1, DexRas1, GenBank accession #NM_016084) was isolated from a normal adult human liver cDNA library using the yeast-based functional screen (Fig. 2; Cismowski et al. 1999). AGS1 expression in the modified yeast strain activated Gαi heterotrimers, but not Gαs, Gα16, or native yeast heterotrimers, indicating a selective regulation of G-proteins themselves. Epistasis analysis confirmed AGS1 action at the heterotrimer itself. Of particular note is that AGS1 activation of Gαi signaling in yeast was eliminated in the presence of a Gαi2 mutant incapable of stably binding GTP (Gαi2-G204A), and it was antagonized by coexpression of RGS proteins that increase the GTPase activity of Gαi (RGS4 and RGS5), consistent with AGS1 directly facilitating GTP binding on Gα (Cismowski et al. 1999). Such studies provided a crucial insight into how AGS1 was functioning in the context of the G-protein signaling pathway.

Surprisingly, AGS1 is a Ras-related protein with all the conserved Ras-like motifs required for nucleotide binding/hydrolysis and membrane localization (Fig. 4). AGS1 also contains unique N- and C-terminal extensions not seen in the majority of Ras family members, as well as a short internal insert region (Fig. 4). Each of these extensions in AGS1 con-

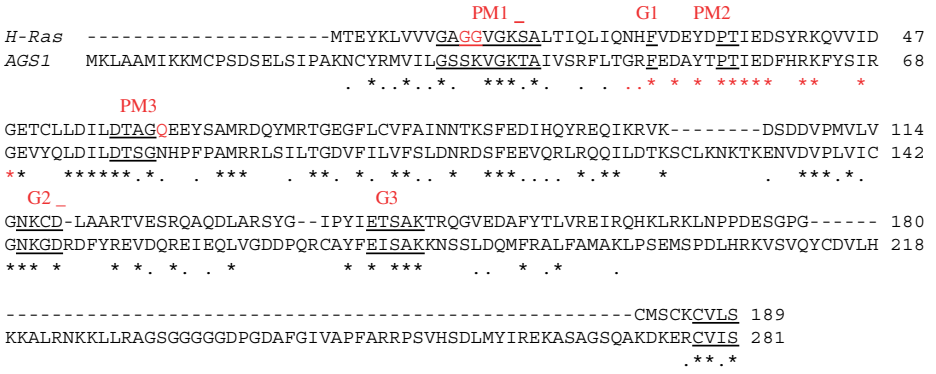

Fig. 4 Sequence alignment of H-Ras and AGS1. Human H-Ras and AGS1 were aligned using the ClustalW program (MacVector 7.0) and manual adjustment. The three conserved GDP/GTP binding motifs (G1–G3), phosphate–magnesium binding motifs (PM1–PM3) and *CAAX* motifs are *underlined*. Mutation of amino acids in *red* result in constitutively active Ras. The H-Ras effector domain is indicated by the *red alignment symbols*. The *asterisks* below the sequence indicate amino acid identity, whereas the *dots* indicate similarity. The *single red overlines* (S17, D119 in Ras) indicate residues that when mutated in Ras behave as a dominant negative.

tains numerous basic amino acids, and the C-terminal extension contains a glycine-rich domain. AGS1 function appears to require guanine nucleotide binding, as mutation of glycine residues required for stable P-loop formation (G31V or G36V; Saraste et al. 1990) renders AGS1 inactive (Cismowski et al. 1999). Nonmammalian AGS1 orthologs exist in zebrafish (AAH56272) and *D. melanogaster* (AAF50620; this entry has an additional 150 amino acids at the N-terminus, which possibly reflects incomplete genome annotation). The function of these candidate orthologs is unknown. Within the Ras superfamily, AGS1 is more closely related to Rap proteins and has some of the general structural features observed in κB-Ras, NOEY2, Rig, Rheb, and Rerg in terms of N-and C-terminus extensions.

Several amino acids in AGS1 within the nucleotide binding/hydrolysis motifs differ from their counterparts in Ras, and mutation of these residues in Ras leads to constitutive activity (Barbacid 1987; Feig and Cooper 1988), suggesting that AGS1 may be constitutively active. AGS1 is active in yeast-based assays without any apparent stimuli, and AGS1 purified from yeast extracts is largely GTP bound (Cismowski et al. 2000). It is presently unclear whether these amino acid variations are sufficient to render AGS1 constitutively active in mammalian cells or how posttranslational modifications of AGS1 influence its level of basal and stimulated activity.

AGS1 was initially isolated as a dexamethasone-inducible cDNA (Dexras1) in mouse AtT-20 corticotroph cells (Kemppainen and Behrend 1998) and subsequently as a blood-loss-inducible cDNA from rat kidney (Yen 1998) and as a binding partner of the adaptor proteins CAPON (Fang et al. 2000) and Nck-2 (Tu and Wu 1999). AGS1 was also identified as an mRNA that cycles with the circadian rhythm (Takahashi et al. 2003). A closely related human homolog of AGS1 (Ras homolog enriched in striatum, Rhes; RASD2; tumor endothelial marker 2, TEM2; NM_014310) with 64% amino acid identity to AGS1 was identified as a thyroid-hormone-inducible gene (Falk et al. 1999; St. Croix et al. 2000; Vargiu et al. 2001). Though AGS1 and Rhes are a distinct branch within the Ras superfamily of small G-proteins, Rhes does not function as a Gαi activator in the yeast screen (Cismowski, unpublished observation).

