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# THE ACTION OF MOLECULAR CHAPERONES IN THE EARLY SECRETORY PATHWAY

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■ **Abstract** The endoplasmic reticulum (ER) serves as a way-station during the biogenesis of nearly all secreted proteins, and associated with or housed within the ER are factors required to catalyze their import into the ER and facilitate their folding. To ensure that only properly folded proteins are secreted and to temper the effects of cellular stress, the ER can target aberrant proteins for degradation and/or adapt to the accumulation of misfolded proteins. Molecular chaperones play critical roles in each of these phenomena.

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## INTRODUCTION

In addition to a number of growth factors and other serum proteins, the liver secretes about 1% of its total weight in albumin each day. Although the secretory capacity of other cells may be lower, every eukaryotic cell exports a variety of proteins either to meet its nutritional needs (proteases and glycanases), for cell-cell communication (growth hormones and pheromones), or for defense (toxins and antibodies). The first compartment in which this heterogeneous assortment of factors matures is the endoplasmic reticulum (ER). In addition, nearly all proteins that ultimately reside in this and other compartments of the secretory pathway or in the plasma membrane are first translocated (imported) into the ER. To fulfill its role as the primary gateway to the secretory pathway, the ER houses enzymes that process polypeptides and catalyze protein folding. Also associated with the ER is a group of molecular chaperones, defined here as proteins that facilitate the folding of nascent polypeptides. In this review, we discuss the mechanism of action of ER-associated chaperones, emphasizing studies undertaken in the yeast *Saccharomyces cerevisiae*. When appropriate, specific experiments are discussed using other organisms.

## MECHANISM OF ACTION OF THE MAJOR CLASSES OF CHAPERONES INVOLVED IN PROTEIN SECRETION

The stress-inducible 70-kDa heat shock proteins (Hsp70s) and the constitutively expressed heat shock cognate proteins (Hsc70s) belong to a family of molecular chaperones that bind and release polypeptides in an ATP-dependent cycle (32). Early partial proteolysis experiments suggested that Hsc70 could be divided into three regions: a 44-kDa amino-terminal ATPase domain and a 15-kDa peptide-binding domain, followed by a poorly conserved, 10-kDa carboxy-terminus (40, 257). Structural studies on the peptide-binding domain indicated that peptides are trapped in a channel that is gated by a flexible helical lid (166, 286). When ATP is bound, the helical lid pivots to expose this channel and Hsc70 exhibits weak affinity for substrates. Transient interactions with peptide can stimulate ATP hydrolysis and thus trigger a conformational change in the peptide-binding domain that increases the stability of the peptide-Hsc70 complex by closing the helical lid to trap the bound substrate. The exchange of ADP for ATP is then critical for release of the substrate (151, 219). This cycle of substrate binding and release, combined with the preference of Hsc70s for hydrophobic stretches of amino acids (18, 70, 211) that may become solvent-exposed in unfolded proteins, enable Hsc70s to interact transiently with polypeptides as they progress through the secretory pathway.

Hsc70s are inherently weak ATPases (0.03–0.27 $min^{-1}$), but their activity is greatly enhanced by members of the GrpE and DnaJ (Hsp40) families. Identified first in bacteria, GrpE stimulates the exchange of ATP for ADP on DnaK (138), thus facilitating release of substrate and permitting the commencement of a new binding cycle (241). However, a GrpE-like exchange factor does not appear to be critical for the early secretory pathway. In contrast, DnaJ chaperones,

which stimulate the ATPase activity of Hsc70 and thus promote stable substrate binding (151, 219), are critical at many steps in the eukaryotic secretory pathway. In some cases, DnaJ chaperones may even deliver specific substrates to Hsc70s (126, 136, 160, 240, 267) and anchor Hsc70s at the ER or mitochondrial membrane (24, 206).

The DnaJ (Hsp40) family of chaperones is defined by the presence of an ~70 amino acid sequence called the J domain that mediates Hsc70 interaction. Structural studies of the J domains from bacterial DnaJ (106, 193, 242), human Hdj1p (203) and the SV40 and polyomavirus large T antigens (14, 131) indicate that this domain folds into four $\alpha$-helices, two of which are packed tightly against each other to form a finger-like projection. An invariant HPD motif is positioned in a loop at the tip of this finger between the second and third $\alpha$-helices and is critical for interactions with the Hsc70 ATPase domain (59, 68, 247, 254). Additional regions of DnaJ chaperones may also contact a site near or at the substrate-binding domain of Hsc70s (80, 237, 238). With the exception of the J domain, DnaJ family members share little else in common. There are three broad subtypes, defined by their similarity to the bacterial DnaJ protein (41). Type 1 DnaJ homologs are the most similar to DnaJ. In addition to the J domain, they contain a 30–40 amino acid glycine/phenylalanine-rich region that facilitates Hsc70 interaction (128, 240, 255), and a cysteine-rich region that binds zinc and forms a peptide-binding pocket (5, 240). Type II DnaJ family members lack the cysteine-rich region and Type III proteins lack both the cysteine-rich and glycine/phenylalanine-rich regions. *Escherichia coli* DnaJ was recently observed to bind preferentially to hydrophobic, 8-amino acid motifs (212), providing further evidence that this chaperone can deliver polypeptide substrates to Hsc70s.

