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CHAPTER 1

Heterologous Protein Secretion by *Bacillus* Species: From the Cradle to the Grave

Susanne Pohl and **Colin R. Harwood**[1]

Contents

Abstract

The Gram-positive bacterium *Bacillus subtilis* and some of its close relatives are widely used for the industrial production of enzymes for the detergents, food, and beverage industries. The choice of these organisms is based almost exclusively on the high capacity of

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their secretion systems that are, under the right conditions, able to secrete proteins at grams per liter concentrations. In contrast, there are relatively few examples of *Bacillus* species being used for the cytoplasmic production of proteins.

The range of proteins that are capable of high-level production and secretion is limited by a combination of characteristics of both the target protein and the host bacterium. The secretion pathway includes checkpoints that are designed to validate the authenticity of pathway substrates. Although many of these checkpoints are known, only some can be overcome by reengineering the host. As a result, the yield of heterologous protein production is extremely variable. In this review, we consider the *Bacillus* protein secretion pathway from the synthesis of the target protein (cradle) to its emergence at the outer surface of the complex cell wall (grave), and discuss the roles of the various checkpoints both with respect to the target protein and their role on cell homeostasis.

I. INTRODUCTION

Members of the genus *Bacillus* are prodigious producers of industrial enzymes such as proteases, α-amylases, and other macromolecular hydrolases (Harwood, 1992). This reflects the fact that, in their natural habitat, the ability to breakdown and utilize soil detritus—particularly that derived from plants (protein, starch, pectin, cellulose, etc.)—provides important sources of nutrients. Because bacteria generally do not take up macromolecules, *Bacillus subtilis* and its close relatives naturally secrete a wide range of hydrolytic enzymes into their environment. The ability of this Gram-positive bacterium to secrete proteins directly into the culture medium at high concentrations is, in part, a reflection of the fact that its cytoplasm is surrounded by a single membrane system, in contrast to the double membrane found in Gram-negative bacteria. From a commercial point of view, the purification of proteins from the culture medium rather than from the cytoplasm is considerably more cost-effective, often leads to improved structural authenticity and reduces the likelihood of the co-purification of endotoxins and other potential contaminants. Given the commercial advantages of their secretory systems, it is surprising that more use has not been made of *Bacillus* species for the production of heterologous proteins. In practice, attempts to use this group of bacteria for the manufacture of heterologous proteins have met with mixed success. The reasons for this are complex, but relate to the intrinsic properties of both the target proteins and the secretion systems themselves. In particular, given the importance of protein secretion to cell growth and integrity, the native secretion systems include a series of quality control checkpoints designed to avoid potentially lethal blockages.

II. EXPRESSION AND SECRETION VECTOR SYSTEMS

A wide variety of expression systems have been described for *Bacillus* species and these have to be combined with targeting sequences for proteins that are required to be secreted. Both self-replicating (mono- and bi-functional) and integrating plasmids have been developed, although the former is necessary for the highest levels of protein production.

Originally, *Bacillus* cloning vectors were based on *Staphylococcus aureus* antibiotic resistance plasmids, such as pUB110 (Km^R), pT181 (Tc^R), and pC194 (Cm^R). These mono-functional plasmids, and the vectors derived from them, replicate using the rolling circle mode of replication and consequently tend to accumulate single-stranded replication intermediates and to suffer from both structural and segregational instability (Bron, 1990; Gruss and Ehrlich, 1989). The identification of theta replicating plasmids, such as pAMβ1 and BS72, led to the development of a newer generation of *Bacillus* vectors and *Escherichia coli–Bacillus* bifunctional vectors that are both segregationally and structurally stable (Bruand *et al.*, 1991; Jannière *et al.*, 1993; Titok *et al.*, 2003). A good example is pMTLBS72, which contains the replication origins of pBR322 for maintenance in *E. coli*, and pBS72 for maintenance in *B. subtilis*. Like all bifunctional vectors, they require antibiotic resistance genes that are selectable in both hosts.

An alternative to autonomously replicating plasmid vectors is to use integration vectors, exploiting the high frequency of recombination between homologous DNA sequences in many widely used strains of *B. subtilis*. For the most part, integrated vectors are stable provided the site of integration is carefully chosen. *B. subtilis* integration vectors are based solely on *E. coli* origins of replication (usually *colE1*-based) since they use regions of homology between the vector and the host chromosome to facilitate integration via single or double crossover recombination events (Harwood *et al.*, 2002).

Industrial strains of *Bacillus* are able to direct the synthesis of extracellular proteins to concentrations in excess of 20 g/l, representing a combination of optimized expression elements, developed strains, media, and growth regimes. Although these systems are not generally available, there is now a range of promoter systems have been developed for the controlled, high-level expression of proteins from *B. subtilis*. Some of these have been adapted from *E. coli* system, others from *Bacillus* species and other Gram-positive bacteria.

The widely used P_{spac} promoter was constructed by fusing the 5′-sequences of a promoter from the *B. subtilis* phage SPO1 and the 3′-sequences of the *E. coli lac* promoter, including the operator (Yansura and Henner, 1984). P_{spac} expression is dependent on the inactivation of a constitutively expressed lactose repressor by IPTG. The P_{spac} promoter functions in plasmid and chromosomal locations and, when present in

multicopy situations, can direct the synthesis of a protein to a significant proportion of total cellular protein. However, this promoter is not sufficiently strong and its inducer is too expensive for large-scale fermentations.

Also widely used are xylose-inducible promoters based on the *XylR* repressor. Since these promoters originate from *B. subtilis* and related organisms, they have been used to control gene expression without modification (Gartner *et al.*, 1992). When used on high-copy-number expression vectors, an additional copy of the *xylR* gene is usually included to maintain a balance between the number of repressor molecules and operator sites. Although genes in the xylose regulon are usually subject to catabolite repression, the catabolite responsive element (Cre) is not included in the vectors. *XylR*-controlled promoters direct moderately high levels of expression and have the advantage that the inducer, xylose, is relatively cheap.

Constitutive promoters associated with catabolite repressed genes such as the α-amylase genes from *B. licheniformis* or *B. amyloliquefaciens* can be used as non-inducible expression systems by batch-feeding a catabolite repressing carbon source so that the fermenter operates at substrate limiting concentrations. Under these conditions, *B. subtilis* will continue to produce the target protein for several days.

Expression systems continue to be developed for *B. subtilis*. For example, the lactose (*lac*) operator system for controlling gene expression has been combined with the very strong vegetative (σ^A) promoter upstream of the *B. subtilis groESL* operon, encoding the heat-shock protein GroES and GroEL (Phan *et al.*, 2006). More recently, Chen *et al.* (2010) have adopted the T7 expression system for *B. subtilis*. They used an integration vector to insert the gene encoding the T7 RNA polymerase under the control of the P_{spac} promoter, together with the *lacI* gene under the control of the P_{penP} promoter, into the *wprA* gene (encoding a cell wall protease—see later). By flanking the antibiotic and ColE1 *ori* genes of the integration vector with FRT phage integration sites, the inserted DNA was made markerless by the induction of the cognate FLP recombinase gene on a suicide plasmid.

In general, *B. subtilis* has little or no advantage over *E. coli* for the intracellular production of heterologous protein, except for the lack of the highly immunogenic lipopolysaccharides (LPS), traces of which have to be removed from proteins that need to be injected into humans and animals. The main advantage of using *B. subtilis* is its potential for high-level protein secretion with subsequent recovery from the culture medium. Proteins that are targeted for secretion require a targeting signal in the form of a signal peptide and, for proteins that are not naturally secreted, a signal sequence needs to be incorporated into the vector in such a way as to fuse the signal peptide in-frame with N-terminus of the target protein.

Numerous attempts have been made to maximize the secretion of heterologous proteins by identifying optimal *Bacillus* signal peptides. However, while a specific signal peptide may be optimal for the secretion of one particular target protein, it is often found not to be optimal for another, indicating that as yet understood characteristics of both the signal peptide and the mature protein together influence secretion (Brockmeier *et al*., 2006). For general purposes, the signal peptides from the *B. amyloliquefaciens* α-amylase (AmyQ) and *B. subtilis* alkaline protease (AprE) have been used in many secretion/expression vectors (Olmos-Soto and Contreras-Flores, 2003; Phan *et al*., 2006).

III. *BACILLUS* SECRETION PATHWAYS

Between 5% and 10% of the proteins encoded by bacteria are secreted across the cytoplasmic membrane using the ubiquitous Sec-dependant (Sec) (Driessen and Nouwen, 2008; Holland, 2004) and twin-arginine translocation (TAT) (Berks *et al*., 2005; Robinson and Bolhuis, 2004) pathways. The Sec pathway is responsible for the secretion of the majority of these proteins, while the TAT pathway is required for the smaller numbers of proteins that need to be folded prior to translocation. In the case of Gram-negative bacteria such as *E. coli* and *Salmonella enterica*, the need to translocate proteins across a double membrane system has resulted in the evolution of a variety of specialized, substrate-specific, protein secretion pathways (e.g., types I, II, III, IV, V, and VI), some of which require the involvement of the Sec pathway (e.g., types II, IV, and V pathways) (Papaniko *et al*., 2007). In contrast, Gram-positive bacteria generally lack these specialized pathways, except for homologues of the relatively poorly understood ESAT-6 secretion pathway (ESX; Bitter *et al*., 2009), substrate-specific Sec pathways associated with homologues of the SecA protein (Rigel and Braunstein, 2008) and phage-associated holin-like proteins for the secretion of endolysins (Borysowski *et al*., 2006). Only the Sec pathway is currently being exploited for the secretion of heterologous proteins from *B. subtilis* and its relatives, and consequently this review focuses exclusively on this pathway and various attempts that have been made to improve the secretion of foreign protein from these organisms (Brockmeier *et al*., 2006; Sarvas *et al*., 2004; Tjalsma *et al*., 2004).

IV. SUBSTRATE RECOGNITION

A crucial early event in the Sec secretion pathway is the identification, by cytoplasmic components, of substrates that are destined for secretion. Proteins that are targeted for translocation across the cytoplasmic

membrane are identified by their possession of an N-terminal extension, the signal peptide, that is removed during the latter stages of secretion (Bendtsen *et al.*, 2005; Nielsen *et al.*, 1997). The signal peptides of bacterial Sec pathway substrates exhibit a similar structural organization: they are usually between 20 and 30 amino acids in length, and have a positively charged amino terminal (N) region, followed by a hydrophobic (H) central region and a short cleavage (C) region containing the target site for signal peptidase (Fig. 1.1). The signal peptides fall into two distinct types, as defined by the class of signal peptidase responsible for the cleavage event that releases the mature protein and their final location. Type I signal peptidases cleave the most abundant class of secretory substrates, which include proteins associated with the cell wall or which are released into the culture medium. *B. subtilis* encodes five chromosomally encoded Type 1 signal peptidases, namely SipS, SipT, SipU, SipV, and SipW. None

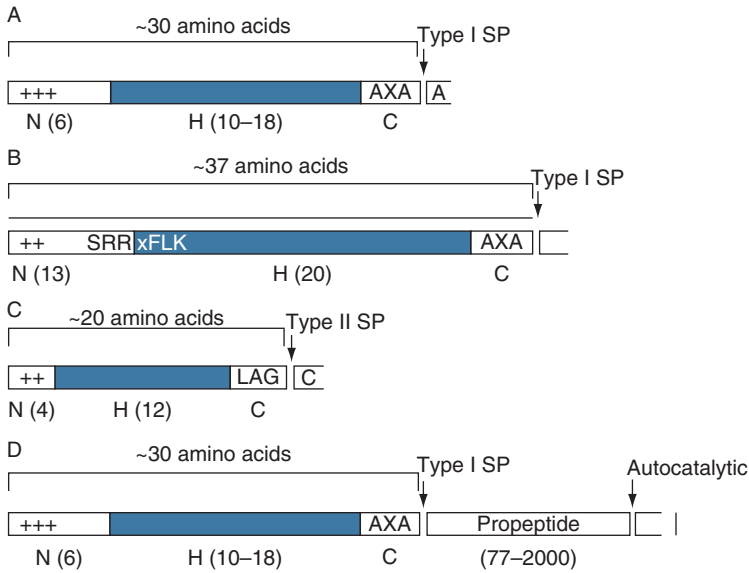

FIGURE 1.1 The main features of *Bacillus* signal peptides and propeptides. The N-terminal (N), hydrophobic (H), and cleavage (C) regions are identified by contrasting shading with their average lengths indicated in brackets. A. Sec-dependent signal peptide cleaved by a Type I signal peptidase (SP); B. TAT-dependent signal peptide, cleaved by a Type I signal peptidase (SP); C. Lipoprotein signal peptide cleaved by the Type II signal peptidase (SP); D. The signal peptide and propeptide (prepropeptide) at the N-terminal end of a secretory protein requiring the propeptide for folding on the *trans* side of the cytoplasmic membrane. The signal peptide is removed by a Type I enzyme and the propeptide either autocatalytically or by a coexisting protease.

are essential, although either SipS or SipT must be present to maintain viability (Tjalsma *et al.*, 1999) and are therefore regarded as paralogues of the single Type I signal peptidase (Lep) of *E. coli*. SipU, SipV, and SipW are minor signal peptidases that appear to be involved in the secretion of specific substrates. The type I signal peptides (Fig. 1.1) of Gram-positive bacteria are, on average, longer (~30 amino acid) and more hydrophobic than those of Gram-negative bacteria (~25 amino acids). They have similar consensus cleavage sequences (AXA↓) and the signal peptides of *B. subtilis* tend to be functional in *E. coli* and vice versa, albeit with differing efficiencies (Zanen *et al.*, 2005).

The absence of a membrane-enclosed periplasm means that *Bacillus* species have a higher proportion of lipoproteins than their Gram-negative counterparts (Tjalsma *et al.*, 2004). Because lipoproteins have to be diacylglycerol-modified prior to attached to the outer surface of the membrane, they are targeted by distinct signal peptides that are recognized and cleaved by the single Type II signal peptidases (LspA). Consequently, while topologically similar to Type I signal peptides, Type II signal peptides share discrete characteristics that include shorter N and H regions and a different consensus cleavage site, referred to as a Lipobox: [LITAGMV]-[ASGTIMVF]-[AG]-↓C-[SGENTAQR] (Fig. 1.1; Sutcliffe and Harrington, 2002; Tjalsma and van Dijl, 2005). The amino acid at the N-terminus of the mature lipoprotein is invariably a Cys residue that, when lipo-modified, serves to tether the protein to the outer leaflet of the cytoplasmic membrane (Juncker *et al.*, 2003).

V. INTRACELLULAR CHAPERONING AND PILOTING TO THE SEC TRANSLOCASE

Although bacterial signal peptides have been well characterized, surprisingly little is known about the intracellular events associated with targeting and the subsequent piloting of their cargo proteins to the Sec translocase, particularly in Gram-positive bacteria. The process requires three key elements; (a) the identification of the target secretory protein, preferably as it emerges from the ribosome; (b) its interaction with chaperone proteins that prevent it folding into a secretion incompetent state; (c) its piloting to the membrane-bound translocase.

There is considerable uncertainty about the intracellular events associated with protein secretion in bacteria. Since there are few relevant studies in *B. subtilis*, and for the most part both these bacteria share the same components, we discuss recent progress in *E. coli* (Luirink and Sinning, 2004; Zhang *et al.*, 2010). The three key intracellular players are the Signal Recognition Particle (SRP), SecA, and SecB (Fig. 1.2). The current view is that the SRP is required for the cotranslational targeting

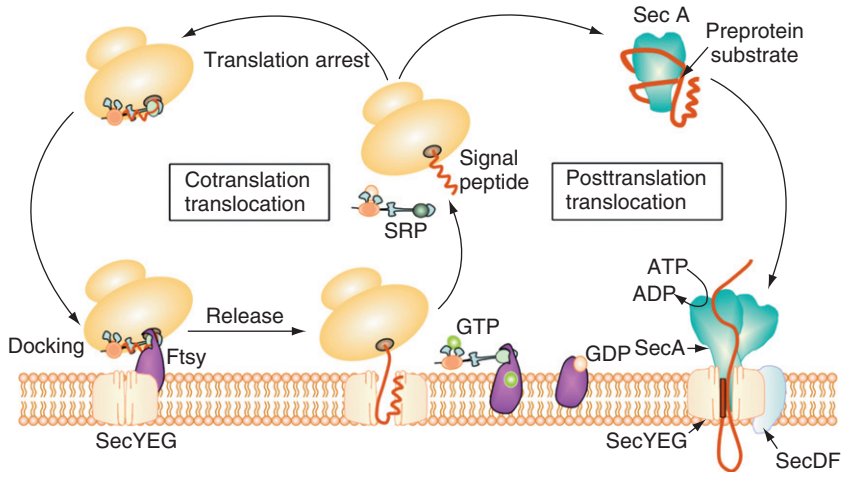

FIGURE 1.2 Diagrammatic representation of the cytoplasmic chaperoning and targeting pathways of *Bacillus subtilis* based on the Signal Recognition Particle (SRP) and SecA cycles.

of integral membrane proteins to the inner membrane via the Sec translocase. SRP, a ribonucleoprotein complex consisting of 4.5S RNA and Ffh, interacts specifically with signal sequences of nascent membrane proteins emerging from the ribosome (Neher *et al.*, 2008). The resulting complex then docks at the membrane and ultimately the Sec translocase via a membrane-bound receptor, FtsY. The result is a switching of the translocase to a transversal opening mode, and the lateral release of target proteins into the membrane. Ffh and FtsY are members of the SRP-GTPase protein family that are essential for viability (Chen *et al.*, 2008).

Proteins that are targeted beyond the membrane (including membrane-anchored lipoproteins) associate with SecB (Bechtluft *et al.*, 2009), a secretion-specific cytoplasmic chaperone, as they emerge from the ribosome, rather than the SRP. The role of the tetrameric SecB is to maintain the secretory proteins in the essential unfolded (i.e., secretion competent) state required for translocation through the Sec translocase. SecB binds to the mature region of its secretory substrates, in a groove that forms between the interacting dimers (Dekker *et al.*, 2003). How SecB recognizes its cargo is still unclear. The SecB tetramer is organized at a dimer of dimers, with inwardly facing α-helices and outwardly facing β-sheets. Each dimer binds one molecule of the SecA–ADP dimer that interacts with the translocase, via the highly conserved C-terminal SecB binding domain. Once the ternary complex (preprotein/SecB/SecA) interacts with the translocase, the replacement of ADP with ATP leads to the release and recycling of SecB (Luirink and Sinning, 2004).

In the early stages of secretion, it is clear that the SRP and SecB pathways converge and are in competition for substrates. SRP appears to have a preference for signal peptides with more hydrophobic ''H'' regions, while the remaining substrates appear to be directed to the SecB via the ribosome-bound trigger factor.

The intracellular processing of secretory proteins in the Gram-positive bacterium *B. subtilis* is similar except that its SPR RNA is significantly longer (271 nucleotides) than that of its *E. coli* counterpart (114 nucleotides) and contains an *Alu* domain to which an additional histone-like protein, HBsu, is attached (Eichler, 2003; Nakamura *et al.*, 1999). Since *Alu* domains are present in Eukaryal and Archael SRP RNA, both of which undergo translational arrest and subsequent cotranslation translocation, it would be interesting to know if these processes also take place in *B. subtilis*. The second major difference is the absence, in all Gram-positive bacteria, of SecB (or identifiable functional homologue), even though their SecA proteins still encode the highly conserved 22 amino acid C-terminal SecB binding domain. Attempts to identify a direct homologue of SecB, or to show that other intracellular chaperones such as GroEL/ES and DnaK play a major role in secretion, have been unsuccessful. However, one potential protein chaperone has been identified that might fulfill the role of SecB, namely CsaA (Müller *et al.*, 2000; Shapova and Paetzel, 2001). Although the evidence for its role in secretion needs to be strengthened, CsaA has been shown to interact with SecA, to bind to peptides and is upregulated under secretion stress (Linde *et al.*, 2003; Müller *et al.*, 1992; Vitikainen *et al.*, 2005).

The absence of SecB, together with the more hydrophobic nature of the signal peptides of Gram-positive bacteria, has led some to suggest that SRP provides the chaperone activity for all integral membrane and secreted proteins and others to suggest that SecA alone can perform this function for secretory proteins (Zanen *et al.*, 2005, 2006). Depletion of *B. subtilis* Ffh reduces Sec-dependent secretion, although experiments designed to elucidate the precise role and substrate specificity of SRP pathway have provided ambiguous results. This is presumably due to the pleiotropic effects of the depletion of Ffh on the insertion of the translocase itself into the cytoplasmic membrane. Until the molecular processes associated with these early cytoplasmic events are better understood, currently there are few opportunities to improve this stage of secretion by rational intervention.

Potentially, some of these issues can be circumvented by the use of the Twin Arginine Transporter (Tat) pathway (Berks *et al.*, 2005). In this case target proteins are characterized by a signal peptide that includes R–R at the junction of the N- and H-regions. The Tat pathway is adapted for the secretion of folded proteins, which potentially obviates the need for cytoplasmic chaperoning. However, although there are examples of the

use of the Tat pathway for the secretion of heterologous proteins, most notably of the green fluorescent protein, there are currently only limited reports of this pathway being used for the commercial production of such proteins (Meissner *et al.*, 2007; Schaerlaekens *et al.*, 2004). The few studies that have compared the Sec and Tat secretion pathways for the secretion of heterologous proteins generally show that the Sec pathway is more efficient with respect to yield, although the secretion of GFP by the Tat pathway of *B. subtilis* resulted in an authentically fold and active protein as compared to GFP secreted via the Sec pathway (Meissner *et al.*, 2007).

VI. SECA AND THE SEC TRANSLOCASE

The Sec translocases of *E. coli* and *B. subtilis* show extensive similarities and studies on both systems have contributed to our understanding of how the Sec translocase functions (de Keyzer *et al.*, 2003). In addition to its role as a cochaperone, SecA provides the motor component of the Sec translocase. It drives secretory substrates through the pore component of the translocase in a posttranslational manner, deriving its energy from the binding and hydrolysis of ATP. SecA is therefore an ATPase that interacts with both the intracellular and translocase components of the pathway. The motor function of SecA is associated with its DEAD motor, comprising nucleotide-binding folds (NBFs) 1 and 2, which reflect its origins as an RNA helicase motor (Papanikou *et al.*, 2005; Rocak and Linder, 2004). Both NBFs are required for SecA's ATPase activity, the catalytic activity of NBF1 being regulated by NBF2 and the intramolecular regulator of ATP hydrolysis (IRA1) located in the helical scaffold domain (HSD), immediately downstream of NBF2. Between NBF1 and NBF2 is the preprotein cross-linking domain with separate binding sites for the signal peptide and mature domains of the preprotein.

It is the preprotein/SecA–ADP complex (with or without SecB depending on species) that is delivered to the translocase. Interaction with the SecY component of the translocase leads to a cycle of conformational changes in SecA that result in the release of ADP, the attachment of ATP, and the penetration of SecA and 10–12 residues of its cargo deep into the translocase. ATP hydrolysis then allows SecA to decouple from its cargo, ready to initiate another cycle of its motor activity.