Influence of AGS1 on G-protein signaling in mammalian systems

As suggested from the studies on the yeast functional screen, purified AGS1 does indeed function as a guanine nucleotide exchange factor for Gαi/o, activating both free and heterotrimeric Gα subunits (Cismowski et al. 2000). In contrast to the GTP-bound AGS1 observed with expression in yeast (Cismowski et al. 2000), AGS1 expressed in mammalian cells appears to be mainly GDP bound (Fang et al. 2000; Graham et al. 2001). This observation suggests that there are mammalian-specific proteins that may directly regulate the nucleotide binding and/or hydrolysis properties of AGS1. Mutation of A178 to V in AGS1, part of the conserved SAK motif in Ras implicated in nucleotide hydrolysis, was reported to render AGS1 constitutively active (Graham et al. 2001), though this mutation did not greatly alter the in vivo GDP/GTP binding ratio of AGS1. Transiently transfected AGS1 specifically activates the extracellular signal-regulated kinase ERK1/2 pathway in mammalian cells, and this activation is blocked by pertussis toxin pretreatment or cotransfection with either GαT or the C-terminus of β-arrestin kinase (Cismowski et al. 2000; Graham et al. 2002), both of which sequester released Gβγ. AGS1 expression also blocks ADP ribosylation of Gαi by pertussis toxin and inhibits cAMP accumulation in response to forskolin or a constitutively active Gαs (Graham et al. 2002, 2004). These data are consistent with AGS1 functioning in a manner similar to that of a Gαi-coupled GPCR. In AtT-20 cells, the A178V mutant of AGS1 inhibits both basal and cAMP-dependent peptide hormone secretion, and this regulation appears to depend on C-terminal prenylation of AGS1 at the conserved CAAX site found at the carboxyl terminus of several Ras proteins (Graham et al. 2001).

However, AGS1 somewhat surprisingly blocks signaling through an agonist-activated GPCR when transiently cotransfected with a Gαi-coupled GPCR (Takesono et al. 2002; Graham et al. 2002). A similar disruption of GPCR activation of a G-protein-mediated event was observed for Rhes except that Rhes modified GPCR coupling to Gαs, but not Gαi, and this disruption of receptor coupling was blocked by inhibition of phosphatidylinositol-3 kinase (PI3K; Vargiu et al. 2004). The mechanism of inhibition of GPCR-mediated signaling by AGS1 is unclear. Via interaction of AGS1 with G-proteins, AGS1 may limit access of a GPCR to G-proteins, or it may initiate a signal reducing the efficiency of receptor–G-protein coupling.

AGS1 may also mediate signal input to heterotrimeric G-proteins following activation of cell surface receptors other than the typical GPCR, and it is implicated in N-methyl-D-aspartate (NMDA) receptor signaling via the adaptor protein CAPON and neuronal nitric oxide synthase (nNOS). AGS1 forms a trimeric complex with CAPON and nNOS in rat brain (Fang et al. 2000); this association is postulated to facilitate the S-nitrosylation of AGS1 at cysteine-11 and to increase guanine nucleotide exchange on AGS1 (Fang et al. 2000; Jaffrey et al. 2002). This provides a potentially interesting mechanism for AGS1 activation, and AGS1 may mediate the activation of ERK1/2 by NMDA, which is pertussis-toxin-sensitive and thus involves heterotrimeric Gαi/Gαo (Chandler et al. 2001).

In addition to its ability to regulate heterotrimeric G-proteins, AGS1 may have cellular functions independent of G-proteins that involve effector systems analogous to those regulated by other Ras-related proteins. The core effector domain of Ras (^{28}FVDEYDPTIEDSY^{40}) is similar to the corresponding region in AGS1 and Rhes (Fig. 5), although there are notable differences. AGS1 and Rhes may interact with Ras effectors such as Raf, RalGDS, phospholipase Cϵ (PLC), and PI3 kinase, although the substitution of SY in Ras for FH may alter affinities (Herrmann et al. 1996; Winkler et al. 1997; Pacold et al. 2000; Fiordalisi et al. 2002; Herrmann 2003; Nicholas Nassar, SUNY-Stony Brook, personal communication). The AGS1-related protein Rhes actually binds the Ras-binding

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H-Ras   26 N H F V D E Y D P T I E D S Y R K Q V V I D G 48
AGS1    47 G R * E * A * T * * * * * F H * * F Y S * R * 69
Rhes    54 G R * E * Q * T * * * * * F H * * V Y N * H * 76
```

Fig. 5 Alignment of the effector domains H-Ras, AGS1, and Rhes. The *asterisks* indicate identity

domain of PI3K but not that of Raf (Vargiu et al. 2004). A patent issued on AGS1 as a hypoxia-induced cDNA presents data indicating that AGS1 does not bind the Ras-binding domain of Raf (Yen 1998).

Long-term AGS1 expression in some, but not all, cell types causes growth inhibition, and AGS1 expression has prevented the development of tumors in athymic nude mice (Vaidyanathan et al. 2004). These effects appear to be independent of heterotrimeric Gαi-protein because they were not blocked by pertussis toxin. AGS1-mediated regulation of effectors influencing cell growth parameters such as proliferation, senescence, apoptosis, and/or terminal differentiation would be consistent with functions commonly associated with Ras family members (McCormick 1995; Vos et al. 2000; Agell et al. 2002; Cox and Der 2003). The identification of additional AGS1 binding partners that serve as effectors or regulators of nucleotide binding or hydrolysis will facilitate our understanding of the functional role of AGS1.

