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Protein Folding and Secretion: Mechanistic Insights Advancing Recombinant Protein Production in *S. cerevisiae*

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Abstract

The emergence of genomic approaches coupled to recombinant DNA technologies have identified the quality control systems that regulate proteostasis — biological pathways that modulate protein biogenesis, maturation, trafficking, and degradation. The elucidation of these pathways has become of growing importance in therapeutics as loss of proteostasis has been suggested to lead to a number of human diseases including Alzheimer's, Parkinson's Disease and Type II Diabetes. We anticipate that the most successful strategies for protein expression and therapeutics development may involve integration of protein engineering strategies with host manipulation, to exploit the cell's native stress response pathways and trafficking mechanisms. This review will highlight recent findings and mechanistic detail correlated to quality control in the early secretory pathway of *S. cerevisiae*.

Introduction

Protein-based therapeutics comprise a major class of chemical entities advancing drug discovery efforts, and the largest group of molecules that have been FDA approved in recent years. Here, the term “biopharmaceutical” refers to any recombinant protein or nucleic acid used for therapeutic or *in vivo* diagnostic purposes. In a broader sense, this definition encompasses engineered cells or tissue-based products [1]. Vaccines, hormones, interferons, and antibodies (*e.g.* mAbs and antibody fragments, *i.e.* Fabs, scFv's, and sdAb) are common examples of biopharmaceuticals. With more than 200 products approved for distribution [2], the biopharmaceutical market was estimated between 100 – 120 billion US dollars in 2012 [3], and anticipated revenues are expected to exceed 170 billion US dollars in 2014 [4].

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Competing Interests Statement

The authors declare that they have no competing financial interests.

The model eukaryote, *Saccharomyces cerevisiae*, is the preferred expression platform for a range of recombinant proteins such as insulin precursor [5], human serum albumin [6], hepatitis B vaccine [7], and scFv (Supplemental Table S1). *S. cerevisiae* has several attractive intrinsic characteristics that endorse its use for protein expression, including a well-established genome; ease of cultivation; high protein titers (*e.g.* > 1 g/L); well-developed and easily implemented genetic tools; and significant homology to the protein folding machinery, and cellular and metabolic processes of higher eukaryotes.

While proper folding and secretion of heterologous proteins have been attained for many proteins of interest, these successes have been predominantly a result of trial-and-error genetic manipulations to components within the yeast secretory pathway, reviewed extensively by Delic *et al.* [8]. Recent investigations suggest that the inherent complexity of protein homeostasis in yeast is more elaborate than previously conceived. In fact, prominent modulators of proteostasis include positive and negative feedback loops of stress-inducible responses, as well as chaperone networks (*i.e.*, “chaperome”, [9]) that influence substrate selection, protein folding, conformation, and stability. Specifically within the early secretory pathway, the endoplasmic reticulum (ER) maintains a full complement of molecular chaperone and co-chaperones interactions organized in both space and time (Fig 1). ER protein folding, trafficking, and degradation are highly integrated mechanisms, utilize common machinery, and generate complex signals, which ultimately determine a protein’s fate in multiple compartments [10].

In this review, we discuss stress-induced cell signaling responses of *S. cerevisiae*, as well as the molecular chaperone interactions required for effective ER quality control. Select trafficking mechanisms will be highlighted as they relate to protein and cellular engineering. To facilitate the design of new therapeutic strategies, we conclude with exciting perspectives promoting the future impact of systems biology approaches and metabolic engineering.

Cellular responses — global analyses of transcriptional regulation

Unfolded Protein Response—Approximately one-third of the *S. cerevisiae* proteome transverse the secretory pathway en route to various destinations [11]. Within this pathway, an elaborate quality control system regulates ER homeostasis by ensuring the fidelity of protein synthesis and maturation. Yet, a variety of exogenous and endogenous insults – *e.g.*, chemical treatment, nutrient deprivation, and alterations in redox status or calcium concentration – may overwhelm the ER’s processing capacity. During periods of acute ER stress, aberrant proteins accumulate within the lumen, activating an intracellular signal transduction pathway referred to as the unfolded protein response (UPR). To attain ER homeostasis, the UPR transcriptionally up-regulates genes encoding molecular chaperones, ER-associated degradation (ERAD) machinery (described in more detail later in this review), key enzymes of lipid biosynthesis, and protein transport to other compartments [12].