The core of a heterotrimeric integral membrane pore that interacts with SecA is composed of the SecY, SecE, and SecG proteins. SecA, SecY, and SecE are essential for viability, while SecG improves the translocase efficiency at sub-optimal temperatures (Driessen and Nouwen, 2008; van Wely *et al.*, 1999). SecY forms the main component of the pore, with 10 transmembrane domains, while SecE and SecG contribute two to four additional membrane-spanning domains, depending on species.

Interestingly, while SecE from *E. coli* has three transmembrane domains, its *B. subtilis* homologue has just one. Together these proteins form a pore, plugged on the outer surface, through which preprotein substrates are translocated. A second heterotrimeric complex, comprising SecD–SecF–YajC in *E. coli* and SecDF–YrbF in *B. subtilis*, increases translocation efficiency by improving SecA cycling and maintaining the forward momentum of the preprotein (Driessen and Nouwen, 2008).

During or shortly after the secretory preprotein is translocated, the signal peptide is cleaved by a signal peptidase. In the case of *B. subtilis*, SipS or SipT processes most Type I signal peptides, while LspA processes the Type II signal peptides associated with lipoproteins. These otherwise inhibitory signal peptide fragments are subsequently degraded by peptidases SppA and TepA (Bolhuis *et al.*, 1999a).

VII. CELL WALL STRUCTURE AND IMPLICATIONS FOR SECRETION

The environment into which *Bacillus* secretory proteins emerge from the translocase is very different from that found in the periplasm of Gram-negative bacteria. The absence of an outer membrane provides both advantages and disadvantages for the secretion of heterologous proteins. While the absence of an outer membrane means that heterologous proteins are potentially free to pass directly into the culture medium, proteins that are required to be active on or at the cell surface have to be tethered to the cytoplasmic membrane or the cell wall. The latter proteins include quality control proteases (see later), extracytoplasmic chaperones, autolysins, surface layer proteins, and substrate binding proteins. One consequence of this is that Gram-positive bacteria encode a higher proportion of lipoproteins than their Gram-negative counterparts (Tjalsma *et al.*, 2004) and proteins with motifs that direct them to the cell wall via ionic or, occasionally, covalent interactions (Marraffini *et al.*, 2006; Mesnage *et al.*, 2000). The extent to which specific proteins are immobilized at the membrane surface and in the cell wall provides the cell with an environment that is functionally analogous to the periplasm of Gram-negative bacteria.

Secretory proteins emerge from the Sec translocase into an environment that is dominated by the physicochemical properties of the cell wall. The cell walls of Gram-positive bacteria consist of a thick, highly cross-linked semi-porous copolymer of peptidoglycan and anionic polymers that protect the underlying cytoplasmic membrane from the potentially lethal effects of the high intracellular turgor (Thwaites *et al.*, 1991). In addition to this role, the wall plays key roles in cell division and the maintenance of cell shape, is involved in metal ion homeostasis,

and controls various interactions between the cell and its environment (Vollmer *et al.*, 2008).

The peptidoglycan of *B. subtilis*, which typically represents ~40–45% of the wall by weight, is the structural component. It consists of repeating units of the disaccharide *N*-acetyl glucosamine and *N*-acetylmuramic acid, cross-linked with flexible peptides via the C3 residue of muramic acid. *B. subtilis* strain 168 contains two teichoic acids (Weidenmaier and Peschel, 2008): the main polymer is poly(glycerol phosphate) while the minor polymer is poly(glucosyl *N*-acetylgalactosamine 1-phosphate). Both anionic polymers are covalently attached to peptidoglycan at the C6 residue of *N*-acetylmuramic acid (Freymond *et al.*, 2006). Since teichoic acids are phosphate rich (they can contain up to 30% of total cell phosphorus), during phosphate limitation they are replaced with nonphosphate-containing teichuronic acids, while the displaced teichoic acid is utilized as a reserve source of phosphate (Allenby *et al.*, 2005). In addition to the anionic polymers that form the copolymer with peptidoglycan, the cell wall also contains significant amounts of lipoteichoic acid and protein.

The presence of a thick cell wall (ca. 20 layers) comprising up to 50% by weight of anionic polymer means that the environment immediately outside the cell membrane contains a high density of immobilized negative charge, counterbalanced by divalent metal cations (e.g., Ca^{2+}, Mg^{2+}, Fe^{2+}). Because proteins emerging from the Sec translocase are effectively in an unfolded state they are susceptible to illegitimate interactions with a porous structure that is effectively a cation exchange resin (Beveridge and Murray, 1980). This is a challenging environment for proteins to fold into their native structural configuration without forming aggregates with themselves, other proteins, or the cell wall. While intra and intermolecular interactions are likely to lead to inactive proteins, the formation of aggregates at the inner surface of the cell wall is likely to block the linkage of nascent material into the cell wall, leading ultimately to cell lysis.

VIII. POSTTRANSLOCATION FOLDING

It has become clear in recent years that the rate at which proteins fold as they emerge from the translocase is a key element of their productivity (Harwood and Cranenburgh, 2008). This is due to the susceptibility of slowing folding or misfolded proteins to proteolysis by the so-called quality control proteases (see later). Native *Bacillus* secretory proteins have evolved a variety of intrinsic and extrinsic mechanisms to ensure their rapid folding. These chaperones and folding factors include pro-peptides, a peptidyl-prolyl *cis*/*trans* isomerase, disulfide isomerases, and metal ions.

A. Propeptides

A number of *Bacillus* secretory proteins, predominately proteases, are synthesized with a cleavable propeptide located between their signal peptide and mature substrate protein (Fig. 1.1). Propeptides vary considerably in length and function (Shinde and Inouye, 2000). Class I propeptides, such as the propeptide of *B. amyloliquefaciens* subtilisin BPN′, an important commercial enzyme, is 77 residues (residues 31–107), have a significant role in posttranslocational folding, while the Class II propeptide of Barnase, from the same bacterium, may be required for interactions with the intracellular chaperones, GroEL, and may therefore be involved in intracellular stability (Zahn *et al.*, 1996).

Class I propeptides are essential for rapid posttranslocational folding of their cognate mature protein (Yabuta *et al.*, 2002). They function by overcoming large kinetic barriers in the productive folding pathway and are potent competitive inhibitors of the active enzyme in the case of protease substrates (Yabuta *et al.*, 2001). During the translocation of a preproprotein, the signal peptide is cleaved in the usual manner during or immediately following translocation. The propeptide then accelerates posttranslocational folding by stabilizing an intermediate complex, thereby generating a nucleus for folding (Gallagher *et al.*, 1995; Wang *et al.*, 1998). In the case of subtilisin, once the proprotein is folded, the propeptide temporally inhibits its proteolytic activity (Fu *et al.*, 2000). Full subtilisin activity is only achieved after proteolytic self-cleavage and subsequent degradation of the propeptide (Yabuta *et al.*, 2001). In the absence of the propeptide, the protein is trapped in a molten globular-like intermediate folding state (Wang *et al.*, 1998). Propeptide catalyzed folding and propeptide removal are necessary for subtilisin to pass through the cell wall (Power *et al.*, 1986). Although propeptides are intrinsic intramolecular chaperones, they can be provided extrinsically to catalyse the folding of their cognate mature protein *in vitro* in both an intra and intermolecular fashion.

The Class II propeptides have a variety of functions and are often not necessary for their cognate protein to achieve the active and stable configuration. For example, the role of the so-called ''LEISSTCDA'' propeptide from the Nuc nuclease of *S. aureus* appears to be to decrease the rate of intracellular folding, thereby facilitating its interaction with chaperones that maintain its secretion competence (Le Loir *et al.*, 2001).

Various attempts have been made to exploit propeptides, to enhance the secretion of heterologous proteins, albeit with limited success (Chiang *et al.*, 2010). However, ''LEISSTCDA'' propeptide has been shown to increase the secretion efficiency of a number of heterologous proteins from *Lactococcus lactis*, the *Brucella abortus* L7/L12 antigen and

the α-amylase of *Geobacillus stearothermophilus*. However, this has been shown to be the result of increased intracellular stability resulting from the insertion of negatively charged residues in the N-terminus of the mature protein, rather than improved extracellular folding (Le Loir *et al.*, 1998).

B. Divalent cations as folding catalysts

The environment into which secreted proteins are translocated is rich in metal cations adsorbed to the negatively charged cell wall. Because these ions are mobile and in a dynamic equilibrium with the immobilized negative charge of the cell wall, the concentration of free ions at the membrane/wall interface is higher than in the culture medium. This is significant because many *Bacillus* secretory proteins require metal ions for their folding, structural stability, and activity. The role of metal ions in posttranslocational folding has been best studied in the case of the *B. licheniformis* α-amylase (AmyL). AmyL has a triadic Ca–Na–Ca metal-binding site contained within the classic α-amylase structure with a central $(\beta/\alpha)_8$-barrel (domain A), a complex loop (domain B), and a Greek key motif-containing C-terminal domain (domain C) (Declerck *et al.*, 2000). The importance of Ca^{2+} in posttranslocational folding and secretion efficiency was clearly demonstrated using a chimeric form of AmyL, engineered to change its pI from 7.0 to 10.0. The chimeric AmyL required a ~100-fold increase in the Ca^{2+} concentration to achieve the same *in vitro* folding rate as the wild-type enzymes and was considerably more susceptible to cell-associated exoproteases such as Wall-associated protein A (WprA), but not to proteases in the culture medium, indicating the vulnerability of slowly folding secretory proteins to proteolysis (Jensen *et al.*, 2000; Stephenson and Harwood, 1998).

The availability of metal cations at the *B. subtilis* membrane/wall interface can be modulated by the extent of teichoic acid D-alanylation. Alanylation decreases the negative charge of the wall by neutralizing adjacent phosphoryl residues. Inactivation of the *dlt* operon, required for the alanylation of teichoic acids, significantly increases the yield of many secretory proteins, including *B. amyloliquefaciens* and *B. licheniformis* amylases and *Bacillus anthracis* protective antigen (Hyyryläinen *et al.*, 2000; Thwaite *et al.*, 2002). The increase in negative charge resulting from the absence of D-alanylation, particularly at the membrane/cell wall interface, is likely to increase the cell wall's affinity for cations, which would be available to catalyze folding either from solution or by ligand–ligand exchange.

C. Peptidyl prolyl *cis/trans* isomerases

In comparison with Gram-negative bacteria, only a single class of protein has been shown to assist the posttranslocational folding of Gram-positive secretory proteins, namely PrsA-like proteins. *B. subtilis* PrsA is an essential 270-amino acid protein that has sequence similarity to peptidyl-prolyl *cis/trans* isomerases (PPIases) of the parvulin family (Kontinen *et al.*, 1991; Vitikainen *et al.*, 2001, 2004). PPIases increase the rate of folding of proteins with *cis*-prolyl residues and this activity is consistent with a function of PrsA in a posttranslocational stage of secretion.

Depletion of PrsA causes gross morphological alterations and cell death, indicating that PrsA is required for the folding of one or more proteins involved in cell wall synthesis (Vitikainen *et al.*, 2001). A strain of *B. subtilis* encoding a PrsA protein with reduced activity shows a marked reduction in the production of the *B. amyloliquefaciens* and *B. licheniformis* α-amylases (AmyQ and AmyL, respectively), while overproduction of a fully functional PrsA resulted in a dramatic increase in the production of these α-amylases. However, most secretory proteins are unaffected by PrsA, indicating that PrsA targets a limited number of substrates (Vitikainen *et al.*, 2004). Strains depleted of PrsA do not accumulate the unprocessed precursors of their substrates in the cytoplasm and do not influence their rate of translocation. Instead, they show a marked increase in the posttranslocation degradation of PrsA-dependent proteins. These data support the view that PrsA functions as a relatively substrate-specific, cell-associated extracellular folding chaperone.

Interestingly, *B. subtilis* encodes a paralogue of PrsA, namely YacD, which is neither essential nor able to complement PrsA with respect to viability or secretion. The function of YacD is currently unknown.

D. Disulfide isomerases

A major difference in the secretion rather than intracellular accumulation of heterologous proteins is that they are translocated to an oxidized environment that favors disulfide bond formation. This is a major advantage for heterologous proteins that need to form disulfide bonds to fold into their native structure. The emergence of such proteins in an essentially unfolded state from the Sec translocase into the oxidized environment at the membrane/wall interface improves the likelihood of their forming legitimate disulfide bonds. This is in contrast the intracellular accumulation of proteins in the reduced environment of the cytoplasm, which increases the probability of illegitimate disulfide bonds forming during extraction and purification.

Interestingly, however, the secretory proteins of *B. subtilis* generally lack disulfide bonds (Tjalsma *et al.*, 2004), apparently preferring stabilization by

metal cations (see above). The two native *B. subtilis* peptides/proteins of *B. subtilis* that do form disulfide bonds, the bioactive peptide sublancin and pilin-like ComGC protein required for competence development, are secreted via specialized pathways rather than the Sec pathway. Nevertheless, *B. subtilis* encodes three membrane-bound (BdbA, BdbB, and BdbC) and one secreted (BdbD) thiol-disulfide oxidoreductase/isomerase enzymes involved in disulfide bond formation. Some or all of these enzymes are likely to be important for the commercial production of disulfide-bonded proteins since inactivation of the *bdbB* and *bdbC* genes result in a reduction in disulfide formation of PhoA, an *E. coli* alkaline phosphatase that is only active when correctly disulfide bonded (Meima *et al.*, 2002). A parallel observation showed that substantial amounts of PhoA were degraded even in the Bdb-proficient strain. This led to the conclusion that the native disulfide bond-forming enzymes of *B. subtilis* might not be efficient enough to increase the rate of disulfide bond formation in heterologous proteins. Attempts to over produce the *B. subtilis* Bdb enzymes were not successful and instead, DsbA, a BdbC/BdbD homologue, from either *S. aureus* or the nonpathogenic *S. carnosus*, was expressed in *B. subtilis* with a resulting increase in the amounts of active PhoA (Kouwen *et al.*, 2007). The activity of DsbA was found to be dependent on the presence of redox active compounds in the culture medium. Consequently, the addition of cystine (the oxidized form of cysteine) to the medium further enhanced the activity of PhoA in the medium (Kouwen *et al.*, 2008).

IX. MEMBRANE-BOUND AND EXTRACELLULAR PROTEASES

One of the major factors limiting the use of *B. subtilis* for the production of heterologous proteins has been its production of a diverse set of membrane-bound and extracellular proteases that often lead to the degradation of secreted nonnative proteins. In recent years, we have identified two distinct roles for these proteases; quality control proteases with a key role in cell homeostasis, and feeding proteases that provide sources of nutrients (in the form of amino acids) from proteins and peptides in the environment. Both sets of proteases can adversely affect the secretion efficiency of heterologous proteins (Harwood and Cranenburgh, 2008).

The quality control proteases include two membrane-bound serine proteases, HtrA and HtrB and the wall-bound protease, WprA (Darmon *et al.*, 2002; Stephenson and Harwood, 1998). Quality control proteases are necessary to ensure that secretory proteins do not block the Sec translocase or cell wall growth sites, both events having potentially fatal consequences. These *B. subtilis* proteases, therefore, monitor the ''quality'' of proteins at the membrane/wall interface (Fig. 1.3), degrading those that are misfolded.

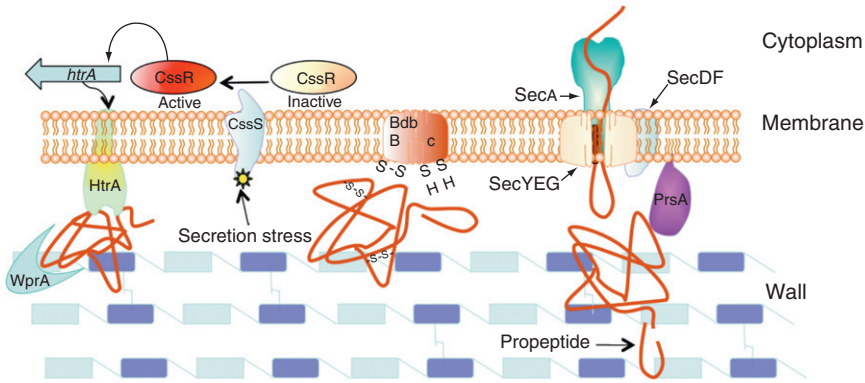

FIGURE 1.3 Diagrammatic representation of the folding and quality control processes that maintain protein authenticity at the membrane/wall interface.

HtrA and HtrB are predicted to be membrane-anchored proteins with large, extracytoplasmic serine protease domains (Darmon *et al.*, 2002). The genes encoding these proteases are induced (via the CssRS two-component signal transduction pathway) in response to secretion and/or physiological stresses (e.g. over production of AmyQ, heat, etc.) that are likely to negatively influence the structure of secretory proteins (Hyyryläinen *et al.*, 2001; Westers *et al.*, 2006). Consequently, CssRS misfolded proteins at the membrane/wall interface irrespective of how they were generated. The resulting production of HtrA and HtrB reduces the potential for these proteins to block the Sec translocase and/or cell wall growth sites. There is evidence that *B. subtilis* HtrA, like that of its *E. coli* counterpart, also has a chaperone-like activity that assists the folding of denatured or misfolded proteins, while targeting unrecoverable proteins for degradation (Antelmann *et al.*, 2003).

WprA is the processed product of the *wprA* gene (Stephenson and Harwood, 1998). Following translocation, the 96 kDa primary translation product is proteolytically cleaved into three products; cell wall binding protein (CWBP) 23 derived from the N-terminus of the primary product, CWBP52 derived from the C-terminus, and a ~21 kDa linker region that connects CWBP23 and CWBP52 in the precursor. Only CWBP23 and CWBP52 are detectable in the cell wall and culture supernatant, the linker protein is rapidly degraded following the original processing event. CWBP52 is a serine protease involved in the degradation of nonnative secretory proteins, whereas CWBP23 is most likely to be a propeptide involved in both the folding of CWBP52 and the control of its activity. The *wprA* gene is under the regulation of YvrGHb, a two-component signal transduction pathway that controls the expression of genes for the major

cell wall autolysins, *lytB* and *lytC* (Serizawa *et al.*, 2005). However, the signal responsible for the induction of this system is not known.

The presence of the quality control proteases HtrA, HtrB, and WprA appears to be a major barrier to the production of certain secreted heterologous proteins, particularly those that fold slowly. Consequently attempts have been made to inactivate each of the genes encoding these proteins individually and jointly (Jensen *et al.*, 2000; Stephenson and Harwood, 1998; Vitikainen *et al.*, 2005). Mutations in either of the genes encoding HtrA and HtrB have little or no obvious effect on growth or the yield of secretory proteins, probably because the absence of one leads to the increased synthesis of the other. In contrast, an *htrA–htrB* double null-mutant exhibits a marked sensitivity to heat, oxidative stress, and secretion stress and shows a noticeably reduced growth rate and yield of secretory proteins (Noone *et al.*, 2001).

Mutants in *wprA* have no obvious growth phenotype, but show enhanced production of native wall proteins and certain heterologous proteins (Stephenson and Harwood, 1998). WprA was implicated in the thermal inactivation of a temperature-sensitive derivative of the membrane anchored signal peptidase SipS showing that, despite its location in the cell wall, it is active at the membrane/wall interface (Bolhuis *et al.*, 1999b).

B. subtilis encodes genes for seven so-called feeding proteases, namely *nprB*, *aprE*, *epr*, *bpr*, *nprE*, *mpr*, *vpr*. Products AprE, Epr, Bpr, and Vpr are serine proteases while Mpr, NprB, and NprE are metalloproteases. While the substrates for the extremely efficient quality control protease are active primarily on misfolded proteins at the membrane/wall junction and in the cell wall, the feeding proteases are adapted to degrade authentically folded proteins in the culture supernatant. Native *Bacillus* exoproteins that have coevolved with these feeding proteases are generally resistant to their activities (Stephenson *et al.*, 2000), however, many studies have shown their frequent negative impact on secreted heterologous proteins (Wu *et al.*, 2002). Consequently various groups have isolated strains that lack both these proteases, often in combination with WprA and IspA, the major intracellular protease. One of the most extensively used strain is WB800 (Wu *et al.*, 2002), that lacks the feeding proteases and WprA.

X. CONCLUDING REMARKS

The absence of an outer membrane, combined with an efficient Sec-dependent secretion pathway, has resulted in the use of *B. subtilis* for the commercial production for industrial enzymes. These enzymes are secreted at high concentrations directly into the culture medium from

which they are relatively easily purified. Generally, the resulting proteins are natively folded and enzymatically active. At face value, *B. subtilis* and its close relatives would therefore appear to be ideal host organisms for the commercial production of a wide range of heterologous proteins. Disappointingly, these bacteria are less successful at secreting heterologous proteins, and yields are often in the milligram or even microgram range per liter of culture medium. Understanding the reasons for the low yields of heterologous protein secretion has been the focus of worldwide laboratory studies. While progress has been made and these organisms can be engineered to increase the production of heterologous proteins, sometimes quite considerably, some basic bottlenecks remain. Despite these limitations in knowledge, Wu and colleagues have shown that *B. subtilis* strains in which the synthesis of intracellular chaperones such as GroES/GroEL and DnaK/DnaJ/GrpE is upregulated can be used to increased the yields of MH-1-single chain antibody (MH-1-SCA) and anti-digoxin-SCA, in the latter case with a concomitant reduction in inclusion body formation (Wu *et al.*, 1998; Wu *et al.*, 2002).

One of the major bottlenecks has been to understand the intracellular chaperoning and targeting mechanisms that deliver secretion competent proteins to the Sec translocase and significantly more work is needed to understand the relative roles of the SRP, CsaA, and SecA. We have, for example, encountered heterologous proteins that simply fail to be translocated and are consequently degraded in the cytoplasm. However, there is a clear evidence that the reason for the low yield of many heterologous proteins is the result of events later in the Sec pathway, and particularly the activities of first of the quality control proteases and then the feeding proteases (Harwood and Cranenburgh, 2008). More can be done to overcome the effects of the feeding proteases since they are not essential for growth. However, attempts to delete all of the quality control proteases lead to cell that grow slowly and are prone to lysis, particularly under conditions of secretion or temperature stress.

Nevertheless, various strategies have improved the yields of many heterologous proteins from *B. subtilis*. A key observation is that the target proteins must fold rapidly as they emerge from the Sec translocase, if they are to avoid blocking the translocase itself or the cell wall growth sites: slowly folding or partially folded proteins expose protease-sensitive sites that are not exposed in the natively folded protein. This is best illustrated by the kinetics of secretion of *B. licheniformis* amylase AmyL (Jensen *et al.*, 2000). Pulse-chase experiments show that during secretion from *B. licheniformis*, virtually 100% of the synthezised protein is recovered from the culture medium. In contrast, when transferred to *B. subtilis*, ~75% of the protein is degraded within seconds of emerging from the translocase, and only 25% is recovered in the growth medium. WprA has been shown to be responsible for a significant portion of this degradation (Stephenson and

Harwood, 1998). The rate of degradation was even more dramatic when a folding mutant of AmyL is used. Significantly, both AmyL and its folding mutant are completely stable in the presence of feeding proteases. These data imply that native secretory proteins have coevolved with their natural host to avoid both the quality control proteases and, in the case of *B. subtilis*, the seven proteases secreted into the culture medium to provide nutrients from the degradation of proteins and peptides in the environment (i.e., feeding proteases).