Role of AGS1 in cell and organ function

AGS1 mRNA is widely distributed in human and rat tissues (Tu and Wu 1999; Fang et al. 2000; Vaidyanathan et al. 2004), although AGS1 protein appears enriched in rat brain relative to other rat tissues (Fang et al. 2000). Of note are the observations of dramatic tissue-specific upregulation of AGS1 by dexamethasone in cultured cells and the intact animal (Kemppainen and Behrend 1998; Tu and Wu 1999; Brogan et al. 2001; Kemppainen et al. 2003), the downregulation of AGS1 (RASD1) in breast ductal carcinoma in situ (Abba et al. 2004), and changes in AGS1 mRNA that occur with blood loss (Yen 1998) and during the circadian cycle in specific brain areas (Takahashi et al. 2003). AGS1 is implicated in a diverse set of cellular functions, and the multiple observations regarding the transcriptional regulation of AGS1 indicate a potentially significant role in signal regulation in response to physiological control mechanisms and/or pathophysiological settings. The dramatic upregulation of AGS1 expression by glucocorticoid treatment and/or blood loss suggests the role of AGS1 in the cellular response to stress. Because glucocorticoids regulate multiple cellular functions, including cell growth, differentiation, and secretion, it is tempting to speculate that some of their actions (i.e., growth inhibition) may be mediated through upregulation of AGS1 (Vaidyanathan et al. 2004; Graham et al. 2001).

Recent studies in mice with an AGS1 gene disruption provide a functional link to the previously suggested role of AGS1 in NMDA signaling and emphasize the potential importance of the transcriptional regulation of AGS1 (Cheng et al. 2004). AGS1 is one of a handful of mRNAs in suprachiasmatic nuclei that cycle with the circadian rhythm (Takahashi et al. 2003). Targeted disruption of AGS1 in the mouse does not appear to affect viability or reproduction, but mice lacking AGS1 exhibited altered regulation of the circadian cycle, and this appears to involve a specific aspect of the cycle regulated by glutamate and neuropeptide Y (Cheng et al. 2004). These data suggest that AGS1 is mediating signal input from NMDA and/or neuropeptide Y receptors to Gαi/Gαo with subsequent activation of ERK1/2. However, as transcriptional regulation of AGS1 may provide important discrete signals for AGS1 function in development and general organ function, system compensation in response to the

absence of AGS1 in these mice may complicate a full understanding of AGS1 function in the animal, and conditional manipulation of AGS1 expression will be an important area for further studies.

AGS3-6—a family of Gαi/Gαo-specific guanine nucleotide dissociation inhibitors

AGS3 or G-protein signaling modulator (GPSM) 1 (GenBank accession #NM_144745), the first identified member of this group, was isolated from the neuroblastoma–glioma hybrid cell line NG108 using a functional screen in a yeast strain expressing Gαi2 sequences in place of the yeast Gα (Cismowski et al. 1999; Takesono et al. 1999). Subsequent screens in the yeast-based system identified AGS4 (GPSM3, GenBank accession #NM_022107), AGS5 (LGN, GPSM2, GenBank accession #NM_013296), and AGS6 (RGS12, GenBank accession #NM_198229). AGS5 or LGN was first identified in Dr. Insel's laboratory as a binding partner of Gαi (Mochizuki et al. 1996), and the ortholog of AGS3 and AGS5 (LGN) is termed PINS in *D. melanogaster* and AGS3.1 in *C. elegans*. AGS4 is identical to G18.1b (160 amino acids, molecular weight 17,864), a protein encoded by a gene within the major histocompatibility complex (MHC) class III region (chromosome 6-open reading frame 9), containing three G-protein regulatory (GPR) motifs (Cao et al. 2004; Kimple et al. 2004). The MHC class III region contains genes that encode proteins of diverse function and is of particular interest in terms of disease susceptibility. BLASTP searches did not reveal any obvious candidate AGS4 orthologs in *C. elegans*, *S. cerevisiae*, or *D. melanogaster*.

Like AGS1, subsequent tests in yeast demonstrated that AGS3-5 functioned at the level of the Gαi2 heterotrimer and were specific for Gαi proteins. However, AGS3 and other members of this group were distinguished from AGS1 in yeast in their ability to function in the presence of either the Gαi2–G204A mutant or coexpressed RGS4 (Takesono et al. 1999), strongly suggesting a mechanism of action distinct from that of AGS1. Members of this group of AGS proteins were also distinguished from AGS1 by their selective interaction with the GDP-bound conformation of Gαi (Takesono et al. 1999; Bernard et al. 2001; Cao et al. 2004).

AGS3 and AGS5 are "mosaic" proteins containing multiple repeats of two distinct domains—7 N-terminal repeats of a tetratricopeptide (TPR) motif and 4 C-terminal repeats of ~20 amino acids termed the GPR or GoLoco motif (Fig. 6; Takesono et al. 1999; Siderovski et al. 1999). The GPR motif functions as a docking site for Gαi. Proteins with GPR domains were also identified by virtue of their ability to interact with Gαi, Gαo, and/or Gαz proteins (Fig. 6; Mochizuki et al. 1996; Luo and Denker 1999; Meng et al. 1999; Jordan et al. 1999). A single GPR motif is found in the GTPase-activating proteins RGS12 and RGS14, providing an interesting platform for dual regulation of Gα subunits during the G-protein activation–deactivation cycle (Snow et al. 1998; Cho et al. 2000; Hollinger et al. 2001, 2003; Kimple et al. 2001; Mittal and Linder 2004; Traver et al. 2004).