The Hsp90 chaperones are abundant, essential proteins that have been most intensely studied for their role in kinase and steroid hormone receptor maturation (reviewed in 35). Although mammalian cells contain both cytoplasmic and lumenal Hsp90s, yeast lack a lumenal Hsp90 homologue. Hsp90 function depends upon two peptide-binding domains at the amino and carboxy termini, both of which can mediate dimerization. The amino terminus also contains a weak ATPase activity (202, 234) that is essential for Hsp90 function (183, 188). Peptide binding (and dimerization) at the amino terminus is regulated by ATP (234). Whereas both peptide binding domains of Hsp90 can prevent aggregation of denatured proteins in vitro, recent evidence indicates that full-length Hsp90 is required to enhance the refolding of denatured substrate in vitro (124). Still controversial, however, is whether Hsp90s are generally involved in protein folding. Support for their involvement in protein folding includes the observation that Hsp90 can maintain aggregation-prone substrates in a refolding-competent state in vitro (21, 74) and that it associates with unassembled immunoglobulin chains in the mammalian ER (158, 217). In addition, Hsp90 can enhance the Hsp70- and Hsp40-mediated folding of luciferase in vitro and in vivo (87, 125, 223). However, the folding of most proteins is unaffected in yeast lacking functional Hsp90 (173).

The peptidyl prolyl isomerases (PPIases) comprise another family of chaperones, with members found in virtually all cellular compartments where protein

folding occurs. The PPIases catalyze the *cis/trans* isomerization of the peptide bond immediately preceding proline residues, which is kinetically unfavorable when uncatalyzed (135). These chaperones can be divided into two structurally distinct classes based on homology: the immunophilin family, whose founding member is the target of the immunosuppressant cyclosporin, and the FK-binding protein (FKBP) family, whose founding member is the target of the compound FK506 (280). PPIases have multiple functions, some with little to do with protein folding, which has led to controversy regarding the requirement for PPIases in folding reactions. However, other observations support their significance for protein folding in the ER. First, PPIases are induced by the unfolded protein response (UPR; see below) (39, 245). Second, the physiological significance of a cyclophilin family member from *Drosophila melanogaster* known as *ninaA* is well established (3, 45, 233). As *ninaA* is specifically required in the folding of a subset of rhodopsins, it is discussed in the final section of this review.

## MOLECULAR CHAPERONES AND PROTEIN TRANSLATION

The influence of chaperones on the maturation of both secretory and nonsecretory pathway-targeted proteins begins during translation. In yeast, chaperones of the Hsp70 (Ssb1/2p; 174) and Hsp40 families (Sis1p, zuotin, Ydj1p; 28, 275, 284) have been implicated in translation by their association with ribosomes and/or by their requirement for translation initiation, efficient protein synthesis, or the translation of heterologous proteins. Although the Ssb chaperones crosslink directly to the nascent polypeptide-ribosome complex and might prevent protein misfolding (194), the mechanisms by which these and possibly other chaperones facilitate translation remain largely unknown. Studies examining early events during protein translation in mammalian and in heterologous systems have uncovered ribosome-nascent polypeptide chain interactions with the Hsp70 and Hsp40 chaperones, as well as with the TriC/CCT chaperonin complex (12, 63, 76, 196). Recently, a ribosome-associated Hsp70-Hsp40 complex formed by Ssz1p/Pdr13p and zuotin was shown to facilitate the translocation of a ribosome-bound mitochondrial precursor protein into the mitochondria in vitro (81). However, a ribosome-associated Hsp70-Hsp40 complex that similarly facilitates preprotein translocation into the ER has not been identified.

## PROTEIN TRANSLOCATION INTO THE YEAST ER IS FACILITATED BY MOLECULAR CHAPERONES IN THE CYTOPLASM AND ER LUMEN

Protein translocation (import) into the ER proceeds either cotranslationally or posttranslationally. During cotranslational translocation, translation is attenuated after the emergence of the signal peptide through the action of the signal recognition

particle (SRP). Upon interaction with the SRP receptor (SR, also known as "Docking Protein"), GTP-dependent release of SRP permits the re-initiation of translation, and the signal sequence of the preprotein is presented to the translocation machinery at the ER membrane (reviewed in 123). During posttranslational translocation, the signal sequence-containing secreted preprotein is synthesized in its entirety before interaction with the translocation machinery (reviewed in 205). One of many advantages provided by the examination of translocation in yeast is that the pathways operate in parallel, and a survey by Ng et al. (176) concluded that signal sequences with greater hydrophobic cores co-opt preferentially the cotranslational pathway, or utilize both pathways.

When genes encoding components of SRP and SR are deleted, yeast grow ~fourfold more slowly than wild-type cells and accumulate several cotranslationally targeted preproteins in the cytoplasm (97, 185). A recent microarray analysis from Mutka & Walter (170) indicates that the ability of yeast to survive the loss of SRP arises from the induction of ER-associated chaperones that may prevent preproteins from aggregating, and from an attenuation of protein synthesis, perhaps permitting the translocation machinery to better couple translation and translocation.