Several studies have shown that increasing the amounts of PrsA can improve the recovery of the limited number of proteins that are substrates for this chaperone. Again this is likely to be a reflection of their increased rate of posttranslocational folding and reduced susceptibility to proteolysis. These include α-amylases, *B. anthracis* protective antigen, and MH-1-SCA (Kontinen and Sarvas, 1993; Williams, *et al.*, 2003; Wu *et al.*, 1998). Similarly, we have shown that the absence of cell wall D-alanylation increases metal ion concentrations at the membrane/wall interface that improves the yield of certain metalloproteins (Thwaite *et al.*, 2002).

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CHAPTER 2

Function of Protein Phosphatase-1, Glc7, in *Saccharomyces cerevisiae*

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Contents

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Abstract Budding yeast, *Saccharomyces cerevisiae*, and its close relatives are unique among eukaryotes in having a single gene, *GLC7*, encoding protein phosphatase-1 (PP1). This enzyme with a highly conserved amino acid sequence controls many processes in all eukaryotic cells. Therefore, the study of Glc7 function offers a unique opportunity to gain a comprehensive understanding of this critical regulatory enzyme. This review summarizes our current knowledge of how Glc7 function modulates processes in the cytoplasm and nucleus. Additionally, global Glc7 regulation is described.

I. INTRODUCTION

Reversible protein phosphorylation is a well-studied method of post-translational protein regulation. The relative activities of protein kinase and protein phosphatase enzymes control phosphorylation of their substrates. Despite their numerical scarcity in comparative gene numbers, protein phosphatases achieve great diversity by association with a variety of regulatory proteins. Among the serine/threonine phosphatases, protein phosphatase-1 (PP1) is perhaps the most extensively studied. The genomes of all eukaryotic organisms studied contain multiple PP1 genes except for budding yeast (*Saccharomyces cerevisiae*) and its close relatives, which have a single gene, *GLC7*, encoding PP1. Therefore, *S. cerevisiae* offers a unique opportunity to comprehensively understand organismal PP1 function in the simplest system in which Glc7 is sui generis. In this review, I will use ''PP1'' to denote PP1 enzymes in general (including Glc7) and ''Glc7'' to denote data discovered for Glc7, which may or may not also be widely applicable to PP1 in other organisms.

The *GLC7* mnemonic comes from the glycogen reduction trait used to first discover it (Cannon *et al.*, 1994; Feng *et al.*, 1991). Like PP1 from larger organisms, Glc7 regulates many processes in addition to glycogen metabolism. These processes will be described later (Sections IV and V). Glc7 function was last reviewed in 1996 (Stark, 1996) and this review will emphasize our understanding since then. Since that last review, a battery of new techniques used to study cell biology and biochemistry have focused on Glc7 function. In particular, genomic-scale methods to comprehensively analyze protein–protein interactions (Gavin *et al.*, 2002; Hazbun *et al.*, 2003; Ho *et al.*, 2002; Tong *et al.*, 2002; Walsh *et al.*, 2002; Yu *et al.*, 2008), examine subcellular localization (Bloecher and Tatchell, 2000; Huh *et al.*, 2003), analyze the phosphoproteome (Alms *et al.*, 1999; Holt *et al.*, 2009), and explore

genetic interactions (Logan *et al.*, 2008) have yielded a wealth of data pertinent to Glc7. These new tools as well as classic methods expose several novel perspectives on PP1 function. Since 1996, our understanding of PP1 function in mammalian and other eukaryotes has obviously also expanded. This review about Glc7 in budding yeast will mention that work when it is related to Glc7 function in *S. cerevisiae*. For general PP1 function, there are several comprehensive reviews (Bollen and Stalmans, 1992; Ceulemans and Bollen, 2004; Cohen, 2002, 2004; Lee *et al.*, 1999; Virshup and Shenolikar, 2009).

II. STRUCTURE OF PP1

Nine X-ray crystallography determined structures of mammalian PP1 have been reported and 10 PDB structures are available. This variety comes from analysis of at least seven inhibitors bound to PP1 and efforts to use the structural information to design more specific inhibitors. The structures of all of these PP1 proteins are practically identical despite the differences in PP1 isoforms, crystallization conditions, and crystal packing contacts. PP1 has two tightly linked domains: an N-terminal domain (residues 1–160) and C-terminal (residues 161–330). A Y-shaped cleft with β sheets is formed where the two domains converge. Three grooves of this cleft are called hydrophobic, acidic, and C-terminal. The catalytic site is at the intersection of the Y and it contains two metal ions, which appear to be Mn^{2+} and Fe^{2+} (Egloff *et al.*, 1995). Consistent with the requirement for Fe^{2+} in the active site, a Glc7 hypomorph, *glc7-E101Q*, is synthetically lethal with iron transport mutations, *fet3* and *mrs4* (Logan *et al.*, 2008).

The Glc7 amino acid sequence is 85% identical to the four human PP1 isoenzymes with most variation in the N- and C-termini. The central section is also shared with related protein phosphatases PP2A, PP2B (calcineurin), and Ppz1,2. Orthology of human PP1 isoenzymes with Glc7 has been seen by complementation of *glc7* by the human PP1 cDNAs; however, not all *glc7* traits were complemented (Gibbons *et al.*, 2007). Similarly, none of the eight *Arabidopsis* PP1 genes completely complemented *glc7* (Cannon, unpublished data). These findings illustrate that the variable residues in PP1 enzymes have significant bearing on the *in vivo* PP1 substrate specificity.

Two general surveys of *GLC7* mutations have been performed: one by alanine scanning (Baker *et al.*, 1997), another by screening for glycogen deficiency (Ramaswamy *et al.*, 1998). A common theme of these two studies was that it was not possible to affect merely a single trait by *glc7* mutations; each mutation affected multiple traits. The biochemical rationalization of this conclusion and the great conservation in sequence is that the PP1 interaction with a large number of other proteins constrains the amino acid sequence to be very similar across species. Therefore, knowledge about proteins interacting with PP1 is key to understanding PP1 function.

III. GLC7 COMPLEXES

A hallmark of PP1 enzymes is that the catalytic subunit always works in a complex. For this review, three types of PP1 interacting proteins are distinguished: regulatory subunits, which make a distinct PP1 holoenzyme; regulators, which modulate the global PP1 activity; and substrates, which interact with PP1 transiently. The Glc7 regulatory subunits are summarized in Table 2.1. Comprehensive two-hybrid and affinity purification methods either initially discovered or confirmed previous characterization of many of these Glc7 regulatory subunits (Gavin *et al.*, 2002; Hazbun *et al.*, 2003; Ho *et al.*, 2002; Tong *et al.*, 2002; Uetz *et al.*, 2000; Walsh *et al.*, 2002; Yu *et al.*, 2008). Given the possibility of false positives in comprehensive studies, only Glc7 regulatory subunits with some additional confirmation (conventional biochemical or genetic analyses) are listed in Table 2.1. Also some Glc7 interactors were excluded from Table 2.1 because they are components of Glc7 containing complexes, which will be discussed in Section III.C, or are clearly Glc7 substrates (Table 2.2) and not a component of a Glc7 holoenzyme. Indeed, there are 195 proteins identified from affinity purifications and 71 from two-hybrid analyses (Breitkreutz *et al.*, 2008; Nash *et al.*, 2007). For historical comparison, only 10 of these Glc7 interacting proteins were known in 1996 (Stark, 1996). Shp1 was previously reported to be Glc7 ''associated,'' but there is no evidence to date indicating a physical association of Shp1 with Glc7. Instead, Shp1 is now considered to be a Glc7 regulator and will be discussed in Section VI.C.

A. RVxF motif Glc7 regulatory subunits

The G_M- and MYPT1-bound PP1 X-ray crystallography structures reveal a frequently used interface between regulatory subunits and PP1 is to a hydrophobic groove (Egloff *et al.*, 1997; Terrak *et al.*, 2004). Mutating residues of this hydrophobic groove in Glc7 reduced affinity to some regulatory subunits and resulted in traits due to reduced Glc7 activity (Wu and Tatchell, 2001). Severe hydrophobic groove Glc7 variants were unable to complement the essential functions of *glc7*Δ to allow viability. Therefore, Glc7 interaction with other proteins through the hydrophobic groove is required for Glc7 to perform dephosphorylations essential for yeast viability.

The interface in many PP1 regulatory subunits has a consensus RVxF primary sequence (Moorhead *et al.*, 2007; Zhao and Lee, 1997). This RVxF motif is recognized in at least 10 Glc7 regulatory subunits (Table 2.1). In all cases tested, mutation of one or both of the valine or phenylalanine residues reduced or eliminated Glc7 binding. Fin1 and Scd5 regulatory subunits contain more than one potential RVxF motif. The two motifs in Scd5 contribute to Glc7 binding to varying degrees (Chang *et al.*, 2002).

TABLE 2.1 Glc7 regulatory subunits

Regulatory subunit	Process regulated	Substrates	Subcellular location[a]	Molecules per cell[b]	Glc7 interface[c]	References[d]
Afr1	Mating septin architecture	–	Mating projections	Not available	KDVRF	Bharucha *et al.* (2008b)
Bni4	Chitin ring assembly	–	Bud neck	Not available	QGVRF	Kozubowski *et al.* (2003), Larson *et al.* (2008), Zou *et al.* (2009)
Bud14	Bud site selection, transcription	–	Cell cortex, nucleus	538	KSVSF	Cullen and Sprague (2002), Knaus *et al.* (2005), Lenssen *et al.* (2005)
Fin1	Mitosis	Dam1, Ndc80, Ndc10	Nucleus	Not available	KLTF, RARF, KDAPF, KASF, KFKL	Pinsky *et al.* (2006), Akiyoshi *et al.* (2009)
Fpr3	Global regulator	–	Nucleolus	–	Unknown	Hochwagen *et al.* (2005), Cannon (unpublished data)
Fpr4	Global regulator	–	Nucleolus	–	Unknown	Ho *et al.* (2002), Cannon (unpublished data)
Gac1	Glycogen metabolism, transcription	Gsy1, Gsy2, Gph1	Cytoplasm	Not available	KNVRF	Wu *et al.* (2001), Lin and Lis (1999)
Gip1	Meiosis	–	–	Not available	Unknown	Tachikawa *et al.* (2001), Tu *et al.* (1996)
Gip2	Glycogen metabolism	Gsy1, Gsy2	Cytoplasm	125	Unknown	Tu *et al.* (1996), Cheng *et al.* (1997)
Gip3	Unknown	–	Cytoplasmic ribosomes	656	Unknown	Pinsky *et al.* (2006)

(*continued*)

TABLE 2.1 (*continued*)

Regulatory subunit	Process regulated	Substrates	Subcellular location[a]	Molecules per cell[b]	Glc7 interface[c]	References[d]
Gip4	Unknown	–	Unknown	227	Unknown	Pinsky *et al.* (2006)
Glc8	Global regulator	–	–	3440	Unknown	Tan *et al.* (2003), Nigavekar *et al.* (2002), Tung *et al.* (1995)
Pan1	Actin cytoskeleton	–	Cell cortex	Not available	–	Zeng *et al.* (2007)
Pex31	Peroxisome size regulation	–	Peroxisome	238	Unknown	Pinsky *et al.* (2006)
Pig1	Glycogen metabolism	Gsy1, Gsy2, Gph1	Cytoplasm	Not available	Unknown	Cheng *et al.* (1997)
Pig2	Glycogen metabolism	Gsy1, Gsy2, Gph1	Cytoplasm	996	Unknown	Cheng *et al.* (1997)
Red1	Meiosis	Red1	Nucleus	Not available	–	Tu *et al.* (1996), Bailis and Roeder (2000)
Pta1	RNA processing	Pta1	Nucleus	3730	Unknown	He and Moore (2005)
Pti1	RNA processing	Pta1	Nucleus	937	Not VxIF	He and Moore (2005)
Ref2	RNA processing, transcription	–	Nucleus	7450	Unknown	Ferrer-Dalmau *et al.* (2010), Walsh *et al.* (2002), Nedea *et al.* (2003)
Reg1	Glucose repression	Snf1, Hxk2, Pda1, Hsp60	Cytoplasm	2560	RHIHF	Sanz *et al.* (2000a,b), Cui *et al.* (2004), Alms *et al.* (1999), Dombek *et al.* (1999), Gadura *et al.* (2006), Tabba *et al.* (2010)

Reg2	Glucose repression	Snf1	Cytoplasm	Not available	RHIKF	Frederick and Tatchell (1996)
Scd5	Vesicular secretory pathway, cortical actin organization	Pan1	Cell cortex, nucleus	704	KDVF, KKVRF	Chang *et al.* (2002), Zeng *et al.* (2007)
Sds22	Nuclear targeting	Dam1, Ndc80	Nucleus, cytoplasm	3870	11 LLR	Peggie *et al.* (2002), Pedelini *et al.* (2007), Hong *et al.* (2000), Ramaswamy *et al.* (1998)
Sip5	Glucose repression	Snf1	Cytoplasm	556	Unknown	Sanz *et al.* (2000b)
Sla1	Cortical actin, endocytosis	Sla1	Nucleus, cell cortex	952	KNIF	Gardiner *et al.* (2007)
Ypi1	Nuclear activity regulator	–	Nucleus	1080	VRW	Garcia-Gimeno *et al.* (2003), Pedelini *et al.* (2007), Bharucha *et al.* (2008a)
Sol1	tRNA nuclear export	–	Nucleus, cytoplasm	1970	Unknown	Pinsky *et al.* (2006)

[a] Huh *et al.*, 2003.
[b] Ghaemmaghami *et al.*, 2003. Compare to Glc7 at 14,600 molecules per cell and sum of 30,308.
[c] The underlined residues were mutated and found to be important for Glc7 binding.
[d] These references report functional characterization rather than mere documentation of Glc7 binding.

TABLE 2.2 Glc7 substrates

Substrate[a]	Function	Dephosphorylated in	Phosphorylated by	References
Dam1	Kinetochore attachment to mitotic spindle	Nucleus	Ipl1	Akiyoshi *et al.* (2009), Pinsky *et al.* (2006)
Fin1	Kinetochore attachment to mitotic spindle	Nucleus	Clb5-Cdc28	Akiyoshi *et al.* (2009), Woodbury and Morgan (2007)
Gph1	Glycogen degradation	Cytoplasm	Tpk1,2,3	Ho *et al.* (2002)
Gsy1, Gsy2	Glycogen synthesis	Cytoplasm	Pho85-Pcl10	Ho *et al.* (2002), Anderson and Tatchell (2001)
Hht1, Hht2	Histone H3	Nucleus	Ipl1, Snf1	Hsu *et al.* (2000)
Hta1, Hta2	Histone H2A	Nucleus	Mec1, Tel1	Bazzi *et al.* (2010)
Ndc10	Kinetochore attachment to mitotic spindle	Nucleus	Ipl1	Sassoon *et al.* (1999)
Npl3	mRNA nuclear export	Nucleus	Sky1	Gilbert and Guthrie (2004)
Pan1	Actin cytoskeleton, endocytosis	Cytoplasm	Prk1	Zeng *et al.* (2007)
Pta1	mRNA processing	Nucleus	–	He and Moore (2005)
Red1	Meiosis	Nucleus	Mek1	Bailis and Roeder (2000)
Sla1	Cortical actin, endocytosis	Nucleus, cell cortex	–	Gardiner *et al.* (2007)
Sui2	Translation initiation	Cytoplasm	Gcn2	Wek *et al.* (1992)

[a] Note that some substrates are also discovered as Glc7 interacting proteins.

The five potential sites in Fin1 were not individually tested, but the Fin1 mutant containing five damaged sites failed to bind Glc7 (Akiyoshi *et al.*, 2009). The crystallographic structures predict that only one RVxF motif can bind to Glc7 at a time, which suggests that regulatory subunits with more than one motif could make multiple distinct Glc7 holoenzymes with perhaps distinctive substrate specificities.

Protein abundance data (Ghaemmaghami *et al.*, 2003) indicates that regulatory subunits vastly outnumber Glc7 (sum of regulatory subunits molecules is at least 30,300 per cell compared to 14,600 Glc7 molecules per cell). Moreover, regulatory subunits vary in abundance and in Glc7 affinity; although the latter assays have not been done in a rigorous comparable fashion. Considering these factors, regulatory subunits compete for a limited pool of Glc7. Such competition has been observed experimentally by artificially increasing one regulatory subunit while observing a trait of a Glc7 holoenzyme containing another subunit (Pinsky *et al.*, 2006; Ramaswamy *et al.*, 1998; Wu *et al.*, 2001). Whether such competition occurs naturally to regulate Glc7 is unknown. However, the mRNAs for Sds22, Reg1, Reg2, and Gac1 regulatory subunits and Glc8 and Shp1 regulators are upregulated in aging cells (Yiu *et al.*, 2008) and perhaps other conditions.

In some cases, the RVxF domain Glc7 regulatory subunit also appears to be a Glc7 substrate. For example, Sla1 and Fin1 bind to Glc7 via RVxF and are dephosphorylated by Glc7 *in vivo* (Akiyoshi *et al.*, 2009; Gardiner *et al.*, 2007). The majority of Glc7 substrates do not have an RVxF motif, so this is not a general feature of substrate recognition. The G_M- and MYPT1-bound PP1 X-ray crystallography structures suggest that substrate sites dephosphorylated must be distal to the RVxF region (Egloff *et al.*, 1997; Terrak *et al.*, 2004).

B. Non-RVxF regulatory subunits

Not all PP1 regulatory subunits bind to the hydrophobic groove using an RVxF domain. In particular, Glc7 regulatory subunits, Sds22 and Pti1 clearly do not (Ceulemans *et al.*, 2002; He and Moore, 2005) and there are probably others. Eleven leucine-rich repeats, which are predicted to form a concave surface in Sds22, bind to PP1 (Ceulemans *et al.*, 2002). We found that yeast Sds22 is similarly bound to Glc7 by the leucine-rich repeats (Ghosh and Cannon, submitted for publication). Mutations in these Sds22 repeats show they are important for functions of Sds22 that are essential for viability (Peggie *et al.*, 2002).

C. Larger Glc7 complexes

A previous paradigm for PP1 was that it existed solely as a collection of PP1 binary complexes with regulatory subunits. This is now clearly not true in mammalian cells and some examples exist for larger Glc7

complexes in yeast. Despite such larger complexes, summary figures showing Glc7 in complex with two other proteins should be interpreted with caution (Sanz *et al.*, 2000a,b). Diagrams of the simultaneous affinity of Glc7 regulatory subunit for Glc7 and a substrate illustrates one manner in which regulatory subunits could define substrate specificity of a particular Glc7 holoenzyme. However, such trimeric associations are transitory and have, in most cases, only been documented by binary interactions rather than documentation of a trimeric or larger complex. Note that such tight affinity of a Glc7 substrate to Glc7 would preclude considerable enzymatic turnover. More generally, the regulatory subunits probably mold the PP1 active site as seen in the PP1-MYPT1 structure (Terrak *et al.*, 2004). A demonstration of this mechanism was seen for a truncation of Gac1, which binds Glc7, but not the substrate Gsy2. Such a mutant Gac1 modifies Glc7 substrate specificity *in vitro* (Wu *et al.*, 2001).

A documented trimeric Glc7–Sds22–Ypi1 complex in yeast translocates Glc7 to the nucleus (Pedelini *et al.*, 2007). Leucine-rich repeats of Sds22 interface with Glc7 and Ypi1 contacts via an RVxF motif. Whether this trimeric holoenzyme is an active nuclear phosphatase or the Glc7 subunit is exchanged for other regulatory subunits there is unknown.

There are at least two instances of Glc7 found in larger complexes. These are the cleavage/polyadenylation factor (CPF) 3′-end processing complex and the kinetochore. The CPF contains Cft1, Cft2, Fip1, Mpe1, Pap1, Pfs2, Pta1, Pti1, Ref2, Rna14, Ssu72, Swd2, Syc1, Ysh1, and Yth1 (Dichtl *et al.*, 2002; He and Moore, 2005; Nedea *et al.*, 2003, 2008). Within the CPF, Glc7 interacts with RVxF motif proteins Ref2 and Pta1 and with non-RVxF motif protein, Pti1 (He and Moore, 2005; Nedea *et al.*, 2003). Poly(A) binding protein (Nab2) and nucleolar RNA helicase Hca4 also bind Glc7 by affinity purification (Batisse *et al.*, 2009; Gavin *et al.*, 2002), but are not currently considered CPF subunits.

IV. CYTOPLASMIC GLC7 FUNCTIONS

A. Glucose repression by Reg1–Glc7

Glucose is the preferred carbon source for *S. cerevisiae*. Transcription of many genes for alternate carbon source utilization and other activities are repressed in high glucose conditions by transcriptional repressor, Mig1, in association with hexokinase-2 (Hxk2) (Ahuatzi *et al.*, 2007; Carlson, 1999). Protein kinase Snf1 phosphorylates Mig1 when glucose concentrations fall, which deactivates Mig1-mediated transcriptional repression by enhancing its cytoplasmic localization. Snf1 also promotes phosphorylation of other substrates including transcriptional activators, Cat8, Sip4, and Adr1 to orchestrate a transcription profile tailored to low glucose

conditions (Gancedo, 2008; Young *et al.*, 2003). Note that the *in vivo* Adr1 protein kinase is unknown (Ratnakumar *et al.*, 2009). The trimeric Snf1 protein kinase complex contains catalytic subunit Snf1, gamma regulatory subunit, Snf4, and three alternate beta subunits: Gal83, Sip1, or Sip2 (Jiang and Carlson, 1997). Snf1 regulatory subunit, Snf4, also binds to Glc7 regulatory subunit, Sds22, although the functional significance is unknown (Ghosh and Cannon, submitted for publication; Ho *et al.*, 2002). Snf1 is activated by phosphorylation on Thr-210 by protein kinases, Elm1, Sak1, or Tos3 (Hong *et al.*, 2003).

When yeast grow in high glucose conditions, Reg1–Glc7 dephosphorylates Snf1 Thr-210 thereby deactivating Snf1 activity (Sanz *et al.*, 2000a). This dephosphorylation is assisted by Sip5, which binds to both Reg1 and Snf1 (Sanz *et al.*, 2000b). Although Reg1 has Glc7 and Snf1 affinity, binding to these two proteins appears to be mutually exclusive (Tabba *et al.*, 2010). Phosphorylation of Reg1 stimulates the activity of the Reg1–Glc7 holoenzyme (Sanz *et al.*, 2000a). Although the Reg1 kinase is not known, Reg1 phosphorylation is enhanced by Hxk2 activity, which is thought to be an early transducer of intracellular glucose (Gancedo, 2008; Santangelo, 2006). Since Reg1 is primarily cytoplasmic, Reg1–Glc7 probably dephosphorylates Snf1 in the cytoplasm during Snf1 nucleocytoplasmic cycles (Dombek *et al.*, 1999, 2004). Derepression of glucose repressed genes leads to deoxyglucose resistance on sucrose medium. The role of Reg1–Glc7 can be assessed using *reg1* deletions or *GLC7* alleles that confer deoxyglucose resistance because they encode Glc7 proteins that reduce Reg1 interaction (Baker *et al.*, 1997; Tu and Carlson, 1995; Wu and Tatchell, 2001).