The AGS3, AGS5, and AGS6 isolates obtained from the yeast screen were N-terminally truncated, but partial cDNAs each contained at least one GPR domain that was required for bioactivity. AGS4 was isolated as a full-length cDNA. Intriguingly, full-length AGS3, though expressed in yeast, was unable to activate Gαi signaling, suggesting a regulatory role for the N-terminal TPR domains. Although the exact nature of this putative regulation is currently unknown, TPR motifs are important for protein–protein interactions (Blatch and Lassle 1999; D'Andrea and Regan 2003). The TPR domains of AGS3 and AGS5 (LGN) interact with potentially critical regulatory proteins (see below) and also are an important

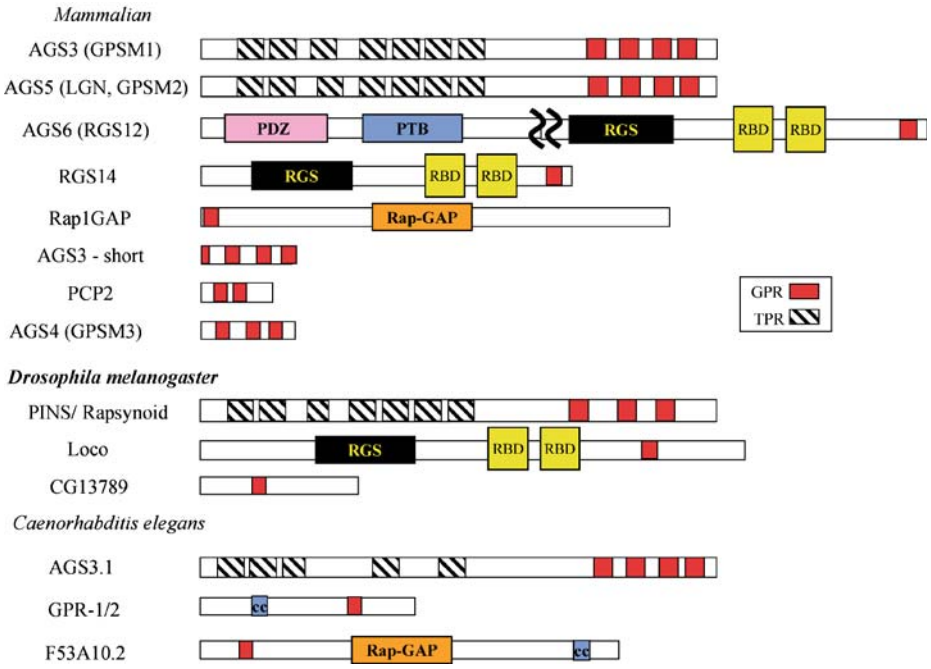

Fig. 6 Proteins with GPR motifs. (*RBD* Ras binding domain, *PTB* phosphotyrosine binding domain, *RGS* regulator of G-protein signaling domain, *PDZ* PSD95/DLG/ZO-1 domain, cc coiled-coil domain). This figure is adapted from the thesis of Dr. Yuri Peterson

determinant of their subcellular location Pizzinat et al. 2001; Du et al. 2001; Kaushik et al. 2003; Pattingre et al. 2003). Therefore in the yeast assays, the lack of activity for full-length AGS3 may be due to its sequestration away from heterotrimeric G-protein, or the protein may assume a conformation in which the GPR domains are masked by intramolecular interaction with the TPR domain of the protein (Du and Macara 2004). As is the case for AGS3 and AGS5, most other GPR-motif-containing proteins have additional domains that may serve a regulatory or additional function (Fig. 6).

Influence of GPR proteins on G-protein signaling

Further study of proteins containing GPR domains as well as isolated GPR domain sequences has indicated that these domains function as GDIs, inhibiting GDP release from Gαi/Gαo and transducin (Peterson et al. 2000; Natochin et al. 2000, 2001; DeVries et al. 2000; Kimple et al. 2001, 2002; Ghosh et al. 2003; Kaushik et al. 2003). Therefore, the function of AGS3 and other GPR proteins as activators of signaling in the yeast assay is likely due to their ability to increase the amount of yeast Gβγ free of GDP-bound Gαi2. The conserved residues within the GPR motif are indicated in Fig. 7, and key residues in the GPR domains of AGS3 required for GDI function have been identified (Fig. 7; Peterson et al. 2000, 2002; Bernard et al. 2001; Adhikari and Sprang 2003; Ja and Roberts 2004). Most of the data from multiple laboratories indicate a selectivity of known GPR proteins for Gαi versus Gαo, although the relative affinities for the two subgroups of G-proteins may differ among individual GPR proteins, and Mittal and Linder (2004) have reported the selective

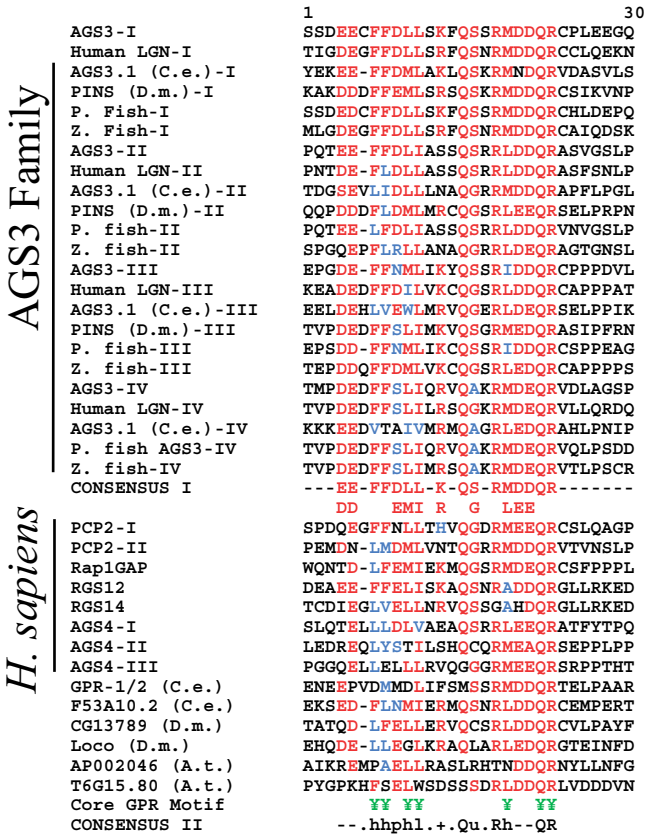

Fig. 7 Sequence alignment of GPR motifs. Consensus amino acids are *red*; chemically similar amino acids are *blue*. Consensus I refers to the conserved sequence defined with the family of AGS3-related proteins. Consensus II refers to the conserved sequence defined with all known GPR proteins. Consensus II amino acid groupings are .=any, +=positive (HKR), –=negative (DE), h=hydrophobic (ACFILMVWY), u=tiny (AGS), p=polar (CDEHKNQRST), and l=aliphatic (ILV). (*C.e. C. elegans*, *D.m. D. melanogaster*, *A.t.. A. thaliana*) The core GPR motif is as defined in Peterson et al. 2002. This figure is adapted from the thesis of Dr. Yuri Peterson.