In yeast, unfolded proteins stimulate the luminal domain of the transmembrane sensor, Ire1p, to initiate its self-association [13,14]; in turn, Ire1p oligomerization facilitates the unconventional splicing of HAC1 mRNA. This spliced RNA variant enables synthesis of the Hac1p transcription factor, which binds to one of three upstream activating sequences, *i.e.*

the unfolded protein response elements (UPREs) [15–17], to alleviate ER stress. To date, Ire1-dependent HAC1 mRNA splicing is the only mechanism identified by which signals from the ER lumen trigger transcriptional changes in yeast. However, the transcription factor Gcn4p and its upstream activator GCN2 also play an essential, modulatory role in the UPR and are required for its induction [17]. Gcn4p, a transcription factor central to the regulation of amino acid biosynthesis [18], binds to Hac1p to form a larger regulatory complex. Patil and coworkers [17] proposed that Gcn4p provides "gain control" by regulating, or modulating, the set point for the UPR, while Hac1p acts as the UPR "switch". Leber *et al.* [19] confirmed that conditions of severe stress (S-UPR) alter the magnitude of the UPR by increasing Hac1p levels via transcriptional regulation. An additional complexity to UPR induction in yeast, identified by Promlek *et al.* [20], is that the transmembrane domain of Ire1p appears to sense membrane aberrancy (*e.g.* a result of lipid or inositol depletion) leading to an active UPR. Ire1p clusters within the ER membrane actively recruiting spliced HAC1 mRNA under conditions of acute ER stress, as indicated by discrete foci in live-cell images [21]; however, conditions of persistent ER stress result in a weak but sustained UPR activation, where Ire1p clusters are dispersed, possibly due to re-associations with BiP (and loss of Ire1p oligomerization) under these conditions [22]. In contrast to its role in UPR activation, the cytosolic kinase domain of Ire1p plays a critical role in stress deactivation. Failure to properly inactivate Ire1p leads to chronic ER stress and reduces cell survival [23]. Collectively, these observations suggest that the UPR serves as a homeostatic feedback loop that integrates signals from multiple sources in a spatially dependent manner, in order to produce a corresponding transcriptional output that finely adjusts protein-folding capacity according to demand.

Although ~5% of the genome (*i.e.* 381 genes) is up-regulated during UPR stress [12], Payne *et al.* [24] demonstrated that ribosomal biogenesis and assembly are translationally repressed – a phenomenon vastly similar to translational attenuation observed in higher eukaryotes following UPR activation. Initial investigations identified UPR targets to the early secretory pathway – specifically the ER or Golgi; while recent reports further revealed an intimate dependence of the late secretory pathway, including export processes and vacuolar function, to achieve homeostatic control [25].

Can ER stress be mitigated by alternative means other than transcriptional regulation? Indeed, ER morphology appears to take many forms while appropriately ameliorating stress. Schuck *et al.* [26] demonstrated that peripheral ER expansion alleviates ER stress independently of the UPR; meanwhile, ER-phagy – an organelle-specific macroautophagy – has been identified in yeast as a means to direct aggregates to the lysosome [27]. Recently, Lipatova *et al.* [28] showed that ER-phagy is regulated by Ypt1p, a key regulator of ER-to-Golgi transport. Even in conditions of extreme duress, the ER stress surveillance pathway (ERSU) ensures the inheritance of a functional ER in budding yeast. The selfless mother cell retains damaged cortical ER but passes undamaged ER to its daughter under the regulation of Slt2p [29].