In addition to its role in Snf1 deactivation, the Reg1–Glc7 holoenzyme inactivates other enzymes unnecessary for growth on glucose (Gancedo, 2008). The most abundant proteins dephosphorylated by Reg1–Glc7 *in vivo* were identified as Hxk2, pyruvate dehydrogenase E1α (Pda1), and mitochondrial chaperonin (Hsp60) (Alms *et al.*, 1999). Reg1–Glc7 promotes fructose-1,6-bisphosphatase (Fbp1) and maltose permease (Mal61) degradation in the vacuole in high glucose conditions (Cui *et al.*, 2004; Gadura *et al.*, 2006). Reg1–Glc7 does not regulate Fbp1 phosphorylation. However, Yck1,2 protein kinase activity is required for Mal61 degradation and Yck1,2 requires Reg1–Glc7 for activity (Gadura *et al.*, 2006). Homotypic membrane fusion required for vacuolar transport is catalyzed by Glc7; however, the Glc7 holoenzyme was not defined (Bryant and James, 2003; Peters *et al.*, 1999).

B. Glc7 regulation of septin processes

Septins are proteins that form hetero-oligomeric filaments at the cell cortex (Longtine and Bi, 2003). They form a scaffold that positions synthetic machinery used to polarize cell growth. Glc7 regulatory subunits,

Bni4, Afr1, and Gip1, each have septin affinity and they mediate three distinct septin localized activities. In mitotic cells, septins encoded by five related genes (*CDC3*, *CDC10*, *CDC11*, *CDC12*, and *SHS1*) form a ring-shaped scaffold at the incipient bud site before bud emergence (Longtine and Bi, 2003). Synthesis of chitin is specifically targeted to these septin rings using vesicle transport and exocytosis (DeMarini *et al.*, 1997). The Bni4-Glc7 holoenzyme regulates the targeting of chitin synthase III (Chs3) to incipient bud sites when Bni4 is phosphorylated by Pho85-Pcl1,2 (Kozubowski *et al.*, 2003; Larson *et al.*, 2008; Zou *et al.*, 2009). Bni4 binds to the septin ring of incipient bud sites and to Chs3 regulatory subunit, Chs4. Merely targeting Glc7 to septin rings via a Cdc10–Glc7 fusion was insufficient to promote proper Chs3 and Chs4 targeting. Bni4 targeted and modulated Glc7 substrate specificity because 70 Bni4 residues fused to Cdc10 were sufficient to direct the proper Chs3 and Chs4 localization (Larson *et al.*, 2008). Bni4-Glc7 substrates responsible for recruitment of Chs3 to the septin rings are unknown; however, there are several possibilities. Chs3 is delivered via transport vesicle fusion, a process that exploits Glc7 in an undefined manner that might involve phosphorylated exocytic proteins (Bryant and James, 2003; Peters *et al.*, 1999). Alternatively, phosphorylated septins could be the relevant substrates. Finally, it is possible that Bin4 itself is the relevant Glc7 substrate for Chs3 recruitment because the 70 residues of Bni4 in the Cdc10–Bni4 fusion that correctly targeted Chs3-bound Glc7 was apparently dephosphorylated by the Cdc10–Bni4·Glc7 holoenzyme (Kozubowski *et al.*, 2003; Larson *et al.*, 2008).

When yeast cells mate, cell growth in the form of a mating projection is directed toward the source of extracellular pheromone. Septin binding protein, Afr1, is a Glc7 regulatory subunit, which targets Glc7 toward mating projections to coordinate polarized mating projection growth (Bharucha *et al.*, 2008b). Afr1-Glc7 organizes the structure of septins in the mating projection, but again the substrates are undefined.

Starving diploid yeast cells undergo meiosis and package the four haploid progeny of the two meiotic divisions into spores. Spore formation involves the docking of post-Golgi secretory vesicles to the spindle pole body after the second meiotic division. Cdc3, Cdc10, and two meiotic septins, Spr3 and Spr28, assist in prospore membrane formation. Gip1-Glc7 is required for septin organization and spore wall formation during meiotic spore maturation (Tachikawa *et al.*, 2001). The sporulation-induced Ysw1 protein binds to Gip1 and can suppress hypomorphic, but not deletions of *GIP1* (Ishihara *et al.*, 2009). Mutations in *GIP1* and *YSW1* reduce sporulation frequency and decrease the number of spores per ascus (Ishihara *et al.*, 2009; Tachikawa *et al.*, 2001). Many *GLC7* mutations also display these sporulation traits (Ramaswamy *et al.*, 1998). Glc7 also controls meiotic transcription and recombination (Sections V.A and V.D).

C. Bud14-Glc7 controls bud-site selection

S. cerevisiae chooses a nonrandom position for the next bud based on mating-type status. Mutations that perturb normal bud-site selection define genes involved in this process. The Bud14-Glc7 holoenzyme modulates bud-site selection because *bud14* mutants have a random budding pattern with elongated buds (Cullen and Sprague, 2002; Ni and Snyder, 2001). Glc7 overexpression also has a notable elongated bud phenotype although no aberrant bud-site selection was reported (Black *et al.*, 1995; Zhang *et al.*, 1995). Bud14 overexpression caused cell cycle arrest after DNA synthesis with large buds (Knaus *et al.*, 2005). This phenotype might be related to the Clb2, mitotic cyclin stabilization reported upon Glc7 overexpression (Pinsky *et al.*, 2009). The preanaphase mitotic spindle was pulled into the daughter bud in many Bud14 overexpressing cells. Normally, the mitotic spindle is partitioned equally between mother and daughter cell. The Bud14-Glc7 holoenzyme stabilizes microtubule attachments to the cell cortex (Knaus *et al.*, 2005). Bud14 is one of the lower abundance Glc7 regulatory proteins (Table 2.1) and its overexpression displaces Glc7 from the nucleus (Pinsky *et al.*, 2006).

The Bud14 regulatory subunit has affinity for cell cortex proteins, Kel1,2 (Ho *et al.*, 2002) thereby positioning the Bud14-Glc7 holoenzyme at the cortex to mediate microtubule attachment. Nuclear Glc7 activity also mediates microtubule attachment to kinetochores and there is evidence for several kinetochore proteins as Glc7 substrates (see Section V.C). In contrast, the cell cortex Bud14-Glc7 substrates are unknown although there are several suspects (Knaus *et al.*, 2005).

D. Scd5-Glc7 regulates endocytosis and actin organization

Cell cortical actin patches are of sites of endocytosis, which is critical for cell viability. Glc7 regulatory subunit, Scd5, binds to endocytic proteins, End3, Pan1, Sla2, and Rvs167, at actin patches to mediate endocytosis and actin organization (Henry *et al.*, 2002; Zeng *et al.*, 2007). When Scd5-Glc7 activity is reduced, actin patches, which are normally predominantly in the daughter cell (bud), were found in the mother cell and aggregates of disassociated actin were visualized (Chang *et al.*, 2002). Scd5-Glc7, which is normally cell cortex associated, can sufficiently function in the cytoplasm to mediate its regulation (Chang *et al.*, 2006). This is perhaps another example of where the regulatory subunit, Scd5, plays a greater role in defining Glc7 substrate specificity rather than merely targeting Glc7 to a particular subcellular location. Both Scd5 and Pan1 are phosphorylated by protein kinase Prk1 and dephosphorylated by Scd5-Glc7 (Zeng *et al.*, 2007). Therefore, similar to the nucleocytoplasmic shuttling of Npl3 by Glc7 (Gilbert and

Guthrie, 2004), the Scd5-Glc7 holoenzyme facilitates a phosphorylation modulated cycle that is used here for endocytosis (Zeng *et al.*, 2007).

Note that Scd5 is essential for yeast viability. Besides Scd5, only two other Glc7 interacting proteins are essential (besides those in large complexes): Sds22 and Ypi1. Overexpression of Glc7 from a multicopy plasmid suppressed a *scd5* mutation designed to prevent Glc7 binding to Scd5 (Chang *et al.*, 2002) and deletion of the Pan1 protein kinase, Prk1, partially suppressed Glc7 depletion (Zeng *et al.*, 2007). These observations illustrate that the essential function of Scd5-Glc7 is to counteract phosphorylations by Prk1. Scd5 shuttles between the nucleus and cytoplasm (Chang *et al.*, 2006). However, the nuclear located Scd5-Glc7 holoenzyme does not regulate endocytosis and solely cytoplasmic Scd5 is not lethal. These findings suggest that nuclear Scd5-Glc7 may perform a nonessential function and that cortical Scd5-Glc7 location is not required for its essential dephosphorylation of endocytic substrates.

V. NUCLEAR GLC7 FUNCTIONS

The majority of Glc7 is found in the nucleus, in particular, the nucleolus (Bloecher and Tatchell, 2000). Chromosome spreads reveal that Glc7 has a global chromatin affinity (Akiyoshi *et al.*, 2009; Hsu *et al.*, 2000). Many Glc7 binding proteins are nuclear localized (Table 2.1); however, many of the nuclear Glc7 activities have not been assigned to a particular Glc7 holoenzyme. Sds22 is an abundant Glc7 binding protein, which has a predominantly nuclear location (Hong *et al.*, 2000; Walsh *et al.*, 2002). The trimeric Sds22–Ypi1–Glc7 complex is thought to transport Glc7 into the nucleus (Bharucha *et al.*, 2008a; Garcia-Gimeno *et al.*, 2003; Pedelini *et al.*, 2007). Indeed, Sds22 and Ypi1 are two of the few Glc7 binding proteins that are essential for yeast viability. RVxF motif containing Ypi1 has a nuclear localization signal and this is required for its activity (Bharucha *et al.*, 2008a). Glc7 and Sds22 do not have recognizable nuclear localization signals; nevertheless, two other pathways for Glc7 nuclear import are possible besides using Ypi1. Glc7 regulatory subunit, Scd5, does enter the nucleus, but this activity is not required for viability (Chang *et al.*, 2006). Additionally, a fusion of the N-terminal 25 residues of Sds22 to β-galactosidase is nuclear localized, showing that Sds22 has intrinsic nuclear targeting activity (Ross-MacDonald *et al.*, 1999).

Ypi1 is homologous to mammalian inhibitor-3, a small heat-stable *in vitro* PP1 inhibitor (Zhang *et al.*, 1998). Similar to I-2, Ypi1 inhibits Glc7 activity *in vitro* and when overexpressed *in vivo* (Garcia-Gimeno *et al.*, 2003; He and Moore, 2005). However, a blanket description of Ypi1 as an inhibitor obscures a more accurate description of its *in vivo* function. First, many PP1 binding proteins act as inhibitors *in vitro* because the binding alters the

PP1 active site and changes its substrate specificity. Therefore, assays using a single substrate may show a reduction in activity, but preference for another substrate may increase. A clear example of this was seen for Sds22 changing the specificity of PP1 from a phosphorylase to a histone H1 preference (Stone *et al.*, 1993). Structural data of MYPT1-PP1 shows one example of how a regulatory subunit accomplishes this feat (Terrak *et al.*, 2004). Second, overexpression of Ypi1, which contains a nuclear localization signal (Pedelini *et al.*, 2007), would be expected to displace Glc7 from the cytoplasm to the nucleus. Therefore, the reduction of glycogen accumulation upon Ypi1 overexpression can be reconciled by displacement of Glc7 from Gac1-Glc7 (Garcia-Gimeno *et al.*, 2003).

A. Glc7 transcriptional regulation

Glc7 activity has negative and positive affects on the production of mature, processed, cytoplasmic mRNA and snoRNA. The only evidence of Glc7 negative regulation of transcription comes from discovery of a hypomorphic *gfa1* allele that required Glc7 attenuation for cell viability (Zheng *et al.*, 1999). *GFA1* encodes glutamine-fructose-6-phosphate amidotransferase, which synthesizes glucosamine-6-phosphate, which is essential for cell wall synthesis. A hunt for mutants dependent on human inhibitor-1 expression for viability yielded a recessive *gfa1* mutant that could survive if Glc7 activity was reduced by various means (Zheng *et al.*, 1999). The hypothesis was that Glc7 dephosphorylation of a transcription factor was required for *GFA1* transcription. Binding sites for Dig1, Ste12, Reb1, and Tec1 transcription factors are found 5′ to *GFA1*. All of these transcription factors are in pathways responsive to protein kinase activity; however, none are known to be deactivated by dephosphorylation. Therefore, the precise details of *GFA1* transcription negative regulation by Glc7 remain unexplained as well as which other genes may be similarly regulated. Glc7 activity is required for meiotic inducer, Ime1, induction (Ramaswamy *et al.*, 1998); again the Glc7 holoenzyme responsible is unknown.

The partially redundant Msn2 and Msn4 transcription factors activate transcription of about 200 genes in response to stress. These stresses, such as heat shock and glucose depletion, reduce phosphorylation of Msn2/4 by cAMP-dependent protein kinase and increase their accumulation in the nucleus to promote transcription (Görner *et al.*, 1998; Jacquet *et al.*, 2003). Nuclear dephosphorylation of Msn2/4 was reported to be via the Glc7-Bud14 holoenzyme, which might be activated by the Ccr4-Not complex (Lenssen *et al.*, 2005). This conclusion was based upon overexpression of Bud14 and is inconsistent with Bud14 reported location at the cell cortex (Knaus *et al.*, 2005). Therefore, Bud14 might be multifunctional or its genetic interaction with the Not complex (Lenssen *et al.*, 2005) could be a case of Bud14 mislocalization upon overexpression. Nevertheless,

Bud14 levels increase upon glucose depletion, which would promote Msn2/4 activation of stress responsive genes.

Like Bud14, two Glc7 regulatory subunits with other well-documented functions appear to have a role in transcriptional regulation. First, there is Ref2, a Glc7 binding protein responsible for Glc7 incorporation into the CPF complex (Section V.B). Ref2-Glc7 is also necessary for *ENA1* transcription in a manner that is distinct from its role in the CPF complex (Ferrer-Dalmau *et al.*, 2010). *ENA1* encodes an ATPase involved in ion homeostasis and Glc7 modulates this process (Ferrer-Dalmau *et al.*, 2010; Williams-Hart *et al.*, 2002). The second example is Gac1-Glc7, which dephosphorylates glycogen metabolic enzymes in the cytoplasm (Wu *et al.*, 2001). Curiously, Gac1 also binds the Hsf1 transcription factor to regulate certain heat-shock responsive genes (Lin and Lis, 1999).

B. Glc7 function in the CPF complex

The 3′ end of mRNA is cleaved and polyadenylated after initial transcription. Both of these activities are catalyzed by the Glc7 containing CPF complex (He and Moore, 2005; Nedea *et al.*, 2008). CPF complex protein Pta1 appears to be a substrate for Glc7 and its dephosphorylation promotes polyadenylation (He and Moore, 2005). When Glc7 is depleted from CPF, the Pta1 and Fip1 subunits are destabilized and the complex loses polyadenylation activity, but not mRNA 3′-end cleavage activity. These data suggest that dephosphorylations catalyzed by Glc7 in this complex control the affinity of several of the subunits to the complex and they are required for polyadenylation activity. Since there are two RVxF domain proteins in CPF, Ref2 and Pta1, there are two distinct Glc7 binding sites. The Pti1 protein does not contain RVxF, so it could bind to Glc7 while either Ref2 or Pta1 was bound.

Export of polyadenylated mRNA from the nucleus requires the RNA binding protein, Npl3, to shuttle between nucleus and cytoplasm. Dephosphorylation of Npl3 in the nucleus by Glc7 increases its mRNA affinity and assists in recruitment of Mtr2 and Mex67, which facilitate nuclear export (Gilbert and Guthrie, 2004). Dephosphorylated Npl3 also stimulates RNA polymerase II elongation by binding to its C-terminal repeats and its binding to the nascent mRNA prevents transcription termination by Rna15 (Dermody *et al.*, 2008). Once in the cytoplasm, Npl3 is phosphorylated on Ser-411 by protein kinase Sky1 and its affinity to mRNA and the other proteins decline. Therefore, mRNA dissociates from Npl3 and the phosphorylated Npl3 migrates back into the nucleus. The phosphorylation status of Npl3 is also used for its autoregulation (Lund *et al.*, 2008). It is unknown whether Glc7 in the CPF complex or a distinct nuclear Glc7 holoenzyme dephosphorylates Npl3 (Gilbert and Guthrie, 2004; He and Moore, 2005).

C. Glc7 promotes microtubule attachment to kinetochores

Soon after PP1 genes were discovered in various organisms, the metaphase arrest trait of PP1 depletion revealed that PP1 was essential for cell cycle progression (Stark, 1996). To ensure faithful segregation of chromosomes, the spindle checkpoint prevents the metaphase to anaphase transition until all chromosomes experience the tension of the mitotic spindle (Lew and Burke, 2003). Multiprotein kinetochores couple centromeric DNA of chromosomes to microtubules of the mitotic spindle. Sister chromatids are coupled by a multiprotein cohesin complex after DNA replication. Kinetochores, which do not sense tension caused by the spindle microtubule force toward the nuclear envelope embedded spindle pole body counteracted by sister chromatid cohesion, emit a signal that prevents separase protease, Esp1, activation required for cohesin cleavage and release of chromatids and anaphase segregation of chromosomes. Clearly, kinetochores unattached to spindle microtubules will cause metaphase cell cycle arrest. Without such arrest, anaphase progression would fail to properly segregate chromatids because they lack bipolar spindle attachment.

Kinetochore proteins must be dephosphorylated by Glc7 for microtubule affinity and a failure in dephosphorylation activates the spindle checkpoint (Bloecher and Tatchell, 1999). In particular, kinetochore proteins, Ndc10 and Dam1, are dephosphorylated by Glc7 (Cheeseman *et al.*, 2002; Sassoon *et al.*, 1999). The Glc7 binding protein, Fin1, targets Glc7 to kinetochores; however, other proteins must also assist in this localization because *fin1* mutants continue to have kinetochore-associated Glc7 and *fin1*Δ mutants are viable (Akiyoshi *et al.*, 2009). Indeed, kinetochore protein Spc105 has an RVxF motif and mammalian PP1 is targeted to kinetochores by binding to the Spc105 homolog, KNL1 (Liu *et al.*, 2010). Fin1 also appears to be a Glc7 substrate and Fin1 dephosphorylation enhances Fin1 kinetochore affinity.

Protein kinase, Ipl1, phosphorylates these kinetochore proteins to enable microtubule detachment in a process that promotes ultimate bipolar attachment of sister chromatids to the spindle (Liu and Lampson, 2009). Therefore, Ipl1 and Glc7 antagonize one another to control the phosphorylation of kinetochore proteins in yeast and orthologs, Aurora and PP1 perform a similar role in mammalian cells (Wang *et al.*, 2008). Both activities are essential for high fidelity chromosome segregation. Glc7 dephosphorylation of kinetochore protein Dam1 promotes mitotic spindle attachment and phosphorylation by Ipl1 promotes detachment (Pinsky *et al.*, 2006). Cycles of attachment and detachment encourage ultimate bipolar attachment. The spindle-induced tension in a productively bipolar-attached kinetochore reduces Dam1 phosphorylation.

Therefore, Glc7 and/or Ipl1 activity is tension regulated; some evidence indicates that Ipl1 is tension regulated (Keating *et al.*, 2009).

Overexpression of Glc7 leads to chromosome gain similar to the trait of *ipl1* mutations (Francisco *et al.*, 1994). Conversely, decreasing Glc7 activity suppresses temperature-sensitive *ipl1* mutations (Francisco *et al.*, 1994; Pinsky *et al.*, 2006). Overexpression of several cytoplasmic Glc7 binding proteins (Bud14, Gip3, Gip4, Scd5, Sol1, Sol2, and Pex31) suppresses *ipl1* by promoting Glc7 translocation from nucleus to cytoplasm (Pinsky *et al.*, 2006). In the screen for high-copy *ipl1* suppressors, three additional genes are noteworthy: *SDS22*, *GLC7Δ186-312*, and *GLC8*. Glc8 will be discussed elsewhere (Section VI.A). Sds22 is predominantly a nuclear protein (Peggie *et al.*, 2002); therefore, its overexpression should not cause Glc7 displacement from the nucleus. High-copy Sds22 suppression of *ipl1* could be explained by Glc7 dislocation from one nucleoplasmic location to another. Alternatively, excess Sds22 could compete for limited Sds22 binding sites on Sds22–Glc7 substrates. We have evidence of this latter mechanism (Ghosh and Cannon, submitted for publication). Glc7 attenuation by the dominant-negative Glc7Δ186-312 has been seen previously (Francisco *et al.*, 1994; Wek *et al.*, 1992). Curiously, the PP1 hydrophobic groove that binds RVxF motif regulatory subunits has been deleted in this truncation (Egloff *et al.*, 1997; Terrak *et al.*, 2004). Therefore, Glc7Δ186-312 is most likely competing with regulatory subunits without an RVxF motif; among the nuclear Glc7 regulatory subunits, only Sds22 and Pti1 fit these criteria.

D. Glc7 reverses cell cycle checkpoints

Cell cycle checkpoints halt cell cycle progression until specific conditions exist. Such machinery improves the fidelity of cell division by ensuring cell cycle steps have a defined order. Protein phosphorylation and other posttranslational transactions are exploited in checkpoint regulation. Protein phosphorylation promotes cell cycle arrest for all studied checkpoints. Currently, Glc7 dephosphorylation has been implicated in reversing two mitotic cell cycle checkpoints: the spindle checkpoint and DNA damage checkpoint; and one meiotic checkpoint.

As indicated above (Section V.C), the spindle checkpoint halts the cell cycle in metaphase until all chromosomes achieve a bipolar attachment to the mitotic spindle. Sister chromatids without tension generate a signal using Bub1,3, Ipl1, Mad1,2,3, and Mps1 to inhibit the ubiquitin-dependent elimination of the separase inhibitor, Pds1 (Kang and Yu, 2009). This collection of checkpoint components includes several protein kinases. Glc7 overexpression induces chromosome missegregation; a trait of spindle checkpoint bypass (Francisco *et al.*, 1994; Pinsky *et al.*, 2009). In contrast to *ipl1* mutations, which show a bud bias for nondisjoined

sister chromatids (Tanaka *et al.*, 2002), Glc7 overexpression has no such bias (Pinsky *et al.*, 2009). This is one of several observations that suggest that Glc7 antagonizes more than one of the spindle checkpoint kinases. Pds1 destruction occurred slightly earlier in Glc7 overexpressing cells, which could explain the observed chromosome missegregation. Loss of kinetochore tension or attachment would normally stabilize Pds1; however, when Glc7 was overexpressed, Pds1 was destroyed on schedule. This observation shows that Glc7 is distinctively reversing the spindle checkpoint. Analogously, one of two fission yeast PP1 isoenzymes also silences the spindle checkpoint (Vanoosthuyse and Hardwick, 2009). Particular Glc7 substrates or Glc7 holoenzymes involved in this checkpoint silencing are unknown; however, Fin1-Glc7 is implicated because when Fin1 is overexpressed it causes premature spindle checkpoint silencing (Akiyoshi *et al.*, 2009).