interaction of the RGS14 GPR motif with Gαi1 and Gαi3. Further studies of G-protein selectivity for different GPR motifs should be an area of fruitful investigation. Whereas G-protein selectivity is observed with the core GPR motif (Peterson et al. 2000, 2002), residues outside the core GPR motif also influence G-protein selectivity, and one component of this selectivity may be achieved by interaction of such regions with the helical domain of Gα proteins (Kimple et al. 2002; Adhikari and Sprang 2003; Mittal and Linder 2004). It is unknown whether other proteins with GPR-like activity have evolved to subserve similar regulatory functions for G-proteins other than Gαi and Gαo. As we gain further understanding into the structural aspects of GPR domain interaction with Gαi and Gαo, it may be possible to design peptides or small molecules that elicit a similar effect on Gαs, Gαq, or other G-proteins.

The identification of proteins that function as GDIs for heterotrimeric G-proteins raises intriguing possibilities regarding cellular roles in both the context of classical GPCR signaling mechanisms and receptor-independent G-protein signaling mechanisms for mammalian systems. In glutathione S-transferase (GST) pull-down assays, AGS3 GPR domains com-

plex with Gαi that is free of Gβγ, and brain AGS3 is coimmunoprecipitated with Gαi3 in a GDP-dependent manner, suggesting that the GPR motif either promotes subunit dissociation independent of nucleotide exchange or that there is a population of Gα that exists free of Gβγ (Fig. 8; Takesono et al. 1999; Bernard et al. 2001; Ghosh et al. 2003). GPR motifs may also interact with Gα–GDP generated during the course of the typical G-protein activation–deactivation cycle and prevent reassociation of Gα–GDP with Gβγ, which would possibly facilitate or increase the duration of Gβγ-mediated signaling events and perhaps participate in cellular adaptations to prolonged activation of Gαi-coupled GPCRs (Watts 2002). Regeneration of a functional heterotrimer would then require dissociation of the GPR protein from GDP–Gα prior to rebinding of Gβγ; it is interesting to speculate that an unknown guanine nucleotide exchange factor may act on the GPR–Gα–GDP complex to facilitate this step (Fig. 8b). In the context of GPCR signaling, the loss of available heterotrimer for receptor coupling may effectively dampen agonist-induced signals, although it is likely to be system dependent (Kinoshita-Kawada et al. 2004; Webb et al. 2005). The impact of AGS3-6 on signaling in standard transfection systems in mammalian systems has not yet provided much insight into these points, indicating the importance of context-specific experimental strategies. Nevertheless, Rap1GAP, which contains one GPR motif, is clearly involved in Gαi- and/or Gαz-mediated signaling events (Meng et al. 1999; Jordan et al. 1999; Mochizuki et al. 1999; Meng and Casey 2002; Lova et al. 2003; Weissman et al. 2004).

Another intriguing scenario is the existence of GPR–Gαi complexes independent of the classic Gαβγ heterotrimer. In both rat brain and model organisms, GPR proteins are coimmunoprecipitated with Gα consistent with the generation of such a complex (Fig. 8a). The GPR–Gαi complex might itself serve as a signaling entity. A more likely scenario is that the GPR–Gα–GDP dimer may be subject to regulation by a guanine nucleotide exchange factor in a manner similar to that of a GPCR acting on Gαβγ (Fig. 8c).

Additional questions to be addressed in the field are the functional impact of binding multiple Gα subunits in proteins with more than one GPR motif and the role of GPR motif in proteins like RGS12 and RGS14 that contain both a GPR motif and the RGS domain responsible for accelerating GTP hydrolysis by Gα. The stabilization of GDP–Gαi following GTP hydrolysis would also contribute to enhanced signaling through effectors regulated by Gβγ signaling pathways (Kimple et al. 2001; Hollinger et al. 2003; Traver et al. 2004). GPR and RGS domains in RGS14 exhibit cooperativity in downregulating Gαi activity, but enhancement of Gβγ signaling was not seen under the conditions tested (Traver et al. 2004). The carboxyl portion of AGS3 contains four GPR motifs, and up to four Gαi–GDP molecules can be simultaneously complexed with the protein (Bernard et al. 2001; Adhikari and Sprang 2003). It will be of great interest to define the structural arrangement of the Gαi subunits in this scaffold.