To promote protein folding and secretion, the UPR is arguably the most well-characterized cellular response in *S. cerevisiae*, yet it affects primarily the early and late secretory pathway. In contrast, the Heat Shock Response (HSR) is predominantly a response to stress

conditions in the cytosol. Analogous to the UPR, the HSR transcriptionally activates molecular chaperones and elements of the ubiquitin-proteasome system in order to relieve cell stress.

Heat Shock Response: The intersection between multiple stress responses—

The Heat Shock Response (HSR) is a highly conserved cellular response to disruptions of proteostasis [30] that impacts many diverse aspects of cell physiology, including oxidant defense, cell-wall remodeling, metabolism, and transport [31]. The primary modulator of HSR is Hsf1p, which recognizes a pentameric heat shock element (HSE) [32] in the promoter regions of target genes. Variations of this non-canonical HSE are widespread in the yeast genome [33], which greatly expands possible Hsf1p targets. Interestingly, the regulation of Hsf1p by posttranslational modifications in higher eukaryotes provides precise and stringent control of the HSR, as opposed to a simple on-off switch [30]. In the model eukaryote *S. cerevisiae*, microarray studies determined that the magnitude of HSR is proportional to the intensity of the stress (*e.g.* temperature shift) [34], thereby implying that the cell is able to detect gradations in temperature, and modulate the transcriptional activation accordingly.

In addition to Hsf1p, thermal stress activates transcription factors Msn2p and its close homolog Msn4p (referred to as Msn2/4). Msn2/4 is regulated by nutrient status and growth phase, *i.e.*, induced after the diauxic shift or in late stationary/quiescent phase of cells [35]. The Msn2/4 regulon targets the stress responsive element (STRE) in the promoter of selected genes [36]. To modulate the HSR, multiple regulatory mechanisms have been proposed recently, including signaling via the cyclic-AMP/protein kinase A pathway [37], Msn2p degradation under periods of prolonged nutrient limitations [38], or Yak1p phosphorylation to activate Msn2/4 under low glucose conditions [39].

While elevated temperature represents the primary insult during the HSR, a secondary consequence involves the production of reactive oxygen species (ROS), *e.g.* hydroperoxides, superoxide anion, thiol-reactive compounds, or heavy metal stress. Oxidative stress reflects an imbalance between the systemic manifestation of ROS and the cell's ability to detoxify reactive intermediates. Therefore, the oxidative stress response (OSR) is defined by transcriptional reprogramming that restores the cell's redox status to a normal range. Skn7p, Msn2/4, Yap1p and its homologs have been identified as transcription factors that play pivotal roles in resistance to oxidative stress [40], whereas gene repression appears to be modulated by the Tup1-Ssn6 complex upon interaction with similar stress response factors, *e.g.* Skn7p and Yap6p [41].

To differentiate the HSR from other stress response pathways, microarray investigations evaluated transcriptional changes in response to heat shock, osmotic stress, and nutrient limitations [40]. These findings establish that ~10% of the genome is remodeled during one or more stresses, highlighting the depth to which stress-based transcription penetrates the global transcriptome [34,42]. Approximately 300 genes were up-regulated, mostly a result of transcription factors Msn2p and Msn4p [34], whereas ~ 600 genes were suppressed. This comprehensive cellular response has now been termed the Environmental Stress Response (ESR), whereas the HSR is considered one subset thereof.

The preceding sections outlined recent progress towards identifying the critical factors of multiple stress response pathways in *S. cerevisiae*, with an emphasis on transcriptional regulation. Recent studies established significant overlap between the HSR and ESR, OSR, and cell wall integrity pathway (CWI, reviewed by Levin [43]). Notably, this overlap potentially explains the phenomenon of “cross-protection” (*i.e.* hormesis), whereby exposure to one stress enhances tolerance of a subsequent stress [40]. In other words, while unstressed cells exhibit moderate resistance to a range of stressors, mildly stressed cells have significantly enhanced tolerance to future insult [44–46].