Cell cycle arrest results from various forms of DNA damage. This damage comes in the form of double-stranded breaks (DSBs), base modification, and others that stall DNA replication. A cascade of protein kinases (Mec1, Tel1, Rad53, and Chk1) is activated by DNA damage (Longhese *et al.*, 2006). Hyperphosphorylation of Rad53 is frequently monitored to measure DNA damage checkpoint response as its phosphorylation coincides with cell cycle arrest in response to DNA damage. Histone 2A (Hta2) in the vicinity of damaged DNA is phosphorylated to mark chromatin for recruitment of repairing factors. Once damage is repaired, Rad53, Hta2, and other proteins are dephosphorylated and cell division proceeds. Protein phosphatases, Ptc2, Ptc3, and Pph3, dephosphorylate Rad53 and other proteins in the cascade to recover from several forms of DNA damage to allow resumption of cell division (Heideker *et al.*, 2007). Similarly, Glc7 is specifically involved in recovery after stalled DNA replication caused by deoxynucleoside triphosphate depletion caused by hydroxyurea (Bazzi *et al.*, 2010). It is intriguing and unknown how different forms of DNA damage exploit distinct protein phosphatases to recover. Hta2 appears to be an *in vivo* Glc7 substrate and other proteins in the cascade are also likely substrates. However, the nuclear Glc7 holoenzyme participating in these activities has not been defined.

In addition to the mitotic cell cycle checkpoints above, additional checkpoint machinery operates during meiosis. DSBs formed in meiosis I initiate recombination of homologous chromosomes. Many proteins that respond to mitotic DSBs also halt meiosis until these breaks are repaired by recombination (Roeder and Bailis, 2000). Additional proteins specific to meiosis also participate in this pachytene checkpoint. Meiotic protein, Red1, phosphorylated by protein kinase, Mek1, generates a signal at meiotic recombination sites that inhibits anaphase I. Glc7 dephosphorylates Red1 to allow meiotic progression once recombination is complete (Bailis and Roeder, 2000; Tu *et al.*, 1996). Fpr3 is normally nucleolar-localized, but it escapes

during meiosis and inhibits Glc7 dephosphorylation of Red1 to delay meiosis until recombination is completed (Hochwagen *et al.*, 2005). Fpr3 function is described elsewhere (Section VI.B).

VI. GLOBAL GLC7 REGULATION

Glc7 is a relatively stable protein with a half-life of over 180 min or two generations under rapid growth conditions (Nigavekar *et al.*, 2002). In contrast to PP1 from other organisms, which have a potentially inhibitory C-terminal phosphorylation (Ceulemans and Bollen, 2004), Glc7 has no known posttranslational modifications. While it is conceivable that phosphorylation of Glc7 substrates could be modulated solely by changes in protein kinase activity, ample evidence shows that Glc7 activity variations play a significant role. Indeed, examples of regulatory phosphorylations of several Glc7 regulatory subunits have been described elsewhere in this review. In this section, substrate-independent regulators of Glc7 activity are described. These interacting proteins have the ability to modulate the total cellular activity of Glc7 up (Glc8 and Shp1) or down (Fpr3).

A. Glc8 is a major Glc7 activator

Assays of Glc7 phosphorylase phosphatase revealed that Glc8 is a major activator of Glc7 activity (Nigavekar *et al.*, 2002). This activation does not modulate the Glc7 protein level or stability. Glc8 is not required for yeast viability, but yeast mutants that require Glc8 to live by synthetic lethal screening were all found to contain alleles of *GLC7* (Tan *et al.*, 2003). These alleles, like *glc7-R121K*, were *not* distinguished by a low protein phosphatase activity or altered affinity to Glc7 regulatory subunits (Nigavekar *et al.*, 2002; Ramaswamy *et al.*, 1998). In a screen for *glc7-E101Q* synthetic interactions, *glc8*Δ was not found (Logan *et al.*, 2008). These findings suggest that particular mutations in Glc7 render it dependent on Glc8 function. A hypothesis is that Glc8 modulates Glc7 conformation and the Glc8-dependent *GLC7* alleles encode Glc7 proteins that demand this Glc8 conformation modulatory function to attain a conformation proficient to dephosphorylate substrates essential for viability. These ideas about Glc8 function are inspired by the chaperone function of the mammalian Glc8 ortholog, inhibitor-2 (I-2) (Alessi *et al.*, 1993). I-2 expression in yeast complements the glycogen-deficient trait of *glc8* and allows viability of *glc8 glc7-R121K* cells. Additional evidence of Glc7 regulation by conformational regulation is discussed elsewhere (Section VI.B).

Details of how Glc8 regulates Glc7 or how mammalian I-2 regulates PP1 are enigmatic. Traits of *glc8* mutants and assays of Glc7 phosphorylase phosphatase activity show that Glc8 activates Glc7 *in vivo* (Cannon

et al., 1994; Cui *et al.*, 2004; Nigavekar *et al.*, 2002; Tung *et al.*, 1995). Glc8 also binds and activates protein phosphatase Ppz1, which has some homology to Glc7, but has distinct functions and features (Venturi *et al.*, 2000). *In vivo* Glc8 activation of Glc7 requires phosphorylation on Glc8 Thr-118, which is homologous to I-2 Thr-72. In contrast, Glc8 and I-2 inhibit both Glc7 and mammalian PP1 phosphorylase phosphatase activity *in vitro* (Ceulemans and Bollen, 2004; Peters *et al.*, 1999; Tung *et al.*, 1995). Additionally, high-copy *GLC8* genes mimic *glc8*Δ as far as *ipl1* suppression (Cannon, unpublished data; Pinsky *et al.*, 2006; Tung *et al.*, 1995). Several factors should be considered to rationalize the high-copy *GLC8* and discrepancies between *in vivo* and *in vitro* results. (1) First, the relative stoichiometry of Glc8 and Glc7 is important. *In vitro* studies of I-2 show that it can reduce PP1 activity by inhibition or inactivation (Bollen and Stalmans, 1992; Cohen, 2002). Inhibition is rapid, reversible, and occurs at low I-2 concentrations. Inactivation requires stoichiometric I-2 levels and is slower. The inactivated PP1·I-2 is reactivated when I-2 is phosphorylated and it does not require dissociation. Although Glc8 is one of the more abundant Glc7 binding proteins (Table 2.1), overexpression of Glc8 could probably push it to inactivation. (2) Clearly, the ionic conditions *in vitro* are different from those *in vivo*. Physiological salt concentrations prevent I-2 inactivation (Bollen *et al.*, 1994). (3) Glc8 overexpression could promote Glc7 displacement from the nucleus like other high-copy suppressors of *ipl1* (Pinsky *et al.*, 2006). Glc8 appears to have a perinuclear location (Morcos and Cannon, unpublished data), which strengthens this possibility. In contrast, I-2 is located in several locations including the nucleus and centrosomes (analogous to spindle pole bodies in yeast) (Kakinoki *et al.*, 1997; Leach *et al.*, 2003). (4) Finally, Glc8 may directly activate Ipl1 like the I-2 activation of the mammalian Ipl1 homolog, aurora kinase (Satinover *et al.*, 2004), so the combination of Glc7 inhibition and Ipl1 activation would be very sensitive to the Glc8 level. This activity of Glc8 has not been tested and might only exist in more evolved members of the I-2 family (Li *et al.*, 2007).

X-ray crystallography of PP1·I-2 greatly extends our understanding of the interaction between these two proteins. It shows that PP1 binding induced a helical structure in two I-2 segments (residues 12–17 and 44–56) and lengthened the NMR-observed helix (residues 130–169) (Hurley *et al.*, 2007). Although these I-2 segments contain some conserved residues, sequence similarity is poor to Glc8 and other homologs (Li *et al.*, 2007). A surprising finding from the PP1·I-2 crystallography was that I-2 residues 44–56 bound in the PP1 hydrophobic groove that binds the RVxF motif common to many PP1 regulatory subunits (Egloff *et al.*, 1997). This was unanticipated for two reasons. First, the I-2 sequence docked there was vastly divergent from the canonical RVxF. Second, PP1 RVxF-harboring mammalian regulatory subunits Nek2 and neurabin

bind to PP1·I-2 in heterotrimeric complexes that have phosphatase activity (Eto *et al.*, 2002; Terry-Lorenzo *et al.*, 2002). Therefore, PP1 interaction with I-2 must tolerate loss of I-2 residues 44–56 binding, perhaps compensated by additional hitherto unknown interactions. The longest I-2 helix (residues 130–169) in the PP1·I-2 X-ray structure shows how I-2 inhibits PP1 activity. This segment of I-2 binds to the acidic and hydrophobic channels of PP1, which normally bind to substrates (Hurley *et al.*, 2007). I-2 bound in this location prevents phosphorylated proteins access to the PP1 active site at the juncture of these grooves. Additionally, PP1·I-2 purified from *Escherichia coli* was missing one or two of the metals (Mn^{2+} and Fe^{2+}) considered essential for catalytic activity. Therefore, I-2 inhibits PP1 via substrate competition and metal displacement.

While the PP1·I-2 crystallography adequately explained PP1 inhibition by I-2, it revealed little about the process of PP1 activation except that it would require increased PP1 metal affinity. The reason for this obscurity is that I-2 residues 57–129 were invisible in the crystal because they were too dynamic. Activation of PP1·I-2 requires I-2 Thr-72 phosphorylation within the invisible interval. Based upon previous biochemical findings and this initial crystallographic glimpse of PP1·I-2 structure, somehow I-2 Thr-72 phosphorylation must change the conformation of PP1 to increase metal affinity (to allow catalysis), displace the I-2 residues 130–169 from the PP1 acidic and hydrophobic channels (to allow substrate binding), and still remain bound to PP1. These details of PP1·I-2 activation cannot be deciphered using crystallography alone, additional methods must be used to monitor the dynamic aspects of PP1·I-2 activation at atomic resolution.

Glc8 activates many Glc7 holoenzymes based on the traits of *glc8* cells. For example, the glycogen-deficient trait of *glc8* mutants shows its role in regulating the Reg1 and Gac1 holoenzymes which regulate cytoplasmic metabolism. Isolation of *glc7* mutants, which are synthetically lethal with *glc8*Δ is evidence that Glc8 activates Sds22- and Scd5-Glc7 holoenzymes with functions essential for viability. Nevertheless, Glc8 function is not required for viability in otherwise wild-type cells. Clues to when Glc7 requires assistance by Glc8 is currently only available by analyzing when the cell induces Glc8 or promotes its activating phosphorylation.

The cyclin-dependent protein kinase, Pho85, phosphorylates Glc8 Thr-118 thereby activating it *in vivo* (Tan *et al.*, 2003). Of the 10 cyclins of Pho85, Pcl6 and Pcl7 are the most active in this regard; however, Pcl8 and Pcl10 also have activity. Pcl6 and Pcl7 also activate Pho85 for glycogen synthase and phosphorylase phosphorylation (Wang *et al.*, 2001). Little is known about the regulation of these Pho85 cyclins. Pcl6 is stabilized by

elongin C (Elc1) (Hyman *et al.*, 2002), Pcl7 appears to fluctuate in the cell cycle with a maximum at S phase (Lee *et al.*, 2000), and Pcl10 is induced by galactose (Ren *et al.*, 2000). Both Pcl6 and Pcl7 are phosphorylated *in vivo* (Holt *et al.*, 2009); however, the impact of those modifications is unknown. Glc8 is glucose repressed and both Pcl6 and Glc8 are induced by transient anaerobiosis (Lai *et al.*, 2005; Nigavekar *et al.*, 2002). Together, these data suggest that the Glc8 is induced and activated under stress conditions like nonglucose carbon sources. These are conditions when Glc8 activation of Glc7 may be beneficial.

B. Proline isomerases Fpr3 and Fpr4 are Glc7 inhibitors

Although a majority of Glc7 is found in the nucleolus (Bloecher and Tatchell, 2000), no Glc7 regulatory subunit has been assigned to target to this location, except Fpr3 and Fpr4. In mammals, protein NOM1 has this function (Gunawardena *et al.*, 2008). The related yeast nucleolar proline isomerases, Fpr3 and Fpr4, bind to Glc7 (Cannon, unpublished data; Davey *et al.*, 2000; Ho *et al.*, 2002; Hochwagen *et al.*, 2005), but they do not have the RVxF motif or leucine-rich repeats found in other Glc7 binding proteins.

High-copy *FPR3* or *FPR4* genes attenuate the Glc7 activity involved in meiosis (Hochwagen *et al.*, 2005) and mitosis (Cannon, unpublished data). Proline isomerization is a rate-limiting reaction in protein folding and Glc7, like other PP1 enzymes, has noted folding and solubility difficulty (Alessi *et al.*, 1993; Cannon, unpublished data). Indeed, some steps of Glc7 maturation were revealed by the Glc7 and Reg1 association with ribosome-associated ATPase chaperone, Ssb1, which functions with DnaJ-like protein Zuo1 (Dombek *et al.*, 2004; Gong *et al.*, 2009). Therefore, proteins like Glc8 (described above) and Fpr3,4 are likely to modulate Glc7 activity via conformational modulation. Note that all 13 prolines in Glc7 (312 residues total) are conserved with other PP1 enzymes. Therefore, Fpr3,4 could alter the Glc7 conformation via proline isomerization.

However, Fpr3 might have a chaperone function distinct from its proline isomerase activity like other proline isomerases (Arie *et al.*, 2001; Kramer *et al.*, 2004). Indeed, a *Schizosaccharomyces pombe* Fpr4 ortholog has a histone chaperone activity that does not exploit the proline isomerase activity (Kuzuhara and Horikoshi, 2004). This chaperone activity may function stoichiometrically to prevent substrate aggregation (Arie *et al.*, 2001). The need for elevated Fpr3 levels for Glc7 suppression is consistent with such a stoichiometric function. Reductions of Glc7 levels at high Fpr3 induction could be the result of Fpr3-induced Glc7 aggregation and degradation. Alternatively, increased Fpr3 might escape from the

nucleolus and translocate Glc7 to a subcellular location where it is less stable. This is an area that requires more investigation.

C. Shp1 activates Glc7 by an unknown mechanism

Overexpression of Glc7 is lethal to yeast. Mutations of Shp1 suppress this lethality by attenuating Glc7 activity (Zhang *et al.*, 1995). Note that yeast Shp1 is distinct from mammalian tyrosine phosphatase, SHP1 (Cyster, 1997). There is no evidence that Shp1 physically interacts with Glc7 (Breitkreutz *et al.*, 2008). Therefore, the Shp1 modulation of Glc7 activity appears to be indirect. Shp1 is a Cdc48 adaptor protein that has reported membrane fusion and protein degradation functions (Hartmann-Petersen *et al.*, 2004; Kondo *et al.*, 1997; Schuberth *et al.*, 2004). Shp1 could potentially either promote or inhibit protein degradation (Rumpf and Jentsch, 2006). It is currently unclear how Shp1 functions to suppress Glc7 although the steady-state levels of Glc7 (Zhang *et al.*, 1995), Glc8, Pcl6, or Pcl7 (Cannon, unpublished data) are not affected. One possibility is that Shp1 modulates the activity of Glc7 regulatory proteins by controlling subcellular location or posttranslational modification. More work is needed to clarify Shp1 regulation of Glc7.

VII. CONCLUSIONS

This review attempted to summarize our current knowledge of Glc7 function in *S. cerevisiae*. By bringing powerful genetic, biochemical, and cell biology analysis techniques to bear on budding yeast, we now have an appreciation the diverse cellular arenas in which Glc7 function is exploited. Findings from Glc7 investigations frequently presage or substantially strengthen conclusions about PP1 function in mammals. Glc7 control of kinetochore spindle attachment and reversal of various cell cycle checkpoints are notable examples.

Many questions remain about Glc7 function however. Of particular interest is the degree to which PP1 activity is constant or regulated for controlling the phosphorylation of specific substrates. Numerous examples of protein kinase activity changes are known. Now that several Glc7-regulated processes are known in some detail, a focus on how Glc7 regulatory subunits change the activity of Glc7 holoenzymes would be timely. In particular, questions such as do these regulatory subunits change in abundance, are they altered by posttranslational modifications to alter their impact on Glc7 activity, and what are the inputs to these changes?

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CHAPTER 3

Milliliter-Scale Stirred Tank Reactors for the Cultivation of Microorganisms

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Contents

Abstract

This review focuses on recent developments in the field of miniaturized stirred tank bioreactors for application in high-throughput bioprocess development. Different reactor concepts and their potential for parallel bioprocess development are discussed. A detailed description of important engineering state variables, their measurement at small-scale and their implication for scale-up and scale-down of bioprocesses are given. Examples of two different parallel cultivations at small-scale are presented: one with *Escherichia coli* and the other one with the filamentous microorganism *Streptomyces tendae*. It is shown that results obtained in

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parallelized milliliter-scale stirred tank reactors can be scaled up to the laboratory- and/or pilot-scale in a highly reliable manner. This helps to reduce development times for bioprocesses significantly. Finally, directions for future research are presented.

NOMENCLATURE

A	constant
CFD	computational fluid dynamics
d	stirrer diameter (m)
D	reactor diameter (m)
DCW	dry cell weight (g L^{-1})
DO	dissolved oxygen (%)
GFP	green fluorescence protein
H	filling height of the reactor (m)
HTBD	high-throughput bioprocess design
k_La	oxygen transfer coefficient (s^{-1})
M	torque (N m)
MTP	microtiter plate
n	impeller speed (min^{-1})
Ne	Newton number; power number
OTR	oxygen transfer rate (g L^{-1} h^{-1})
P	power (W)
P_g	gassed power consumption (W)
Re	Reynolds number
STR	stirred tank reactor
V	reaction volume (L)
v_s	superficial gas velocity [=(gas flow rate)/(cross-sectional area of the reactor)] (m s^{-1})
vvm	volumetric air flow per volume of broth per minute
α, β	constants
ΔC	driving force for mass transfer
ε_{max}	maximum local energy dissipation (W kg^{-1})
$\varepsilon_{\varnothing}$	power input per unit mass (W kg^{-1})
η	dynamic viscosity of fluid (Pa s)
ρ	liquid density ($kg\ m^{-3}$)
ω	angular velocity (s^{-1})

I. INTRODUCTION

The development of bioprocesses generally comprises three sequential steps: design of the biocatalyst (screening, characterization, modification), optimization of the reaction conditions (e.g., medium design), and process

development up to the pilot- and production-scale. The first two steps are mostly performed with simple uncontrolled batch reactors like shaken microtiter plates (MTPs) and/or shake flasks, whereas controlled stirred tank reactors (STRs) are used for the development of production processes. Since the number of potential biotechnological reactions has significantly increased throughout the past years, the parallel operation of as many reactors as possible is highly desirable. Furthermore, the evaluation of these reactions under production process conditions in an early stage would be favorable to obtain scalable results.

The STR is still the most important reactor in the biotechnological industry. The scale-up from uncontrolled shaken reactors to highly controlled STR is usually hindered by reaction engineering limitations. Furthermore, the majority of simple batch reactors offers no possibility for online measurement and control of important state variables like pH and dissolved oxygen (DO) concentration. Hence, for the conversion of process results achieved in widely applied parallel systems like MTP and/or shake flasks into pilot- or production-scale reactors, many additional experiments in sequentially operated laboratory STR have to be done. This is an extremely labor-intensive and time-consuming challenge that strongly reduces the throughput and is very often the bottleneck for the development of bioprocesses. This led to the development of various small-scale bioreactor systems for High-Throughput Bioprocess Design (HTBD; Micheletti and Lye, 2006; Weuster-Botz *et al.*, 2007).

This review focuses on milliliter-scale stirred tank bioreactors since they are the method of choice for controlled ''HTBD'' with the same process performances as laboratory- and pilot-scale STRs. Other milliliter-scale reactors like MTP, shake flasks, or bubble columns have been reviewed extensively elsewhere (Betts and Baganz, 2006; Fernandes and Cabral, 2006; Kumar *et al.*, 2004; Weuster-Botz, 2005). Furthermore, systems for the cultivation of mammalian cells are also not reviewed, since they represent a special field of bioprocess engineering.

The review starts with an overview of the most important milliliter-scale STR. Next, important engineering state variables which have been reported for small-scale reactors and their implications on process design and scale-up are discussed. Application examples of cultivations at the small-scale are also presented. In the last part, conclusions as well as an outlook are given.

II. MILLILITER STIRRED TANK REACTORS

A. Stirred tank bioreactors for bacteria and yeast

The advantages of using stirred tank bioreactors for early-stage process development and cell characterization led to many different approaches for scale-down. In this chapter, reactors are described in which the

cultivation of bacteria (e.g., *Escherichia coli*) or yeast (e.g., *Saccharomyces cerevisiae*) have been demonstrated so far. The most important systems and their main characteristics are summarized in Table 3.1. The working volume (100 μL–100 mL) and the degree of parallelization (1–48 parallel bioreactors) vary considerably. In general, two main strategies for the scale-down of bioreactors can be identified: first small-scale STR that is geometrically similar to laboratory- and pilot-scale STR and second is new reactor concepts that have been especially developed for small-scale cultivations.

Miniature STRs that are geometrically similar to laboratory- and/or pilot-scale STR have been developed by Lamping *et al.* (2003), Betts *et al.* (2006), and Gill *et al.* (2008a). These types of reactors are normally equipped with miniaturized spargers and Rushton turbines as impellers. Online state variables such as pH and DO can be monitored using small probes that are either mounted on the headplate or at the bottom of the bioreactors. Other probes, for example, for optical density, have also been reported (Gill *et al.*, 2008a). One advantage of small-scale conventional bioreactors is that known scale-up principles from STR can be applied, since geometrical characteristics (e.g., aspect ratio H/D or d/D) and process parameters (e.g., aeration rate) can be kept constant throughout the different scales. Mostly, fluid dynamics will also be similar. Furthermore, an almost identical process setup can be applied since all components from the larger scale also exist at the small-scale. Gill *et al.* (2008a) showed that *E. coli* and *Bacillus subtilis* can be grown with good reproducibility in their system and that the results can in principle be scaled up to a liter-scale bioreactor.

One of the major drawbacks of the mentioned miniature STR is the difficulty to highly parallelize these types of reactors, because no technically convincing and inexpensive method has been found so far to, for example, control gas flow in the range of mL min^{-1}, dose titration agents, or feed substrates. Furthermore, the connection of all tubing and electrical connectors is very time consuming and limits the parallel operation of many reactors. The cleaning of small-scale equipment can also be relatively labor intensive.

Weuster-Botz *et al.* (2002) introduced parallel stirred-columns at the 200 mL scale which are equipped with one magnetically driven Rushton turbine and run with an impeller speed of 100–900 min^{-1}. The system is a hybrid between a conventional bubble-column and a STR. Parallel operations of up to 16 columns with online measurement of pH, DO, and substrate feeding have been reported. A further parallelization of the system might, however, be difficult with regard to the above-mentioned problems known from small-scale conventional STR (e.g., connecting tubing and operating all the probes).