GPR proteins in the context of a signal complex

One major control mechanism for the signaling role of proteins containing GPR motifs is the regulation of the subcellular location of such proteins. This is illustrated by elegant studies in the model organisms *D. melanogaster* and *C. elegans*, the relocation of AGS5/LGN in neuronal cells exposed to NMDA, and the localization of AGS5/LGN to spindle poles and the midbody (Miller and Rand 2000; Parmentier et al. 2000; Yu et al. 2000; Schaefer et al. 2000, 2001; Bellaiche et al. 2001; Du et al. 2001; Blumer et al. 2002; Kaushik et al. 2003). The additional motifs present on many of the GPR proteins (for example, TPR, PTB and PDZ domains; Fig. 6) may serve as binding scaffolds for Gα complexes that include either

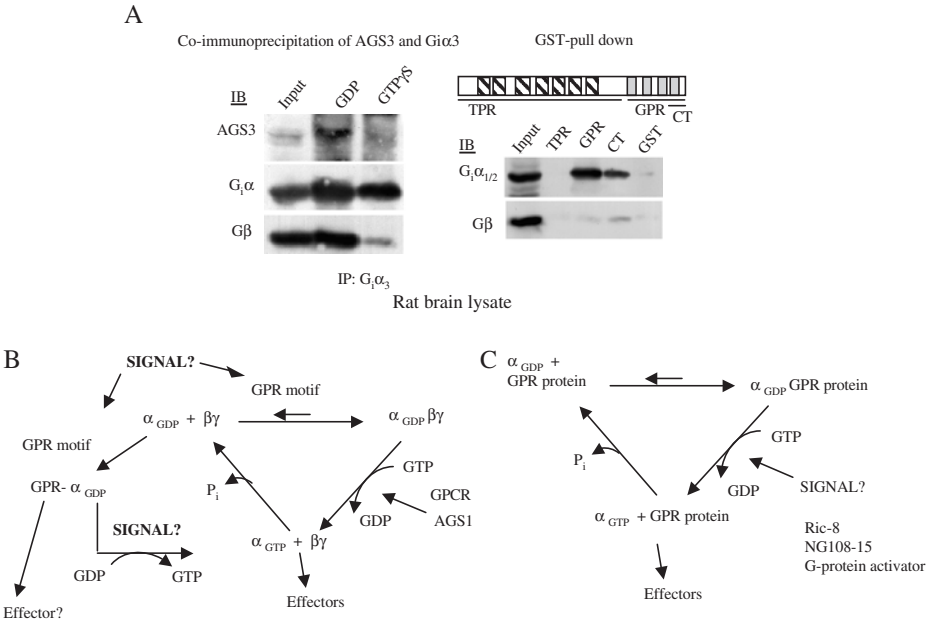

Fig. 8 Interaction of AGS proteins with G-proteins and potential influence on the G-protein activation and deactivation cycle. **A** Interaction of AGS3 and Gαi3. Left: Rat brain (2.5 mg) lysates were preincubated with 30 μM GDP or 30 μM GTPγS/25 mM $MgCl_2$ at 24°C for 30 min. Lysates were then precleared and Gαi3 immunoprecipitated as described (Bernard et al. 2001). Membrane transfers were first blotted with AGS3 P-32 antisera and then stripped and sequentially reprobed with Gαi and Gβ antisera. Input lanes contained 5 μl of the NP-40 lysate used for immunoprecipitation. (*IP* immunoprecipitation, *IB* immunoblot) **B** Right: Glutathione S-transferase (*GST*) pull-down assays of G-proteins from rat brain lysates. GST fusion proteins containing the tetratricopeptide repeat (*TPR*) domain or GPR domain or the one GPR domain at the carboxyl terminus (*CT*) were used in pull-down assays as described (Bernard et al. 2001). **B**, **C** Two scenarios illustrating how proteins containing GPR motifs may regulate G-protein signaling. This figure is adapted from Bernard et al. 2001 and Blumer and Lanier 2003. Refer to text for additional details.

nucleotide exchange factors or other regulatory proteins. Several recent lines of investigation suggest that this may indeed be the case, providing intriguing clues about the different cellular functions of GPR-containing proteins. The TPR domains of AGS3 anchor it along with GDP-bound Gαi3 on the endoplasmic reticulum (ER) of the intestinal cell line HT-29 (Pattingre et al. 2003, 2004), where it may influence the regulation of macroautophagy by Gαi3 (Ogier-Denis et al. 1995, 1996, 1997). A second example of regulated subcellular localization of GPR proteins is provided by the interaction of the *D. melanogaster* AGS3/LGN ortholog PINS (partner of inscuteable) with inscuteable, which assembles a complex of the proteins with Gα at the cell cortex and is involved in asymmetric cell division (Miller and Rand 2000; Parmentier et al. 2000; Yu et al. 2000; Schaefer et al. 2000, 2001; Bellaiche et al. 2001). A related but distinct example is illustrated by the localization of AGS5/LGN to the spindle poles and the midbody in nonasymmetrically dividing cells (Du et al. 2001; Blumer et al. 2002), suggesting a conservation of GPR–Gα function in controlling mitosis and cytokinesis in higher eukaryotes. AGS5/LGN is targeted to the spindle poles by interaction with the nuclear mitotic apparatus protein (NuMA), which binds to the TPR domains of AGS5/LGN and influences the interaction of the GPR domains with G-proteins (Du et al. 2001; Du and Macara 2004).