Quality control mechanisms within the secretory pathway

The high flux of proteins into the ER together with the complicated multidomain nature of secreted proteins necessitates an extremely specialized environment to regulate proteostasis. Aided by resident ER chaperones (*e.g.* Kar2p/BiP, Lhs1p) and foldases (*e.g.* Pdi1p), luminal and membrane proteins fold precisely to their native conformations. In general, the ER chaperone network facilitates proper assembly and minimizes aggregation, whereas co-chaperones (*e.g.* Sec63p, Jem1p, Scj1p) are thought to recruit molecular chaperones based on the needs of the client protein (Fig 1), as recently reviewed [10]. Within the ER, proteins may undergo further posttranslational modifications, including N- and O-glycosylation, disulfide bond formation, and oligomerization, which are often essential for protein quality and function.

The N-glycosylation pathway modifies peptides with oligosaccharide addition at the N4 nitrogen of asparagine in the consensus Asn-Xaa-Ser/Thr sequence, typically located within flexible domains (*e.g.* loops); in yeast, fewer glycosylation sites are located towards the C-termini [47]. This branched (“N-linked”) carbohydrate structure improves protein stability and represents a recognition site during protein folding events, altering the rate of ER exit through interactions with lectin-binding chaperones [48]. High substrate specificity of glucosylidases and oligosaccharyltransferases necessitates finely-tuned spatiotemporal order of glycosylation and protein maturation within the secretory pathway [for review, see 48]. N-glycans in yeast consist of heterologous high-mannose structures (*e.g.* > 50 mannose residues attached [49]), whereas human N-glycans are mainly complex or hybrid-type.

Due to the different N-glycan structure, recombinant therapeutic glycoproteins produced in yeast may cause detrimental effects, including immunogenic reactions and rapid clearance from the bloodstream due to interactions with human mannose receptors. To produce therapeutic glycoproteins, considerable interest has focused on glycan remodeling by disrupting yeast-specific glycosyltransferases (*e.g.* OCH1) that facilitate the loss of hypermannosylated structures [50], or in combination with MNN1 and MNN4 result in an intermediate N-glycan structure identical to human glycoproteins [51,52]. Sequential studies successfully introduced essential genes for the synthesis, transport, and transfer of human sugars in *S. cerevisiae* [51,53–57], enabling all sugar nucleotides to be available for the production of human-type glycans, offering the potential for human-like glycosylation in yeast.

Yeast O-glycosylation is characterized by stepwise addition of shorter glycan structures (*e.g.* < 5 mannose residues) to Ser or Thr, catalyzed initially by O-mannosyltransferases (MT)

[58]. Engineering mammalian O-glycosylation systems in yeast is not as advanced as N-linked glycosylation, as a result of the lethality associated with suppressing O-mannosylation within the host, where the deletion of MT genes affects cell growth and the cell's structural integrity. In contrast to the oligomannosyl-glycans in yeasts, O-glycosylation in humans is mucin-type specific. Recent advancements have introduced humanized yeast platforms by attaining mucin-type glycosylation [54,55] and O-fucosylation [59]. Novel systems incorporating glycan engineering as well as the potential impact for therapeutic design have been meticulously detailed in reviews by De Pourcq *et al.* [60], Chiba and Akeboshi [61], Vogl *et al.* [62]. We believe that engineering approaches utilized in these studies combined with improved methods for detection of the glycoproteome [47] will further the utility of yeast as a platform for pharmaceutical applications including both screening and production.

Intriguing observations recently shed light on the intimate links between N-glycosylation and cell stress pathways. For example, the hexosamine biosynthetic pathway (HBP) generates metabolites for protein N- and O-glycosylation, and ER stress can activate this pathway by an XBP1-linked mechanism in mammalian cells [63,64]. The yeast analog of XBP1, HAC1, is the predominant transcription factor of UPR activation. To our knowledge, causal relationships between yeast glycosylation precursors and HAC1 have not been examined, yet may offer an improved understanding of post-translational modifications and regulation. Under conditions of nutrient deprivation in *S. cerevisiae*, up-regulated proteins will contain clusters of similarly-charged amino acids that replace N-glycosylation. A provocative analysis demonstrated that like-charged residues, in place of N-glycosylation sites, could impart local protein stability and function [65]. Transient interactions with yeast oxidoreductase Ost6p may modulate the modification of N-linked glycosylation sites in direct proximity to cysteine residues [66], suggesting another link protein folding and glycosylation [67].