TABLE 3.1 Overview of the miniature stirred tank bioreactors that have been reported for the cultivation of microorganisms and their key specifications

Device	Reference	Working volume [mL]	Impeller type	Maximum, k_La [s^{-1}]	Number of parallel reactors	Maximum reported DCW [g L^{-1}]	Published growth of microorganisms
Bioreaction block	Puskeiler *et al.* (2005a), Weuster-Botz *et al.* (2005)	8–14	Magnetically driven gas-inducing impeller	>0.4	48	36.9 (*E. coli*)	*E. coli*, *B. subtilis*, *S. cerevisiae*
Bioreaction block for mycelium and pellet-forming microorganisms	Hortsch *et al.* (2010)	8–12	Magnetically driven paddle impeller	0.15	48	20	*S. tendae*
Cellstation	Kostov *et al.* (2001), Harms *et al.* (2006)	35	Motor driven stirrer bar	0.1	12	~ 3	*E. coli*
Microbioreactor	van Leeuwen *et al.* (2010)	0.1	Magnetically driven stirrer bar	N/A	2	~ 6	*C. utilis*
Miniature stirred bioreactor	Betts *et al.* (2006), Lamping *et al.* (2003)	7	3 motor driven six-bladed Rushton turbines	0.13	1	4	*E. coli*
Miniature stirred bioreactor system	Gill *et al.* (2008a)	100	Magnetically driven six-bladed Rushton turbine	0.11	16	9 (*B. subtilis*)	*E. coli*, *B. subtilis*
Multiplexed microbioreactor system	Szita *et al.* (2005), Zhang *et al.* (2006)	0.15	Magnetic stirrer bar	0.02	8	~3 (*E. coli*)	*E. coli*, *S. cerevisiae*
Parallel-operated stirred-columns	Weuster-Botz *et al.* (2002)	200	Magnetically driven six-bladed Rushton turbine	0.34	16	N/A	*E. coli*

A completely new approach for the miniaturization of bioreactors was the development of a STR on a milliliter-scale using a gas-inducing impeller for mixing and oxygen supply (Puskeiler *et al.*, 2005a). Up to 48 of the disposable bioreactors can be operated in parallel, with a working volume of 8–15 mL, using a magnetic inductive drive in a bioreaction block which also contains heat exchangers, a head-space cooler, and a sterile gas cover (Weuster-Botz *et al.*, 2005). Figure 3.1 shows the principle of the gas-inducing impeller. The impeller rotates on a hollow shaft with impeller speeds in the range of 1800–3000 min^{-1}. Due to the rotation, gas is sucked in via the hollow shaft from the head space of the bioreactor and medium is sucked in from the bottom. The gas- and liquid phases are mixed in the center of the impeller and are transported through the diagonal outward pumping channels, ensuring an even and sufficient oxygen supply throughout the whole reactor. Thus, separate sparging and controlling of a gas flow is not necessary. The reactors are equipped with baffles to enhance turbulence in the liquid. Fluorometric sensors for pH and DO are integrated in the bottom of each bioreactor, allowing online monitoring and control of these important state variables. The readout is performed by six fluorescence readers, each with eight separate

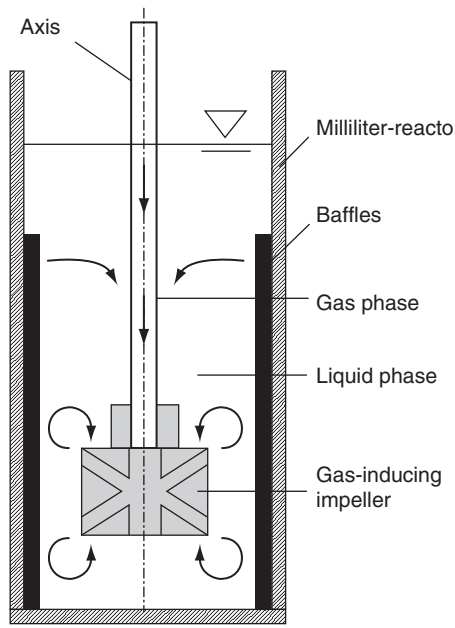

FIGURE 3.1 Principle of the gas-inducing impeller for the cultivation of microorganisms on a milliliter-scale. The magnetically driven impeller rotates on a hollow shaft and sucks in the gas phase which is then dispersed into the culture medium.

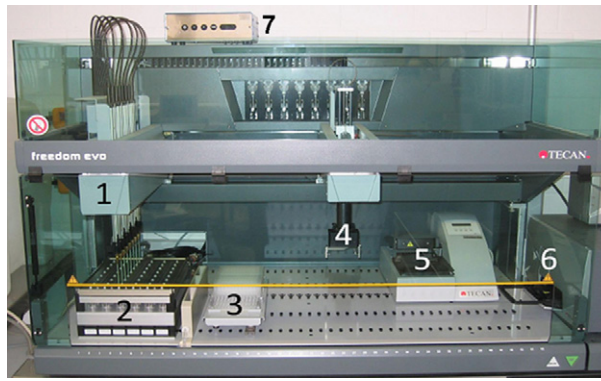

FIGURE 3.2 Bioreaction block with 48 parallel bioreactors integrated in a liquid-handling system for automatic sampling and process control. 1: Liquid-handler; 2: Bioreaction block; 3: Carrier for MTP; 4: Robotic arm for MTP; 5: MTP washer; 6: MTP reader; 7: Impeller control unit.

excitation light sources and eight receiver photodiodes that are placed beneath the bioreaction block. Figure 3.2 shows the bioreaction block in an automated experimental setup with a liquid-handling system. The liquid-handler can be used to automatically take samples as well as for realizing fed-batch processes and controlling pH individually for every single reactor. An additional MTP photometer allows the at-line analysis of, for example, optical density, substrate, and/or product concentrations (Knorr *et al.*, 2007; Vester *et al.*, 2009). Cultivation of different types of microorganisms with good reproducibility and the possibility to scale-up the results obtained in the milliliter-scale have been published for this bioreactor system (see also Section IV).

Another novel system offers 12 parallel STRs with a nominal volume of 35 mL attached to a rotating carousel which allows sequential sampling and monitoring (Harms *et al.*, 2006). Each reactor can be stirred independently with impeller speeds in the range of 10–1000 min^{-1} and oxygen is introduced via surface aeration. For online monitoring, optical sensor patches for pH, DO, and green fluorescence protein (GFP) are attached at the bottom of each reactor (Harms *et al.*, 2002; Kostov *et al.*, 2001). No automatic feeding of substrates or titration agents has been reported so far.

A further downsizing of stirred bioreactors with a working volume of 150 μL has been published by Szita *et al.* (2005) and Zhang *et al.* (2006). The system uses magnetically driven stirrer bars for good mixing with impeller speeds in the range of 200–800 min^{-1}. Oxygen is introduced via surface aeration through a gas-permeable membrane (Zanzotto *et al.*, 2004). Online variables such as pH and DO are measured with fluorescent

sensor spots and optical density can be determined with optical transmission measurement (Zanzotto *et al.*, 2006). The authors showed parallel operation of eight reactors with good reproducibility of the cultivations.

Recently, van Leeuwen *et al.* (2009) introduced a small reactor based on the geometry of a well of an MTP with a working volume of 100 μL. A small magnetically driven stirrer bar run at 200 min^{-1} is used for homogenization of the liquid phase. The headspace of each reactor is flushed with humidified air/oxygen and surface aeration takes place. An electrochemical sensor array is used for online monitoring of pH, DO, and biomass via conductivity (van Leeuwen *et al.*, 2010). Additionally, the produced CO_2 can be monitored by stripping it from the exhaust gas with a scrubber where a change in conductivity can be measured (van Leeuwen *et al.*, 2009). This method, however, seems to be difficult to parallelize for many reactors.

Since the working volumes of the two latter approaches are very small, continuous sampling is not possible. This limits the possibility for offline analysis, for example, HPLC. Furthermore, substrate feeding and/or pH control might be difficult because very small volumes on a nanoliter-scale would have to be added to the reactors. On the other hand, the small size permits a high degree of parallelization and combination with automated microscale processing techniques such as liquid handling robots that can further reduce labor intensity (Lye *et al.*, 2003).

B. Stirred tank bioreactors for filamentous microorganisms

In addition to bacteria and yeast, mycelium- and pellet-forming microorganisms are another important group of industrial organisms because they produce the majority of antibiotics, perform many biotransformations, and are increasingly used for the expression of heterologous proteins, Parallel bioprocess development is especially important for these microorganisms since process times often exceed 100 h. Here, the operation of parallel STRs will thus have the potential to reduce process development times drastically.

However, the cultivation of mycelium- and pellet-forming microorganisms at small-scale requires special consideration, because the complex morphology of the cells and process parameters affects each other. In fact, shear forces and their distribution inside the reactor play an important role in these cultivations since they can have an influence on the morphology of the cells and subsequently alter their productivity (Smith *et al.*, 1990). Another important issue is the strong increase of the viscosity of the culture broth, especially if mycelium is formed. A non-Newtonian shear-thinning behavior can be observed (viscosity decreases with increasing shear rates). The reason for this behavior is the intertwined mycelial structure which reversibly gets pulled apart and aligns if the

shear rate is increased (Nienow, 1990). This influences the reactor performance with respect to mixing, heat, and mass transfer processes. Oxygen transfer is especially likely to become a limiting factor since the oxygen transfer coefficient can decrease significantly during cultivation of filamentous microorganisms (Badino *et al.*, 2001). Finally, the extensive wall growth of such microorganisms poses a large challenge, especially in small-scale stirred tank bioreactors with a high surface-to-volume ratio. For none of the above described reactor systems (see Section II.A), it has so far been demonstrated that they can be used to cultivate mycelium- and pellet-forming microorganisms.

Only one mL-scale STR for mycelium-forming microorganisms has been recently published by Hortsch *et al.* (2010). The system uses a novel magnetically driven paddle impeller that rotates with impeller speeds in the range of 600–1600 min^{-1} in an unbaffled reaction vessel with a working volume of 8–12 mL (Fig. 3.3). Due to the rotation of the impeller, a lamella is formed which spreads out along the reactor wall. Thus, an enhanced surface-to-volume ratio of the liquid phase is generated where oxygen is introduced via surface aeration. Furthermore, the fast moving liquid lamella efficiently prevents wall growth. The impeller and the bioreactor are designed to geometrically fit into the bioreaction block described by Weuster-Botz *et al.* (2005) where the operation of 48 disposable parallel STRs is possible with online measurement and control of DO and pH.

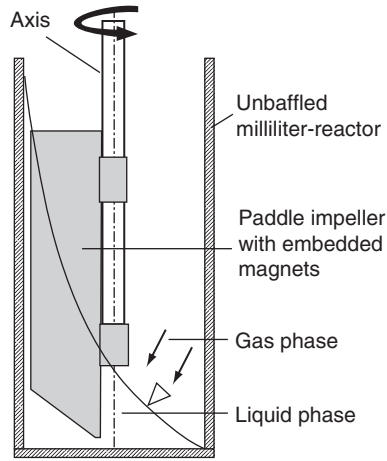

FIGURE 3.3 Scheme of a paddle impeller for the cultivation of mycelium and pellet-forming microorganisms on a milliliter-scale. The magnetically driven one-sided paddle impeller rotates freely on an axis in an unbaffled reaction vessel and forms a fast rotating liquid lamella.

III. ENGINEERING CONSIDERATIONS

A. Oxygen transfer

Sufficient oxygen supply is crucial for most microbial cultivations. Mostly the oxygen transfer capacity of the small-scale reactor systems is the limiting variable in aerobic processes. The oxygen transfer rate (OTR) is defined as follows:

$$\mathrm{OTR} = k_\mathrm{L}a\Delta C. \tag{3.1}$$

The driving force for mass transfer (ΔC) is the difference between the oxygen concentration in the liquid at the gas–liquid interface (e.g., air bubbles) and the oxygen concentration in the bulk liquid phase (culture broth), whereas the volumetric oxygen mass transfer coefficient ($k_\mathrm{L}a$) is characteristic for each bioreactor system. Hence, under the same process conditions, a higher $k_\mathrm{L}a$ also results in a higher OTR.

In technical STR, $k_\mathrm{L}a$ between 0.05 and 0.3 s^{-1} can be achieved under standard process conditions (Middleton, 1985; Van't Riet, 1979). The scale-up of aerobic bioprocesses is often done by keeping the $k_\mathrm{L}a$ constant throughout the different scales to avoid oxygen limitation. However, reported $k_\mathrm{L}a$ of the various small-scale bioreactors vary considerably, ranging from 0.02 to 0.4 s^{-1} (Table 3.1). This has to be carefully taken into consideration during process design at small-scale. Surface aerated systems normally have a significantly smaller $k_\mathrm{L}a$ compared to a system where bubble aeration takes place because of the smaller gas/liquid exchange area.

Different methods for measuring $k_\mathrm{L}a$ in STRs have been reported that can in general also be applied to small-scale reactors (Linek *et al.*, 1987, 1989, 1990; Puskeiler and Weuster-Botz, 2005). Small optical or chemical oxygen sensors with low response times are normally used for the online measurement of the DO concentrations.

The highest $k_\mathrm{L}a$ in milliliter-scale stirred tank bioreactors have so far been reported by Puskeiler *et al.* (2005a) with a gas-inducing impeller. Figure 3.4 shows the measured $k_\mathrm{L}a$ of this system as a function of the impeller speed, compared to values measured in a laboratory STR. The $k_\mathrm{L}a$ increases with increasing impeller speed as more gas is sucked in via the hollow shaft from the headspace of the reactors. The achievable $k_\mathrm{L}a$ are even higher compared to laboratory and pilot STR, making the system useful for a broad range of aerobic bioprocesses and even at high cell density cultivations (Puskeiler *et al.*, 2005b).

In STR, the following empirical correlation for $k_\mathrm{L}a$ is usually applied, with the gassed power consumption (P_g), reaction volume (V), superficial gas velocity (v_s), and the empirical constants A, α, and β:

$$k_\mathrm{L}a = A\left(\frac{P_\mathrm{g}}{V}\right)^{\alpha} v_\mathrm{S}^{\beta}. \tag{3.2}$$

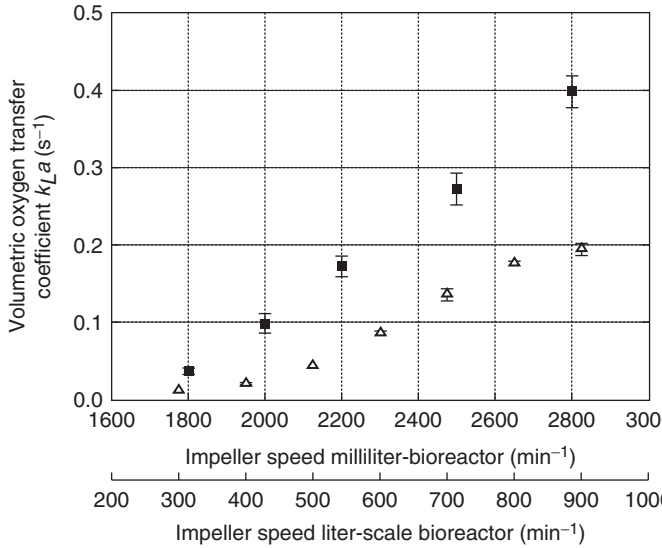

FIGURE 3.4 Volumetric oxygen mass transfer coefficient ($k_L a$) as a function of impeller speed for a milliliter-scale stirred tank bioreactor equipped with a gas-inducing impeller (■) ($V = 12$ mL) in comparison to a liter-scale stirred tank bioreactor equipped with Rushton turbines (Δ) ($V = 2000$ mL; 2 vvm; bioreactor: KLF2000, Bioengineering AG, Wald, Switzerland) in 0.5 M Na_2SO_4 using the dynamic sulfite method.

The applicability of Eq. (3.2) for milliliter-scale STRs has been demonstrated by Lamping *et al.* (2003) and Gill *et al.* (2008b), who both found a good correlation between calculated and measured data. The correlation can be useful for the design and scale-up of bioprocesses, but it can only be applied for small-scale reactors that are geometrically similar to conventional STR and where gas is introduced via active sparging.

During the cultivation of filamentous microorganisms, the viscosity of the culture broth increases significantly, resulting in a decrease of the $k_L a$ because the viscous media offers resistance to oxygen transfer from the gaseous to the liquid phase and hinders the dissipation of gas bubbles. Specific data on $k_L a$ in viscous Newtonian and non-Newtonian fluids in milliliter-scale STR have not been reported so far. Hortsch *et al.* (2010) mention that their surface aerated system has advantages compared to bubble aerated systems since the specific gas/liquid exchange area is only slightly affected by an increasing viscosity. However, it is known from standard STR that $k_L a$ can decrease up to 20 times during cultivations with filamentous microorganisms (Badino *et al.*, 2001), and similar behavior in milliliter-scale reactors can be assumed.

B. Power input

The volume-related power input is a further important process variable that is often used for the scale-up or scale-down of bioprocesses. It can have a significant influence on the morphology and/or aggregate structure of microorganisms or substances that are involved in the cultivations (Juesten *et al.*, 1996). Furthermore, in large-scale cultivations, the available power of the drive is very often the limiting parameter and has to be taken into account, especially in viscous media (Junker *et al.*, 2008).

To measure the power requirement of stirrers in liquid media, usually the generated torque (M) is measured and power can be calculated as follows with angular velocity (ω) and impeller speed (n):

$$P = M\omega = M2\pi n. \tag{3.3}$$

In milliliter-scale reactors very low values of power and torque, respectively, have to be measured, thus specific sensor devices and experimental setups are necessary. Gill *et al.* (2008b) as well as Hortsch and Weuster-Botz (2010) both used special small-scale torque sensors that can measure torques in the mN m range to characterize their milliliter-scale stirred tank bioreactors.

Figure 3.5 shows the measured power consumption of a milliliter-scale stirred tank bioreactor in comparison to a standard STR. Power

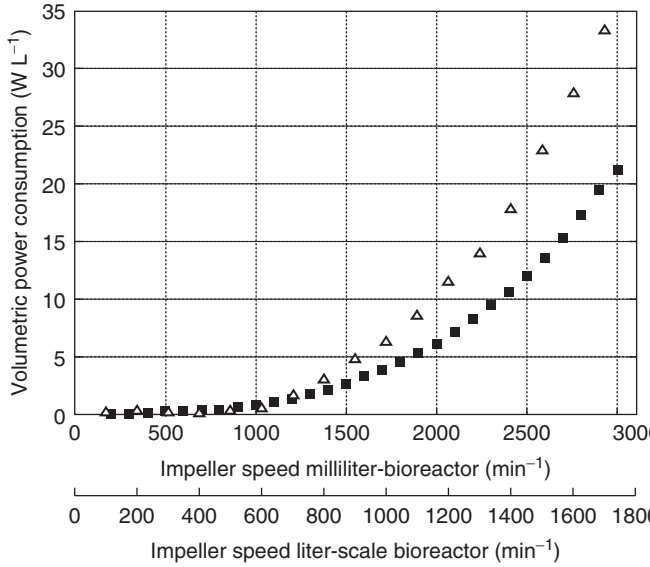

FIGURE 3.5 Volumetric power consumption as a function of impeller speed for a milliliter-scale stirred tank bioreactor equipped with a gas-inducing impeller (■) (V = 12 mL) in comparison to a liter-scale stirred tank bioreactor equipped with Rushton turbines (Δ) (V = 2000 mL; bioreactor: KLF2000, Bioengineering AG, Wald, Switzerland) in water.

consumption increases as expected with increasing impeller speed. On both scales, the same characteristics and similar power consumptions can be measured. With the help of these data, a reliable scale-up is possible by adjusting the impeller speeds on both scales to keep the relevant power inputs constant.

The power consumption in aerated systems is always lower than in unaerated systems because of the lower density of the medium and due to the formation of cavities behind the impeller blades (Hewitt and Nienow, 2007). For standard STRs, empirical equations to calculate the gassed power consumption can be found (Zlokarnik, 2005), whereas on the milliliter-scale gassed power consumptions can be determined experimentally as it has been shown by Gill *et al.* (2008b) and Hortsch and Weuster-Botz (2010).

The volumetric power input of small-scale reactors may also be estimated by using computational fluid dynamics (CFD), as published by Lamping *et al.* (2003) and Puskeiler *et al.* (2005b). However, the simulated data always have to be interpreted with caution, since some approaches tend to underestimate the power input (Gentric *et al.*, 2005).

The volumetric power consumption can be described in a nondimensional form with the Newton number (power number *Ne*) and Reynolds number (*Re*) calculated as follows:

$$Ne = \frac{P}{\rho n^3 d^5} \tag{3.4}$$

and

$$Re = \frac{\rho n d^2}{\eta} \tag{3.5}$$

The obtained power characteristic (Newton number as a function of the Reynolds number) helps to identify flow regimes in reactors. In reaction vessels equipped with baffles two flow regimes can be identified: the *laminar* flow regime where *Ne* decreases linearly with increasing *Re* and the *turbulent* flow regime where *Ne* is independent of *Re*. Since stirred tank bioreactors are usually operated in the turbulent flow regime, it is important to know the Reynolds number at which the changeover of the flow regimes begins, as well as the corresponding Newton number. It has to be pointed out that the power characteristic has to be measured individually for every bioreactor system since it depends on the type of impeller used as well as on the geometrical setup of the whole reactor.

For water-like fluids, Gill *et al.* (2008b) reported turbulent flow for $Re > 8000$ with $Ne = 3.5$ for their milliliter-scale STR. Hortsch and Weuster-Botz (2010) measured a similar Newton number of $Ne = 3.3$ and turbulent flow for $Re > 3000$. Both values are close to the commonly

estimated Newton number of $Ne = 4–5$ for six-bladed Rushton turbines (Zlokarnik, 2005). The results show the advantage of the stirred milliliter-scale bioreactors, since the power characteristic is similar to a standard STR. From an engineering point of view, scale-up of fermentations from these types of bioreactors should be much more precise and reliable compared to shaken screening systems like MTP or shake flasks.

C. Maximum local energy dissipation

Power is not distributed uniformly into the reaction medium in bioreactors. Hence the maximum local energy dissipation (ε_{max}) is the critical process parameter to describe the hydromechanical forces in reactors (Henzler, 2000; Hinze, 1955). The order of magnitude of this parameter has to be known to avoid large discrepancies between different scales. This is especially important for processes where agglomerations or mass transfer limitations occur, and for cultivations of microorganisms with varying morphology because, here, depending on the maximum local energy dissipation, either pellets or mycelium may be formed (Weuster-Botz, 2005).

The direct measurement of this parameter is extremely difficult, therefore indirect model particle systems are commonly used (Hoffmann *et al.*, 1992). In miniaturized bioreactors, almost no data concerning the maximum local energy dissipation are available so far. The only quantitative data have been reported by Hortsch and Weuster-Botz (2010) and Hortsch *et al.* (2010) for their milliliter-scale bioreactor systems. They used a clay/polymer flocculation system where the particle size of the flocs decreases with time due to shear forces in the fluid until an equilibrium particle size is reached. The measured equilibrium particle diameter is hence a function of the hydromechanical forces in bioreactors. The maximum local energy dissipation in the milliliter-scale stirred tank bioreactors is often reduced at the same mean power input per unit mass ($\varepsilon_{ø}$) compared to standard STR. The power input per unit mass ($\varepsilon_{ø}$) can be calculated as follows:

$$\varepsilon_{ø} = \frac{P}{\rho V}. \tag{3.6}$$

This gives values of $\varepsilon_{max}/\varepsilon_{ø} \approx 10$ (Hortsch and Weuster-Botz, 2010) and $\varepsilon_{max}/\varepsilon_{ø} \approx 6$ (Hortsch *et al.*, 2010), respectively, compared to $\varepsilon_{max}/\varepsilon_{ø} \approx 16$ usually reported for laboratory-scale STR. Hence, the milliliter impellers distribute power more homogenously in the reaction medium. This behavior is in good agreement with literature where a decreasing $\varepsilon_{max}/\varepsilon_{ø}$ with increasing ratio of impeller diameter to reactor diameter is reported (Henzler, 2000). Especially, the impeller developed by Hortsch *et al.* (2010) ensures a homogenous distribution of the power in the liquid

which is advantageous for shear sensitive microorganisms like, for example, fungi or actinomycetes. Based on experimental data, impeller speeds can be easily adjusted to achieve the same maximum local energy dissipation at different scales.