LKB1, a serine/threonine-specific kinase, was also identified as a binding partner for the TPR domains of AGS3 (Blumer et al. 2003), suggesting that phosphorylation may be another control mechanism for influencing GPR interactions with G-protein and providing a regulatory mechanism for signal input or subcellular location. Both endogenous AGS3 and AGS5/LGN are phosphorylated (Blumer et al. 2003), although it is not clear what may regulate this phosphorylation within the cell. LKB1 is involved in the regulation of cell cycle progression and polarity (Spicer and Ashworth 2004) and is suggested to form a complex in mammalian cells with AGS3 and Gα, leading to phosphorylation of the GPR domains of AGS3 (Blumer et al. 2003). In vitro phosphorylation of the GPR domain in LKB1 immunoprecipitates from COS-LKB1 transfectants was inhibited by the addition of a consensus GPR peptide, and the introduction of a phosphate moiety on a serine within the GPR motif reduced the apparent ability of the GPR peptide to alter GTPγS binding to purified G-protein (Blumer et al. 2003). Although not yet fully developed, the concept that phosphorylation of the GPR motif would influence its interaction with Gα is an attractive regulatory mechanism. Indeed, endogenous AGS4 was determined to be phosphorylated at S59 (Zarling et al. 2000), which is just upstream of the first GPR motif and analogous to the site of phosphorylation in RGS14 upstream of the GPR motif, where it was suggested to influence the affinity of the interaction of the GPR motif with G-protein (Hollinger et al. 2003).

Tetratricopeptide repeat domains are clearly important docking sites for binding proteins and can also dimerize with other TPR domains. It is interesting to note that TPR domains in Ser/Thr protein phosphatase type 5 interact with Gα12 and Gα13 (Yamaguchi et al. 2002), whereas TPR1, which contains three TPR motifs, was isolated in a yeast 2-hybrid screen with Gα16 and was also shown to bind Ha-Ras in a conformation-dependent manner (Marty et al. 2003). Finally, it is also likely that there are other regulatory proteins that interact with the GPR domains of AGS3-6, and this will be a fruitful area for further investigation.

Role of GPR proteins in cell and organ function

With the identification of unexpected cellular roles for the GPR family of proteins, intriguing clues are emerging regarding the functional role of these proteins in various tissues (Fig. 9). Whereas AGS3 expression is tissue-specific, AGS5/LGN is widely expressed (Blumer et al. 2002). A distinct variant of AGS3 lacking the TPR domains is enriched in heart, whereas full-length AGS3 is enriched in brain (Takesono et al. 1999; Pizzinat et al. 2001). Expression of this short cytosolic form of AGS3 in heart is developmentally regulated, although its role in G-protein signaling in the myocardium is not yet determined. AGS5 (LGN) in retina is postulated to play a role in regulating phototransduction through its interaction with the G-protein transducin (Kerov et al. 2005; Nair et al. 2005).

One of the most extensively studied functional roles for GPR proteins relates to asymmetric cell division and the generation of daughter cells of different size and fate at early stages of development (Knoblich 2001; Arhinger 2003; Willard et al. 2004). Both *D. melanogaster* (PINS) and *C. elegans* (AGS3.1) have AGS3/AGS5 orthologs, and *C. elegans* has two additional proteins with a single GPR domain termed GPR1/2. A series of experiments in each organism demonstrated that asymmetric cell division was regulated by either PINS or GPR1/2 and that GPCR signal input was not required for this regulation (Srinivasan et al. 2003; Gotta et al. 2003; Colombo et al. 2003; Couwenbergs et al. 2004; Afshar et al. 2004; Hess et al. 2004). Recent studies in *C. elegans* indicate that GPR1/2, Gαi/Gαo subunits, a guanine nucleotide exchange factor (GEF; Ric-8), and a GTPase activating protein (RGS7) control a dynamic, segregated series of signaling events that differentially control

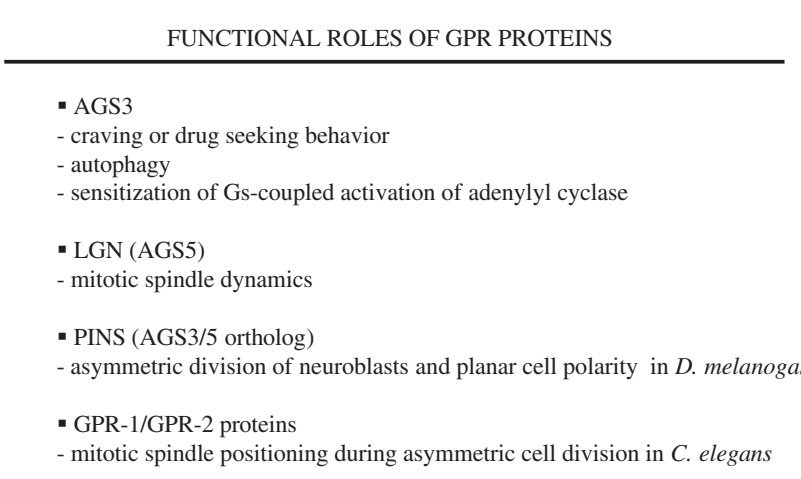

Fig. 9 Functional roles of GPR proteins. Details and references are provided in the text.