Yeast Pdi1p is an essential folding catalyst that introduces disulfide bonds into its substrates and rearranges (or reduces) pre-existing ones. Besides its oxidoreductase activity, Pdi1p also acts as a molecular chaperone. In contrast, Ero1p is the major yeast disulfide-generating flavoenzyme that directly transfers disulfide bonds to Pdi1p. Kim and coworkers [68] recently demonstrated that Pdi1p is the key regulator of Ero1p activity, and that a balanced activation/inactivation of Ero1p establishes ER redox homeostasis. Moreover, utilizing an ER-localized redox-sensitive fluorescent protein sensor (*i.e.* eroGFP) to detect real-time changes in ER redox status, Merksamer *et al.* [69] ultimately determined that misfolded proteins trigger oxidative stress.

ER quality control (ERQC) mechanisms play a key role in ER stress tolerance – to detect and eliminate the deleterious effects of aberrant proteins. Three ERAD pathways have been classified by the location of the misfolded lesion, *i.e.* ERAD-L (L, luminal), -C (C, cytosol), and -M (M, membrane). The terminal step of each ERAD pathway incorporates the core ubiquitin-proteasome system (Fig 1), as extensively reviewed [70]. As with most ubiquitin-proteasome substrates, the final destruction signal is polyubiquitin, attached by a specialized E3 ligase (*e.g.* Doa10p and Hrd1p). The Doa10p complex of yeast ubiquitinates ER membrane proteins with malformed cytosolic domains, as well as soluble cytosolic proteins

bearing destruction signals. By analogy, the Hrd1p complex ubiquitinates proteins bearing transmembrane legions or damaged luminal domains. Interestingly, ER lectin Yos9p maintained a critical role in the recognition of luminal misfolded glycoproteins [71,72], whereas Gauss *et al.* [73] recently demonstrated that Pdi1p played a direct role in the clearance of unfolded glycoproteins from the ER via interaction with the mannosidase Htm1p. Consequently, the implication has been that ERAD-L substrates depend not solely on substrate misfolding, but also on the presence of substrate sugars. Contrary to this notion, Denic *et al.* [74] demonstrated that Yos9p, Hrd3p, and Kar2p form a luminal surveillance complex that recognizes aberrant proteins independent of their glycosylation status. Downstream of ERAD substrate recognition, Hrd1p appears to coordinate retrotranslocation and degradation across the ER membrane [75].

It has been accepted widely that ERAD substrates are recognized and processed by ER receptor sites. Alternatively, substrates may traffic to the Golgi before they are retrieved for ERAD. Certain misfolded proteins bypass ERQC in yeast and are transported directly to the vacuole (*i.e.* yeast lysosome) for degradation [76]. This pathway, referred to as the ER-to-vacuole pathway (Fig 2), is used to divert misfolded luminal substrates to the vacuole under conditions of severe stress [77]. This observation begs the question, do alternative modes of membrane or luminal protein secretion exist in lieu of the conventional anterograde transport, *i.e.* ER-to-Golgi? In addition to the ER-to-vacuole pathway, the ER has been implicated in the formation of pre-autophagosomal structure (PAS) and the isolation membrane (IM) of autophagy [78]; cytoplasm-to-vacuole targeting pathway [79]; and ER-mitochondria encounter structures (ERMES) [80] as a means of phospholipid exchange and Ca^{2+} regulation during cell stress [81,82]. Moreover, post-ER quality control mechanisms focused on the trans-Golgi network [83], plasma membrane [84], and endosomal systems [85] also play a role in protein sorting.