IV. APPLICATION EXAMPLES

In this chapter, two different parallel cultivations in milliliter-scale STRs will be described.

The first example is the cultivation of *E. coli* BL21(DE3) in the milliliter-scale system described by Puskeiler *et al.* (2005a) and Kusterer *et al.* (2008). In this cultivation, 48 parallel STRs were operated at a working volume of 10 mL with complex medium to ensure good growth of the microorganisms. The critical process parameter for this cultivation is the volumetric oxygen transfer coefficient k_La (see also Section III.A) since high cell densities and hence a high oxygen uptake by the microorganisms takes place. The optical sensors for DO and pH at the bottom of each reactor ensure a continuous online monitoring of these important process variables and enable control of DO by changing the impeller speed. Furthermore, the optical density of the medium was measured at-line by automatically taking samples every 15 min with a liquid handler and analyzing them in an MTP reader.

Figure 3.6 shows the results of the growth of *E. coli*. The mean dry cell weights (DCWs; estimated based on the optical density) of the batch cultivations showed a typical growth curve (Fig. 3.6A). Due to the high oxygen transfer capability of the system, DCWs of more than 14 g L^{-1} were obtained within 4 h. The standard deviation (indicated by the error bars) between the 48 parallel reactors was small ($\pm 7\%$). Hence a very good reproducibility of the cultivations was observed.

With the help of optical sensors, the reduction in DO concentrations was monitored online (Fig. 3.6B). The signal decreased from 100% air saturation down to about 5% at a process time of 2.5 h. At this process time, the stirrer speed was increased from 2800 to 2900 min^{-1} to avoid oxygen limited growth. The DO subsequently increases rapidly to $>20\%$. At the end of the process, the DO continuously increases again due to exhaustion of the main carbon sources in the medium. The standard deviation of the DO signals in all 48 bioreactors was small and nearly constant throughout the whole process ($\pm 5\%$ air saturation). This example shows the advantage of controlled milliliter-scale cultivations in advanced bioreactor systems compared to simple uncontrolled batch cultivations, where oxygen-limited growth cannot be detected online, and thus oxygen limitation cannot be avoided.

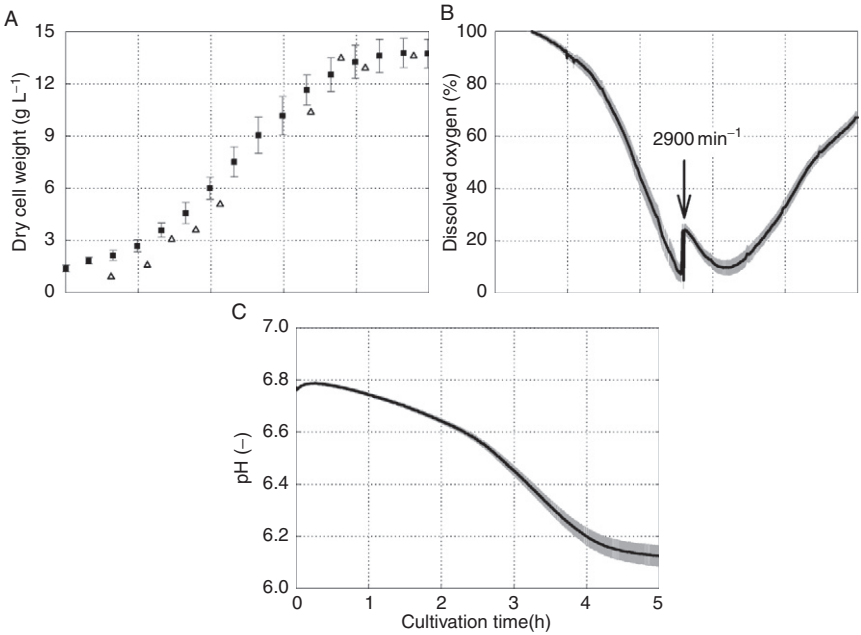

FIGURE 3.6 Forty-eight parallel batch cultivations of *Escherichia coli* on a milliliter-scale in a bioreaction block equipped with gas-inducing impellers ($V = 10$ mL, $T = 37$ °C). Mean and standard deviation of at-line measured dry cell weight concentrations of the milliliter-scale cultivations (■) in comparison to the reference batch cultivation in a pilot-scale stirred tank bioreactor equipped with Rushton turbines (Δ) ($V = 23$ L, $T = 37$ °C) (A). Mean and standard deviation (gray area) of online measured dissolved oxygen (B) and pH (C) as function of process time in the milliliter-scale stirred tank bioreactors.

The pH of all 48 parallel batch cultivations is shown in Fig. 3.6C. The online signal decreases from pH 6.8 to 6.1 at the end of the process due to the production of acetic acid. The standard deviation is very small at the beginning of the process and increases slightly at the end of the process to a standard deviation of 0.04. There was no pH control in the example shown, this can however be easily done with the help of a liquid handler for pH sensitive processes.

The second example describes the cultivation of the mycelium-forming actinomycete *Streptomyces tendae* (*S. tendae*) in the milliliter-bioreactor described by Hortsch *et al.* (2010). *S. tendae* exhibits the typical behavior of a filamentous microorganism like variable morphology, shear-thinning culture broth, and extensive wall growth. Furthermore, *S. tendae* is able to produce the pharmaceutically interesting fungicide nikkomycin Z (a competitive inhibitor of chitin synthase), which recently entered clinical Phase IIa (Nix *et al.*, 2009).

In contrast to most bacterial cultivations, the volumetric power consumption or the maximum local energy dissipation is often used as a scale-up criterion for processes involving filamentous microorganisms. It is important to keep the morphology of the microorganisms the same throughout the stages. Another reason is the available power of the drive, which is very often the limiting process parameter in large-scale cultivations, especially in highly viscous media (Junker *et al.*, 2008).

The parallel unbaffled milliliter-bioreactors were operated with 10 mL complex culture medium and impeller speed was set to 1200 min ^{1}, corresponding to a mean power input of ~3 W L ^{1}. Figure 3.7 shows the offline measured state variables of the milliliter-scale cultivations compared to a 200-fold bigger laboratory-scale STR. Almost the same biomass concentrations were measured on both scales with a maximum DCW concentration of ~20 g L ^{1} (Fig. 3.7A). Due to the high biomass concentrations, the rheological behavior of the culture broth changes from a Newtonian aqueous solution at the beginning to a highly viscous

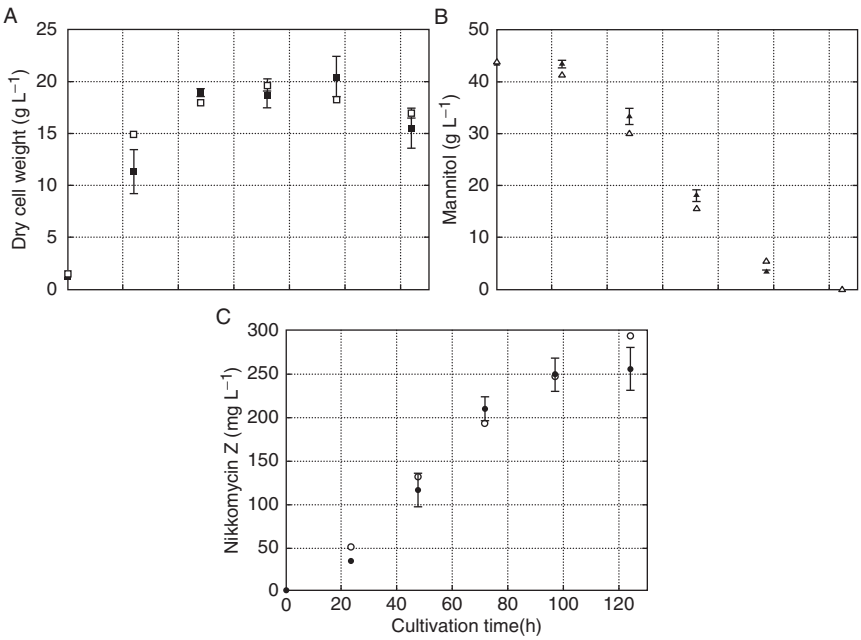

FIGURE 3.7 Mean of dry cell weight concentration (■;□) (A), mannitol concentration (▲;Δ) (B), and produced nikkomycin Z (●;○) (C) during parallel cultivations of *Streptomyces tendae* W42-0 in 12 mL-scale stirred tank bioreactors (closed symbols; $n = 1200$ min^{-1}; $V = 10$ mL; $T = 29$ °C) compared to the reference cultivation in one L-scale stirred tank bioreactor equipped with Rushton turbines (open symbols; $n = 800$ min^{-1}; $V = 2000$ mL; $T = 29$ °C).

non-Newtonian shear-thinning broth for the rest of the process. Throughout the whole process, no oxygen limitation or extensive wall growth of the microorganisms was observed.

The carbon source mannitol was metabolized after the growth phase, which was finished at a process time of about 30 h and the production of the fungicide nikkomycin Z started (Fig. 3.7B and C). Once more, the measured concentrations were in very good agreement on both scales and the same process kinetics were observed. *S. tendae* produced up to about 300 mg L^{-1} nikkomycin Z at the milliliter- and liter-scale with high parallel reproducibility, indicated by the small error bars in the graphs. This example shows that cultivation and scale-up of bioprocesses with mycelium-forming microorganisms is possible. This is especially important for processes running over cultivation times of 100 h and more, as it would be the case here. Thus, process development times can be reduced drastically by these milliliter-scale stirred tank bioreactors.

V. CONCLUSIONS AND FUTURE PROSPECTS

The recent developments in the field of milliliter-scale stirred bioreactors described in this review clearly demonstrate the usefulness of such systems for ''HTBD.'' The reduced reaction volume, the parallelization, and the automation of stirred tank bioreactors have the potential to significantly reduce process development times and assure a cost efficient bioprocess design. In future more and more automated, fully monitored and controlled milliliter-scale reactors will be available, where almost the same process performances as in laboratory and pilot-scale reactors will be possible.

The development and optimization of new microanalytical methods for online or at-line measurement and consequently control of important state variables like, for example, DO, pH, or optical density especially enables cultivations comparable to conventional laboratory- and pilot-scale stirred tank bioreactors. Highly advanced systems even offer the possibility to run and optimize fed-batch processes on a milliliter-scale by, for example, combining the small-scale system with a liquid handler. This is important since the majority of industrial bioprocesses are run in fed-batch mode. Furthermore, the use of disposable miniaturized bioreactors become more and more popular because, especially on the small-scale, cleaning can be a major obstacle to the whole process. Furthermore, disposable reactors are in general increasingly used for bacterial cultivations (Eibl *et al.*, 2010).

However, it has to be stated that for many milliliter-systems there is still a lack of knowledge of important engineering state variables. The volumetric power input and maximum local energy dissipation have only

been reported for a few systems. For a robust scale-up, the characteristics and the limits of parallel reaction systems must be known and taken into consideration. More scientific work on scale-up and/or scale-down issues and their application remains necessary.

In the last years, an increasing number of alternative approaches for the parallel cultivation of microorganisms in microfluidic devices on a microliter-scale were published (e.g., Balagadde *et al.*, 2005; Lee *et al.*, 2006; Maerkl, 2009; Steinhaus *et al.*, 2007). Originally, most of these chip-platforms were used for medical purposes and/or the cultivation of mammalian cells, but several devices are available for the cultivation of bacteria and yeast (Schäpper *et al.*, 2009). The main advantage is the cost-efficient highly parallelized cultivation of microorganisms. Due to progress made in the analytical equipment, online process monitoring may also become possible for such small liquid volumes. However, the scale-up and scale-down capabilities of these reactors with respect to ''technical'' cultivations in standard STRs remain unclear and important engineering parameters are not known. To date, these systems therefore constitute a useful tool for automated screening tasks, but seem less suitable for process development. Finally, the development of complementary miniaturized downstream processing technology is necessary as the number of parallel cultivations increases and only little work on product recovery and purification has been published so far (Jackson *et al.*, 2006; Shapiro *et al.*, 2009).

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CHAPTER 4

Type I Interferon Modulates the Battle of Host Immune System Against Viruses

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Abstract

Type I interferon (IFN), as its name implies, 'interferes' with virus replication by activating numerous genes. Further, virus-induced type I IFN regulates the magnitude and functions of cells directing the host immune system. Importantly, recent exploration into how type I IFN operates following virus infection has advanced our understanding of its role with respect to modulation of host innate and adaptive immune responses. Such activities include the activation of antigen-presenting dendritic cells and the localization, expansion or differentiation of virus-specific T lymphocytes and antibody-producing B lymphocytes. However, type I IFN not only benefits the host but can also induce unnecessary or extremely pathogenic immune responses. This review focuses on such interactions and the manner in which type I IFN induces dynamic changes in the host immune network, particularly adaptive immune responses to viral invasion. Manipulating the type I IFN-mediated host immune response during virus infections could provide new immunotherapeutic interventions to remedy viral diseases and implement more effective and sustainable type I IFN therapy.

I. INTRODUCTION

Type I interferon (IFN) is renowned as the most powerful of antiviral molecules, because it effectively obstructs the replication of numerous viruses. Its inhibitory activity was initially uncovered by Isaacs and Lindenmann (1957), who coined the name (interfere + -on). Since then, its potency has been reaffirmed in multiple experimental systems *in vitro* and *in vivo* and occasionally even in virus-infected humans. The success of genetics technology for creating knockout (ko) mice deficient in type I IFN signaling highlighted the importance of type I IFN for protection of the host from virus-induced pathogenicity *in vivo*. Type I IFN is a cytokine family, which includes IFN-α (13 subtypes in mice), IFN-β, IFN-ε, IFN-κ, and IFN-ω; therefore, deleting the gene encoding the known receptor subunit (IFNAR1) for all these components blocked the activity of the entire system (Muller *et al.*, 1994). Mice lacking the cognate receptor for type I IFN became highly susceptible to severe infections with multiple viruses such as vesicular stomatitis virus (VSV), Semliki Forest virus (Muller *et al.*, 1994), and the A/WSN/33 strain of influenza virus (Garcia-Sastre *et al.*, 1998). Similarly, other strains of influenza virus propagated much faster in mice deficient in a single IFN-β gene with higher virulence when compared to wild-type (wt) mice (Koerner *et al.*, 2007). Although viruses appear to have devised multiple strategies to evade or counteract the type I IFN response, the results obtained from experiments with IFNAR ko mice confirmed that the type I IFN system is required for effective host protection from diverse viral diseases. Multiple research

fields deal with type I IFN responses to virus infections. These specialties include investigations of (1) molecular mechanisms for type I IFN induction pathways, such as recognition of viral components by Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide oligomerization domain (NOD)-like receptors (NLRs); (2) the JAK/STAT signaling pathway; (3) IFN-stimulated genes (ISGs) such as PKR; (4) viral evasion strategies of type I IFN induction, signaling, or function of ISGs; and (5) the effect of type I IFN on host adaptive immune responses. This review documents recent results from work on type I IFN's interaction with the host immune system, particularly the responses of dendritic cells (DCs) and T and B lymphocytes to virus infections.

II. REGULATION OF DC RESPONSES BY TYPE I IFN RELEASED FOLLOWING VIRUS INFECTIONS

A. Production of type I IFN from DCs

Identification of plasmacytoid DCs (PDCs) as major IFN-producing cells (IPCs) has advanced study of IFN generation and the specification of cell types that mediate its synthesis (Colonna *et al.*, 2004). Although all nucleated cells can produce type I IFN, PDCs are especially abundant producers upon virus infection (Barchet *et al.*, 2005; Fig. 4.1A). They synthesize up to 10 pg of type I IFN/cell and are 10–100-fold more efficient than other cell types. For example, the antiviral activity of type I IFN-producing PDCs was compared to that of myeloid DCs. The myeloid DCs were shown to be susceptible to H5N1 influenza virus infection, and that susceptibility was reversed by pretreatment with type I IFN, whereas type I IFN-producing PDCs were resistant to the viral infection (Thitithanyanont *et al.*, 2007). Similarly, after infection with the coronavirus, mouse hepatitis virus (MHV), splenic PDCs, but not myeloid DCs, produced significant amount of type I IFN and rapidly contained MHV replication (Cervantes-Barragan *et al.*, 2007). However, the expression of IFNAR on macrophages and conventional DCs was pivotal for the control of fatal cytopathic MHV infection (Cervantes-Barragan *et al.*, 2009). These findings suggest that PDCs release type I IFN, which acts directly on conventional DCs and macrophages to protect the host from coronaviral pathogenicity. Depletion of PDCs by injecting mice with antibodies that react with a bone marrow (BM) stromal cell antigen 2 (BST-2), such as 120G8 or PDC antigen-1 (PDCA-1), substantially decreased the level of type I IFN following virus infection *in vivo* and increased the animals' susceptibility to this infection (Swiecki and Colonna, 2010). Further, the CpG-dependent OVA-specific CD8 T cell response in spleens was

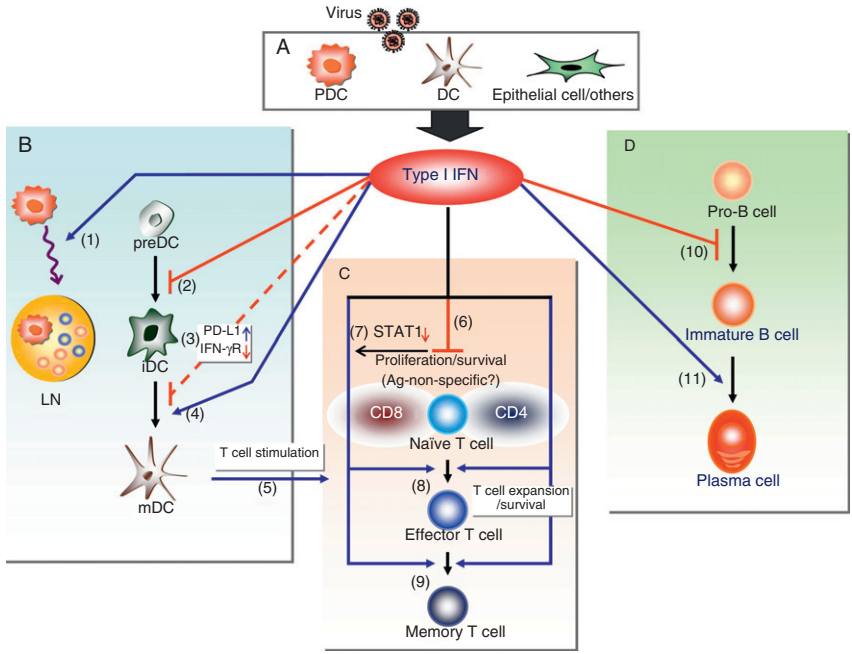

FIGURE 4.1 The effect of type I IFN on differentiation and function of DCs, T cells, and B cells. (A) Following virus infection, cells including PDCs, DCs, and epithelial cells produce type I IFN, which affects the differentiation and function of DCs (B), T cells (C), and B cells (D). B. Type I IFN promotes migration of PDCs (1) as well as transit of T and B cells into lymph nodes leading to transient lymphopenia in the bloodstream (not shown); inhibits development of committed immature DCs (iDC) from precursor DCs (preDC) via STAT2-specific signaling (2); could inhibit DC function by increasing PD-L1 expression or downregulating the receptor for IFN-γ (3); increases the maturation of DCs (4) and upgrades the capacity of mature DCs (mDC) to stimulate T cells (5). C. Type I IFN inhibits T cell proliferation and induces T cell apoptosis under certain conditions (6); virus-specific CD8 T cells, but not CD4 T cells, display low levels of STAT1 and evade type I IFN's antiproliferative activity (7); type I IFN is critical for viral antigen-specific T cell expansion and survival (8), and the formation of memory T cells (9). D. The effect of type I IFN on B cell responses: type I IFN inhibits the development of B cells (10) but enhances antibody-mediated B cell responses to viral infections and the differentiation of B cells into plasma cells (11).

drastically impaired by depletion of PDCs from mice treated with 120G8 antibody (Honda *et al.*, 2005), revealing the importance of PDCs for the induction of adaptive immunity. However, although BST-2 is exclusively expressed on PDCs and plasma cells, it was inducible on multiple cell types by type I or type II IFN or by virus infection (Blasius *et al.*, 2006),

prompting reevaluation of results from experiments with antibody-mediated PDC depletion (Swiecki and Colonna, 2010).

PDCs express large amounts of IRF7; the expression and activation of IRF7 are essential for type I IFN production (Honda *et al.*, 2005). These cells respond efficiently to stimulation with TLR7 or TLR9 ligands by producing type I IFN massively in murine systems. However, following infection with lymphocytic choriomeningitis virus (LCMV), PDC depletion failed to abrogate type I IFN production (Dalod *et al.*, 2002). Indeed, myeloid DCs ($CD11c^{high}Ly6C^{}B220^{}$) isolated from spleens of mice infected with LCMV Clone 13 (Cl 13) released high levels of type I IFN (Diebold *et al.*, 2003). Similarly, influenza virus lacking NS1 as well as synthetic dsRNA poly(I:C) induced substantial amounts of type I IFN from splenic DCs or BM-derived DCs in GM-CSF-supplemented culture, indicating that non-PDCs could become plentiful IFN producers under certain circumstances such as specific virus infections. Measles virus (MV) was reported to block signaling for type I IFN induction mediated by TLR7 and TLR9 on PDCs (Schlender *et al.*, 2005), whereas type I IFN's synthesis was detected when GM-CSF-driven conventional DCs were infected with MV (Hahm, 2009). Thus, the nature of infecting viruses strongly affects the cell types synthesizing type I IFN and the amount produced.

Type I IFN is upregulated and detected in sera for several days upon LCMV Cl 13 infection *in vivo* (Zuniga *et al.*, 2007). However, the cytokine's presence is nearly undetectable in sera during chronic infection, although the virus might continue to replicate in cells and, thus, persist in the host. This process is attributable to the negative regulatory circuits of type I IFN synthesis such as activation of suppressor of cytokine signaling-3 (SOCS-3) viral inhibition of type I IFN signaling, that is, suppression of the host's innate immune response (Martinez-Sobrido *et al.*, 2009), persistence of the virus in a specific target tissue, and/or decreased virus amplification efficiency during the state of persistence. However, when DCs from spleens were isolated at 30 days postinfection with LCMV Cl 13, which preferentially infects DCs via α-dystroglycan receptor, type I IFN mRNA was detected in those DCs, suggesting the sustained local production of type I IFN by the infected cells during viral persistence (Hahm *et al.*, 2005; Truong *et al.*, 2009). Type I IFN was suggested to inhibit DC development, presumably explaining the decreased number of DCs observed during the LCMV Cl 13 persistence (Lee *et al.*, 2009). However, elevated IFN-β contributes to the upregulation of MHC class I detected in the CNS and the peripheral tissues during LCMV persistence and seemed to continuously display antiviral activity, since the virus titer increased in IFNAR-deficient LCMV carrier mice (Truong *et al.*, 2009). Thus, the precise role of locally produced type I IFN during chronic virus infections needs further investigation.