pulling forces on the mitotic spindle (Couwenbergs et al. 2004; Afshar et al. 2004; Hess et al. 2004). Although the exact sequence of events in terms of signal transfer is not fully worked out, the precise integration of signals from each protein is critical for the generation of daughter cells of different sizes. Genetic screens in *C. elegans* identified Ric-8 as an important component of G-protein signaling in the nervous system (Miller and Rand 2000; Reynolds et al. 2005; Schade et al. 2005). As discussed earlier, the placement of GPR proteins within the cell is an important regulatory step for these events. Of note is that RGS14, which contains one GPR motif, can also localize to centrosomes and that disruption of the RGS14 in mouse is lethal (Martin-McCaffrey et al. 2004; Cho et al. 2005). Further understanding of this fascinating and unexpected functional role of G-proteins will be greatly facilitated by the identification of downstream signaling mechanisms involved in the control of spindle pulling forces. GPR-containing proteins may play similar roles in symmetric cell division in mammalian systems and have a functional impact in terms of stem cell propagation as well as the general control of cell proliferation, and they may be related to recognized roles of G-proteins in development (Malbon 1997).

In addition to their expression at early stages of development, AGS3 and LGN are also clearly expressed in the mature organism. Several laboratories have focused on defining the role of GPR proteins in this context. Due to their ability to provide unique regulatory control of the G-protein activation–deactivation cycle, GPR proteins such as AGS3, which is enriched in brain tissue, are of interest for various behavioral adaptations that involve subtle changes in G-protein signaling within the central nervous system. Indeed, overexpression of AGS3-GPR blocks the sensitization of adenylyl cyclase observed with prolonged activation of a Gα-coupled GPCR (Sato et al. 2004). As part of a broader effort to define the role of altered G-protein signaling in the synaptic plasticity associated with addictive behavior, Bowers et al. (2004) examined the expression of GPR proteins in a rat model of drug-seeking behavior. AGS3 is upregulated in the prefrontal cortex during withdrawal from repeated cocaine administration, and blockade of this increase with antisense oligonucleotides prevented the phenomenon of craving observed with cocaine withdrawal. Of particular note relative to therapeutic manipulation of this signaling pathway was the observation that a membrane-permeable GPR peptide was biologically active and mimicked the behavioral adaptations associated with this animal model. Recent studies by Yao et al.

(2005) similarly implicate AGS3 expressed in the nucleus accumbens in positively regulating heroin seeking behavior through enhancing Gβγ.

AGS2, AGS7, AGS8—a new family of Gβγ-interacting proteins

Although the yeast functional screens used to identify activators of G-protein signaling were designed with a bias toward Gα activators, several isolated cDNAs appeared to be functioning directly through interaction with the yeast Gβγ. As the proteins encoded by these cDNAs bind mammalian Gβγ in vitro (Takesono et al. 1999), a third class of AGS proteins can be defined by their apparent ability to facilitate Gβγ signaling and exclude Gα binding. It is not clear how these signals are processed or whether such proteins actually promote subunit dissociation or prevent rebinding of Gβγ to Gα during basal cycling of the heterotrimer. In either situation, the interaction of these proteins with yeast Gβγ occurs in a manner that still allows effective coupling to downstream effectors.

AGS2, isolated from the screen of a neuroblastoma–glioma NG108 cDNA library, is identical to mouse Tctex1, a light chain component of both the cytoplasmic motor protein dynein and ciliary dynein (Harrison et al. 1998). The interaction of AGS2 with Gβγ suggests a potential regulatory role of Gβγ in dynein function. The demonstration that AGS2/Tctex1 also interacts with multiple signaling molecules, including GPCRs (Campbell et al. 1998; Nagano et al. 1998; Tai et al. 1999; Mueller et al. 2002; Sugai et al. 2003), suggests that cytoplasmic dynein may function to organize signaling complexes including heterotrimeric G-proteins that regulate organelle movement within the cell. Although there is no evidence on this point, there may be a role for this mode of signal input to dynein in the context of the role of GPR proteins concerning spindle pulling forces (Merdes et al. 2000; Gaetz and Kapoor 2004; Hughes et al. 2004).

AGS7 was isolated from a prostate leiomyosarcoma cDNA library and contains a putative ATP-binding domain. AGS8 was isolated from a rat heart model of transient ischemia. Little else is known about the function of these newly identified proteins. Although the role of these Gβγ-binding proteins in mammalian G-protein signaling has not been established, it is possible that they serve either as direct effectors of cell surface Gβγ or as binding partners for a population of Gβγ functioning independent of Gα or GPCRs. Further characterization of this class of AGS protein and identification of other members of this class will undoubtedly shed light on the mechanisms of Gβγ-driven signal transduction. It is unknown whether there is selectivity of such AGS proteins for different types of Gβγ or whether they all interact with Gβγ in the same manner. Recent studies regarding unexpected control mechanisms for Gβγ and its interaction with effectors have further increased the interest in this class of AGS proteins (Scott et al. 2001; Goubaeva et al. 2003; Bonacci et al. 2005).

Conclusions

Accessory proteins that interact with and regulate G-proteins offer unexpected and surprising mechanisms for signal input to G-protein signaling systems. Perhaps of even broader impact is that the discovery of AGS proteins has provided the foundation for the concept that heterotrimeric G-protein subunits are processing signals within the cell involving intrinsic cues that do not involve the classical signal input from a cell surface GPCR. Several questions are currently being addressed in the field. There is interest in the structural aspects

of the interaction of AGS proteins with G-protein subunits. In addition, whereas the subcellular location of AGS proteins is clearly important for their function, key questions remain as to what provides the initial signal input to these proteins. If AGS proteins are critical for regulating G-protein signaling events not typically associated with a GPCR, what are the downstream effectors for such a signaling pathway? As more information is gathered regarding the role of these proteins in different disease states, it is likely that, by virtue of their tissue-restricted expression, selected AGS proteins may be candidate targets for therapeutics targeting G-protein signaling at a postreceptor level.

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