Trafficking mechanisms influencing secretion

Because of the complexity of the stress response pathways, efforts to engineer the secretory pathway have met with mixed success, and often, cellular engineering strategies are empirical approaches that work for a given molecule of interest. One area rarely explored in recombinant protein production includes the evaluation of substrate motifs that either retain or facilitate ER secretion. Export and retrieval of secretory proteins occurs via three well-established sorting mechanisms: (i) ER retention of immature proteins mediated by chaperone interactions, (ii) selective uptake by ER-to-Golgi (anterograde) transport of mature cargo by COPII machinery, and (iii) the retrieval of immature proteins that erroneously exited the ER via Golgi-to-ER (retrograde) transport regulated by COPI machinery. Selective uptake occurs at ER exit sites (ERES) [86]. At these unique spatial domains, the COPII complex consisting of Sar1p, Sec23p–Sec24p and Sec13–Sec31p is localized. Notably, export of secretory proteins relies on signal-mediated sorting mechanisms, where Sec24 and its paralogs (*i.e.* Sfb2p/Iss1p and Sfb3p/Lst1p) facilitate the diversity of trafficking motifs [87] denoted in Supplemental Table S2. Cargo proteins that mediate the secretion of luminal proteins include Erv29p, Erv14p, Erv26p, and Emp46p/Emp47p, as reviewed [87]. In contrast to ER exit motifs, ER retention motifs via COPI machinery and export signals corresponding to quality control mechanisms of the trans-

Golgi network [88] are well-established in literature. The implementation of selective trafficking motifs to enhance recombinant protein secretion is a compelling avenue of investigation, recently demonstrated using model substrate, CPY* [89].

Perspective

Improvements in yeast expression systems and engineered strains [90–92] and the rationale design of protein engineering [93] have led to advances in therapeutic protein production *S. cerevisiae* [90,94]. More recently, a systems-level approach (*i.e.* systems biology) has enabled broader metabolic engineering strategies [95–97] to optimize heterologous proteins of biofuels, metabolites, or renewable chemicals in yeast. Despite potential constraints of the tight coupling of many different biochemical pathways, metabolic engineering relies on increasing a flux towards a specific precursor metabolite, typically by the insertion of heterologous pathways (or components) [95]. The insertion of heterologous pathways has also enabled the implementation of a human-like O-glycosylation pathway in *S. cerevisiae* [59], to produce mucin-type human glycoproteins [55].

To achieve a global understanding of how cellular processes and intracellular molecular interactions perform under external and internal perturbations, we believe that the integration of molecular measurements from many hierarchical cellular levels (*e.g.* mRNA, proteins, metabolites, etc.) will begin to elucidate quality control mechanisms and offer control points for heterologous protein production. From a broader perspective, we propose that biological insights of transcriptional regulation can be established via network analyses; genome-wide screening combined with clustering analyses can identify not only promoter elements or regulatory domains, but also key trafficking motifs; whereas intracellular spatial localization of recombinant proteins can be determined in real-time with fluorescent variants [98,99] to identify alternate routes of protein trafficking. To this end, protein and cellular engineering strategies, guided by biological principles and emerging systems approaches, will facilitate optimal yields of biopharmaceuticals in yeast.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Review highlights ER quality control, associated protein interactions, and trafficking motifs
- Describe changes to the UPR and HSR and their impact on heterologous protein production
- Highlight interactions in yeast quality control for endogenous & heterologous protein expression

Cytosol

Figure 1. ER Quality Control Mechanisms

Selected chaperone interactions facilitate ER protein translocation, folding and maturation, and ERAD. Post-translation modifications including disulfide bond formation and glycosylation are also illustrated.

Figure 2. Quality Control in the Secretory Pathway

In addition to ER-to-Golgi secretion, alternate routes of protein trafficking have been established in *S. cerevisiae*. To alleviate ER stress, organelle morphology may be significantly altered by mechanisms such as ER-phagy or the formation of ER-mitochondria encounter structures (ERMES).