B. Dual opposite effects of type I IFN on DC development versus DC maturation

DCs are the most potent of all antigen-presenting cells and adept at priming naïve T cells (Steinman, 2007). Following the recognition of viral components, DCs mature, migrate to secondary lymphoid organs, and interact with antigen-specific T cells to stimulate them. The capacity of DCs to prime T cells is strongly influenced by the former's maturation status. DC maturation is determined mainly by the expression level of costimulatory molecules such as B7-1, B7-2 and CD40, and MHC molecules (MHC-I and MHC-II). Type I IFN was reported to enhance DC maturation, since the treatment of DCs with type I IFN increased the amounts of those proteins on the surfaces of DCs (Fig. 4.2) and rendered them more efficient in stimulating T cells. Exposure of human conventional DCs and PDCs to recombinant IFN-β before influenza virus infection enhanced the cells' expression of multiple cytokines and ISGs, indicating that type I IFN can prime DC activation to enhance the immune response to such infection (Phipps-Yonas *et al.*, 2008). In support of the type I IFN's DC stimulatory activity, impaired DC maturation was observed when IFNAR-deficient mice were infected with Newcastle disease virus (NDV; Honda *et al.*, 2003). However, migration of IFNAR-deficient DCs into secondary lymphoid tissues and the expression of CCR7 mRNA on the IFNAR-defective DCs were unaltered by NDV infection. These results suggest that type I IFN is critical for DC maturation but not for DC migration upon NDV infection.

Experiments performed with human monocyte-derived DCs revealed two opposite functions of type I IFN. DCs derived from type I IFN-treated

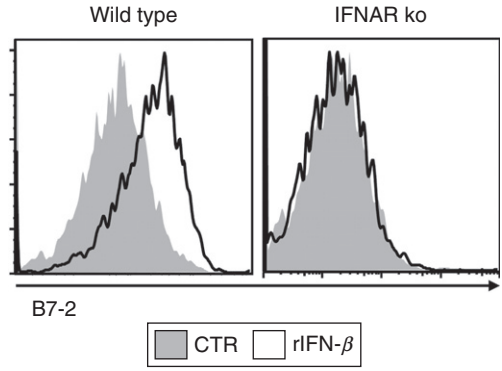

FIGURE 4.2 Type I IFN induces maturation of DCs. Wild-type or IFNAR-deficient BM-derived DCs were untreated (control, CTR) or treated with recombinant IFN-β (rIFN-β) (1000 U/mL). At day 2 after treatment, $CD11c^{+}$ cells were analyzed for the expression of B7-2 by flow cytometry.

human monocytes were not competent in producing IL-12 and inefficient in stimulating T helper (Th) 1 type immune responses (Dauer *et al.*, 2003). In contrast, type I IFN induced a rapid maturation of monocytes into potent DCs that effectively induced IL-15 and promoted a strong Th1 cell response (Santini *et al.*, 2000). Further analysis of murine BM-derived DCs clarified the contrasting effect of type I IFN on DC precursors *versus* committed immature DCs (Hahm *et al.*, 2005). Recombinant IFN-β markedly impaired the development and generation of myeloid DCs or PDCs from DC precursors, whereas it enhanced the maturation of committed immature DCs by elevating the level of MHC molecules and costimulatory molecules (Fig. 4.1B). This result indicates that the differentiation status of DCs (DC precursor vs. committed DC) is a decisive factor in the outcome of type I IFN treatment. Blockade of DC development was also demonstrated by using immune suppressive viruses such as MV and LCMV Cl 13 and was mediated via a STAT1-independent but STAT2-dependent novel IFN signaling pathway. Presumably, these viruses utilize the pathogenic effect of type I IFN via STAT2-specific signaling to suppress the host immune system. Interestingly, induction of a type I IFN-induced protein, adenosine deaminase acting on RNA (ADAR) 1a (p150) is dependent on STAT2 but not STAT1 (George *et al.*, 2008). ADAR1a displays proviral activity by antagonizing PKR activation and enhancing VSV amplification (Li *et al.*, 2010; Nie *et al.*, 2007). Still unknown is whether ADAR1a is induced in DCs to affect the development or activation of DCs upon virus infections.

C. Type I IFN–DC interaction during chronic human virus infections

In the blood of human immunodeficiency virus (HIV)-1-infected donors, the absolute number of myeloid DCs and PDCs was shown to be decreased (Donaghy *et al.*, 2001; Pacanowski *et al.*, 2001). The loss of DC quantity inversely correlated with the viral load in the plasma of HIV patients. Whether the reduction of DC quantity is caused by the inhibition of DC development by type I IFN (Hahm *et al.*, 2005) or by DC apoptosis or necrosis, as observed *in vitro* (Meyers *et al.*, 2007), is uncertain. However, very similar findings were reported when patients persistently infected with hepatitis C virus (HCV) were examined for DC frequency (Kanto *et al.*, 2004) and apoptosis (Siavoshian *et al.*, 2005). An alternative possibility is that the DC quantity in the blood decreased because DCs accumulated, instead, in the secondary lymphoid tissues (Dillon *et al.*, 2008). Although it is unclear whether DC accumulation in the lymph nodes is mediated by type I IFN induced by HIV or HCV infection, type I IFN can cause PDCs to gather in the lymph nodes (Gao *et al.*, 2009).

Viral persistence can be achieved when the pathogen evades or suppresses its host's immune system, especially the DC-mediated adaptive T cell immune response. Eventually, multiple T cells specifically reactive with the infecting virus are deleted or exhausted even though they had expanded soon after infection (Yi *et al.*, 2010). These exhausted T cells become unable to respond to the viral antigen so fail to proliferate, do not synthesize effector cytokines (IL-2, IFN-γ, or TNF-α), and do not perform cytotoxic activity. These exhausted T cells have been detected during chronic infections with HIV, hepatitis B virus (HBV), and HCV and found to express several inhibitory proteins such as programmed death-1 (PD-1), which was initially identified in mice infected with LCMV Cl 13 (Barber *et al.*, 2006). The continual interaction of inhibitory receptor PD-1 on T cells with its ligand PD-L1 on DCs or other cells is critical to sustain T cell exhaustion. In fact, type I IFN treatment was shown to upregulate PD-L1 on DCs during HCV infection (Groschel *et al.*, 2008; Muhlbauer *et al.*, 2006; Urbani *et al.*, 2008; Fig. 4.1B), but why and how type I IFN would induce PD-L1 on DCs remains elusive. Possibly type I IFN produced by the host is designed to induce apoptosis on certain cells via PD-L1 to block viral propagation. Currently, pegylated IFN-α2 and ribavirin are being used as treatment for HCV infection, although this regimen is effective in only approximately 50% of such patients. Tests to discern how PD-L1 blockade affects IFN treatment of patients acutely infected with HCV have provided promising results (Urbani *et al.*, 2008), but further detailed assessment is necessary for patients with chronic infections. Nevertheless, the effectiveness of current antiviral type I IFN therapy could be enhanced by elevating the immune regulatory activity of type I IFN or blocking the adverse pathogenic properties of IFN.

III. TYPE I IFN MODULATION OF T CELL RESPONSES TO VIRUS INFECTIONS

A. Mobilization of T lymphocytes

Upon virus infections, a transient lymphopenia in the bloodstream is frequently observed. This temporary reduction in the quantity of lymphocytes in the blood comes from the swift migration of lymphocytes into secondary lymphoid organs where they take actions in response to pathogenic invasion. Interestingly, transient blood lymphopenia and the subsequent accumulation of T cells as well as B cells and PDCs in the lymph nodes were shown to be largely dependent on type I IFN signaling resulting from viral infection (Gao *et al.*, 2009; Kamphuis *et al.*, 2006). Highly pathogenic H5N1 virus caused depletion of circulating T cells,

which correlated with massive induction of a type I IFN response (Baskin *et al.*, 2009). Since sphingosine 1-phosphate (S1P) receptor signaling is involved in the egress of lymphocytes from lymph nodes (Rosen and Goetzl, 2005), possibly type I IFN acts on S1P signaling to induce lymphocyte retention in lymph nodes (Shiow *et al.*, 2006). However, the lymphopenia in blood following virus infection was shown to be independent of G protein-coupled receptors and chemokines (Kamphuis *et al.*, 2006), excluding the possibility for the involvement of S1P receptor signaling. Thus, further work is needed to reveal the molecular mechanisms of type I IFN-mediated lymphocyte mobilization and the role of lymphocytes' redistribution upon virus infection.

B. Shaping the virus-specific T cell response

Type I IFN was reported to regulate T cell responses positively or negatively yielding contrasting results (Fig. 4.1C). Type I IFN inhibited proliferation of naïve T cells and sensitized T cells for activation-induced cell death *in vitro* (Kaser *et al.*, 1999; Petricoin *et al.*, 1997), whereas this molecule enhanced the expansion of cytotoxic T cells and prolonged the survival of stimulated T cells (Aichele *et al.*, 2006; Biron, 2001; Brinkmann *et al.*, 1993; Marrack *et al.*, 1999). Tough *et al.* showed that the direct injection of mice with poly(I:C) or recombinant IFN-β promoted the generation and survival of $CD44^{hi}CD8^{+}$ cells, indicating bystander memory CD8 T cell amplification and maintenance by virus-induced type I IFN. Recombinant IFN-α/β directly acted on activated CD4 and CD8 T cells to prevent them from undergoing activation-induced death, yet the IFN did not increase the viability of resting T cells (Marrack *et al.*, 1999). In studies of human T cells, recombinant IFN-α drove the development of $CCR7^{high}/CXCR3^{low}$ central memory CD8 T cells (Ramos *et al.*, 2009).

Comparison between IFNAR-intact (wt), and IFNAR-deficient, antigen-specific T cells using LCMV GP33-41 epitope-specific P14 T cell receptor transgenic CD8 T cells extended our understanding of the impact type I IFN signaling has on T cells (Aichele *et al.*, 2006; Kolumam *et al.*, 2005). Upon recognition of LCMV in wt mice so-infected, adoptively transferred IFNAR-deficient P14 T cells retained their proliferative activity and IFN-γ/TNF-α-producing effector functions, but these virus-specific T cells failed to expand efficiently or form memory CD8 cells. Thus, the intactness of type I IFN signaling on CD8 T cells is critical for these cells to survive and expand at the T cell expansion phase but is not necessary for the cells' division *per se*. Although CD8 T cells lacking IFNAR could secrete IFN-γ/TNF-α, the diminished expression of granzyme B from the cells raises a question as to whether type I IFN signaling on T cells is important for complete functional competence of effector CD8 T cells (Kolumam *et al.*, 2005). Further, the expansion of CD8 T cells

deficient in IFNAR appears to be strongly influenced by the pathogenic context of a virus infection. That is, P14 CD8 T cell expansion in wt mice was less dependent on the intactness of type I IFN signaling on the T cells when recombinant vaccinia virus-expressing LCMV glycoprotein was used to infect mice instead of LCMV (Aichele *et al.*, 2006). Similar to CD8 T cells, LCMV-specific CD4 T cells (SMARTA) depended on type I IFN signaling for clonal expansion and survival, but the loss of IFNAR on the CD4 T cells scarcely affected IFN-γ secretion or their capacity to proliferate (Havenar-Daughton *et al.*, 2006). In contrast, upon infection with recombinant *Listeria monocytogenes*-bearing OVA, OVA-reactive CD4 T cells did not require type I IFN signaling for their expansion.

Type I IFN signaling often leads to the inhibition of cell division or induction of cellular apoptosis via transcriptional activation of ISGs. The effectiveness of type I IFN in the treatment of tumors or blockade of viral spread seems to be attributed to this property of type I IFN, since it contributes to removal of unwanted cells (tumor cells and virus-infected cells). This property also accounts for the inhibitory effect of type I IFN on T cells such as inhibition of T cell proliferation and enhanced T cell apoptosis observed under certain conditions. For instance, respiratory syncytial virus (RSV)-induced IFN-α and IFN-λ suppressed CD4 T cell proliferation, and neutralization of cognate receptors for IFN-α and IFN-λ reversed viral inhibition of CD4 T cell division (Chi *et al.*, 2006). Intriguingly, when STAT1 or STAT2 is deficient in T cells, type I IFN does not inhibit mitogen-induced T cell proliferation, but rather facilitates T cells' proliferative activity and enhances T cell viability (Gimeno *et al.*, 2005). Additionally, LCMV-specific $CD8^+$ T cells, but not $CD4^+$ T cells, display a significantly decreased level of STAT1 following LCMV infection, thereby evading type I IFN-mediated antiproliferative activity (Gil *et al.*, 2006; Fig. 4.1C). Therefore, the function of type I IFN is thought to be influenced by multiple factors including the activation/expression of JAK/STAT type I IFN signaling components, the nature of a pathogen, the cytokine milieu, and the cell types in play.

C. CD4 T cell differentiation

Type I IFN also has the feature of affecting CD4 T cell polarization by inducing Th1 cells that produce IFN-γ (Brinkmann *et al.*, 1993; Rogge *et al.*, 1998; Fig. 4.1C). Additionally, type I IFN stimulates activated CD4 T cells to produce IL-10 (Aman *et al.*, 1996). Recent study of human memory CD4 T cells demonstrated that IFN-α acted directly on the memory CD4 T cells in response to recalled antigens and modulated the responses differentially: (1) Upon challenge with tuberculin purified protein derivative, type I IFN enhanced proliferation of CD4 memory T cells with elevated IFN-γ production relative to IL-10 secretion. (2) Challenge with tetanus toxoid

protein or influenza A hemagglutinin protein resulted in slightly inhibited cellular proliferation by type I IFN as well as a decreased ratio of IFN-γ/IL-10 (Gallagher *et al.*, 2009).

IV. TYPE I IFN INTERACTION WITH B CELLS

A. B cell development and migration

At an early stage of B cell development, type I IFN was reported to inhibit IL-7-induced growth of pre-B cells and induce apoptosis of the cells *in vitro* (Wang *et al.*, 1995). Moreover, the development of $CD19^+$ pro-B cells and their B lineage progeny was impaired when newborn mice were injected with IFN-α2/α1 hybrid molecule (Lin *et al.*, 1998). To a similar but lesser extent, type I IFN also inhibited the development of pro-T cells. Other experiments employing oral administration of type I IFN into mice yielded a drastic reduction of B cell numbers and minor alteration of the T cell population in the spleens of these animals (Bosio *et al.*, 2001). Thus, these previous observations suggest that type I IFN inhibits early B cell survival and development and may alter T cells as well (Fig. 4.1D).

Type I IFN can also affect the mobilization of B cells. A recent study showed that type I IFN was produced from PDCs in autoimmune BXD2 mice and contributed to follicular entry of marginal zone precursor B cells in secondary lymphoid organs, promoting antigen transport (Wang *et al.*, 2010). Further, IFN-α enhanced the chemotaxis of human B cells to CCL20, CCL21, and CXCL12, by regulating chemokine receptor signaling and decreasing ligand-induced chemokine receptor internalization (Badr *et al.*, 2005). On the contrary, IFN-β suppressed the migration of B cells purified from spleens of mice to CCL19, CCL21, and CXCL12 in a transwell experiment (Chang *et al.*, 2007). Type I IFN signaling appeared important for B cell accumulation in the lymph nodes early after influenza virus infection by upregulating CD69 on B cells. Studies of the role of virus-induced type I IFN in the development and migration of B lymphocytes, and its impact on viral pathogenesis, however, remain incomplete.

B. B cell-mediated host immune responses

In contrast to the inhibitory effects of type I IFN on B cells at their early developmental stage, several lines of evidence indicate that type I IFN enhances the function of committed B cells in mediating immune responses to virus infections. Type I IFN enhanced CD69 and B7-2 on B cells in lymph nodes upon influenza virus infection, suggesting that type I IFN induces an early signal for B cell accumulation at these sites and mediates B7-2-induced local IgG secretion (Chang *et al.*, 2007;

Coro *et al.*, 2006; Rau *et al.*, 2009). Importantly, intact IFNAR signaling on B cells was shown to be critical for ensuring a sufficient quantity and quality of local antibody production in response to influenza (Coro *et al.*, 2006). Selective deletion of the IFNAR on either T cells or B cells inhibited the type I IFN-mediated antibody response to a soluble protein antigen (Le Bon *et al.*, 2006), denoting that type I IFN signaling on B and T cells contributes to the IFN-mediated stimulation of antibody responses. When BDCA4-coupled magnetic beads were used to deplete type I IFN-producing human PDCs from human peripheral blood mononuclear cells (PBMC), the cells' production of IgG specific for influenza virus was abrogated (Jego *et al.*, 2003). Also, local B cell activation in the draining lymph nodes after West Nile virus infection was dependent on signals through the type I IFN receptor (Purtha *et al.*, 2008). Following VSV infection, genetic deletion of IFNAR on VSV-specific B cells resulted in significant impairment of plasma cell formation and the antiviral IgM response (Fink *et al.*, 2006). Enhancement of human plasma cell differentiation by type I IFN was also demonstrated by a direct treatment with IFN-α; neutralization of the IFN or IFNAR on the cells nullified type I IFN's stimulatory activity (Jego *et al.*, 2003). Further, type I IFN was shown to promote isotype switching and stimulate long-term antibody production and immunological memory in response to chicken gamma globulin (Le Bon *et al.*, 2001; Fig 4.3D). Collectively, these studies underscore the important role of type I IFN in the antibody response and, in particular, antiviral adaptive B cell immunity against virus infections.

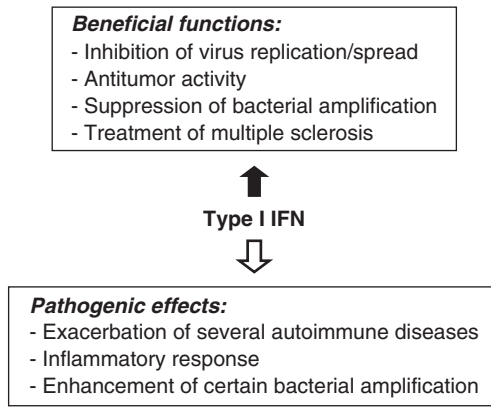

FIGURE 4.3 Diverse functions of type I IFN. Shown are diagrams depicting beneficial or pathogenic activities of type I IFN.

Type I IFN was reported to play a critical role in B cell survival. For instance, signaling by IFNAR was required for enhancement of B cell receptor-dependent B cell responses and increased cellular resistance to Fas-mediated apoptosis (Braun *et al.*, 2002). Badr *et al.* (2010) showed that type I IFN protects both naïve and memory B cells from apoptosis via cellular signaling mechanisms including PI3Kδ/Akt, Rho-A, NFκB, and Bcl-2/Bcl_{XL}.

These stimulatory effects of type I IFN on B cell responses could explain why type I IFN signaling is closely related to the development of autoimmune responses. In support of this notion, type I IFN was shown to promote the proliferation and development of peritoneal B-1 cells, which are an important producer of autoantibodies (Santiago-Raber *et al.*, 2003). As also reported, type I IFN contributed to TLR-mediated naïve B cell activation and antibody production in the absence of B cell receptor engagement (Bekeredjian-Ding *et al.*, 2005; Giordani *et al.*, 2009). Indeed, type I IFN signaling is a factor in strengthening the pathogenesis of systemic lupus erythematosus (Baechler *et al.*, 2003; Thibault *et al.*, 2009).

V. TYPE I IFN IN BACTERIAL PATHOGENESIS

Type I IFN was reported to downregulate the expression of a receptor for type II IFN (IFN-γ) on DCs and macrophages upon *L. monocytogenes* infection, demonstrating that this cytokine can magnify the pathogenicity of bacterial infections (Rayamajhi *et al.*, 2010). That is, type I IFN signaling increased the susceptibility of lymphocytes to infection by *L. monocytogenes*, since the IFNAR ko mice were relatively resistant to that infection (Auerbuch *et al.*, 2004; Carrero *et al.*, 2004; O'Connell *et al.*, 2004). Similarly, the IFNAR ko mice were more resistant to *Francisella novicida* infection than their wt counterparts (Henry *et al.*, 2010). Additionally, influenza virus-induced type I IFN appeared to impair production of the neutrophil chemoattractants KC (Cxcl1) and Mip2 (Cxcl2), rendering the infected mice highly susceptible to bacterial superinfection (Shahangian *et al.*, 2009). These findings were surprising because type I IFN previously proved to protect victims from bacterial as well as viral infections, as evidenced when type I IFN inhibited the replication of *Legionella pneumophila* (Opitz *et al.*, 2006) and facilitated the clearance of *Leishmania* infection (Mattner *et al.*, 2004). Possibly type I IFN incites harmful immune responses in pathogenic conditions and at certain locations (Vilcek, 2006; Fig. 4.3).

VI. PERSPECTIVES

Type I IFN has been used as clinical treatment for such diseases as hepatitis, hairy cell leukemia, condyloma acuminatum, multiple sclerosis, and Kaposi sarcoma (Pitha and Kunzi, 2007; Vilcek, 2006). However, type I IFN is occasionally associated with the induction or exacerbation of such autoimmune diseases as systemic lupus erythematosus and insulin-dependent diabetes mellitus, implying this treatment's pathogenic potential (Baccala *et al.*, 2005; Kunzi and Pitha, 2003; Theofilopoulos *et al.*, 2005). Direct administration of type I IFN has also induced flu-like symptoms in humans, indicative of a harmful inflammatory immune response (Vilcek, 1984, 2006). Interestingly, although several doses of IFN-αA (100, 1000, or 10,000 IU) inhibited virus replication to similar extents, only small amounts (100 IU), but not high doses (1000 or 10,000 IU), of IFN-αA protected mice from lethal influenza virus challenge (Beilharz *et al.*, 2007). These results emphasize the importance of a balanced host immune response in conjunction with the antiviral effect of type I IFN for host protection. Further, if a factor (IFN-mediator) that induces local production of type I IFN is introduced near virus-infected cells, that mediator could block virus propagation locally without causing a systemic inflammatory response *in vivo*. Importantly, the H1N1 influenza virus responsible for 2009s pandemic was described as a weaker inducer of type I IFN from DCs and macrophages than seasonal influenza viruses but was highly sensitive to the IFN's antiviral activity (Osterlund *et al.*, 2010). Thus, discovery of a small molecule(s) or cellular factor(s) that induces local type I IFN synthesis from virus-infected cells, but not from other ordinary cells could yield a product for curing multiple viral diseases.

Although type I IFN's antiviral activity is well established as an inhibitor of viral spread (Isaacs and Lindenmann, 1987; Vilcek, 2006), its regulation of individual immune components that influence innate and adaptive immune responses against invading viral infections requires extensive investigation. That research will not only provide a detailed understanding of host–virus interactions but can also lead to the development of therapeutic interventions to help those afflicted with pathogenic viral diseases.

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