Because the translocation of preproteins utilizing the posttranslational pathway is uncoupled from translation, and because the diameter of the translocation channel in the ER membrane precludes the translocation of folded proteins (see below), the preprotein must translocate in a nonnative conformation. Thus, cytoplasmic molecular chaperones are required for the translocation of preproteins into the ER posttranslationally even when SRP function is proficient.

The first evidence that chaperones facilitate posttranslational protein translocation emerged from both biochemical and genetic studies. Chirico et al. (43) utilized an in vitro assay in which the dependence on yeast cytosol of the translocation of a wheat germ–synthesized yeast mating factor prepheromone, pre-pro $\alpha$ factor (pp$\alpha$F), into yeast ER-derived microsomes was observed (261). The cytoplasmic Hsc70s Ssa1p and Ssa2p were then purified based on their ability to substitute for cytosol (43). The cytosol dependence could also be replaced by denaturation of the substrate in urea prior to the translocation assay, suggesting that the chaperones maintained pp$\alpha$F in an unfolded conformation. Deshaies and colleagues (56) simultaneously found that the depletion of Ssa1p in yeast harboring knockout alleles of the *SSA1*, *SSA2*, and *SSA4* genes (which encodes a third, related Hsc70) led to the accumulation of pp$\alpha$F and a mitochondrial-targeted preprotein in the cytoplasm. Support for the requirement of cytoplasmic chaperones in protein translocation also emerged from examining translocation in other systems. First, although the posttranslational pathway is rarely utilized in higher eukaryotes, short preproteins that cannot interact with SRP during translation required Hsc70 for translocation into mammalian microsomes (288). Second, Gross and co-workers (270) found that *dnaJ* and *dnaK* mutants failed to secrete bacterial preproteins that utilize the Sec-independent secretory pathway, and that overexpression of DnaJ and DnaK facilitated the secretion of Sec-dependent preproteins in *secB* mutants. Thus, as in

yeast, cytoplasmic chaperones apparently compensate for defects in an alternate secretory pathway.

To determine whether the action of Ssa1/2p on posttranslational translocation requires an Hsp40 co-chaperone, Caplan et al. (36) examined yeast containing a temperature-sensitive allele of *YDJ1*, an ER-associated Type I Hsp40, and found that posttranslationally translocated preproteins accumulated in the cytoplasm at the nonpermissive temperature. Both genetic (10) and biochemical (53, 54) data indicate that Ssa1p and Ydj1p interact, an interaction that is required to support translocation (10, 36, 153). These combined results led to a model in which release of the Ssa1p-bound preprotein is catalyzed by Ydj1p at the ER membrane, resulting in the delivery of the preprotein in an unfolded conformation to the translocation machinery.

However, a recent study suggests that chaperones may free preproteins spontaneously before interacting with the translocation apparatus at the ER (196). Consistent with these data, a fusion protein heterologously expressed in yeast was folded in the cytoplasm prior to its translocation (192), and based on studies of luciferase folding in yeast lysates, Bush & Meyer (33) proposed that Ssa1/2p catalyze the folding of preproteins in the cytoplasm before ER targeting. In each case, however, the preprotein must be unfolded again before it can insert into the translocation channel. Whether this process is re-engineered by Ssa1p/Ydj1p or by other chaperones is not clear.

After a nascent secretory preprotein is targeted to the cytosolic face of the ER membrane, its signal sequence is engaged by the Sec61p translocation complex (169, 197, 214), composed of the Sec61p, Sss1p, and Sbh1p proteins in yeast (99). High-resolution electron micrographs of both the yeast and mammalian pore complexes suggest that the Sec61p complex assembles into a tetramer, forming a central pore that is likely to be the channel through which preproteins are translocated (96). Measurements of truncated preproteins containing fluorescently tagged amino acids at various positions within the polypeptide indicate that the mammalian pore may be as large as 80 Å (90), a value somewhat higher than that obtained by the EM studies (11, 96). Regardless, the pore is of sufficient diameter to transport polypeptides in an $\alpha$-helical conformation, or containing some secondary structure, but not the sizes adopted by most fully folded proteins. Because the interior of the pore is an aqueous channel (52, 83), transmembrane domains of integral membrane proteins must laterally diffuse into the lipid bilayer during their translocation (61, 103, 148, 167).

Elegant biochemical studies using proteoliposomes lacking lumenal components indicate that the driving force for cotranslational translocation into the ER can be provided by the ribosome (149). Thus, the nascent polypeptide is "pushed" into the lumen. In contrast, lumenal components most likely "pull" polypeptides into the ER during posttranslational translocation. In yeast, numerous studies have led to the conclusion that the Hsc70 and Hsp40 homologues, BiP (Kar2p) and Sec63p, respectively, are responsible for driving polypeptides into the ER (reviewed in 24). Yeast BiP is ~50% identical to *E. coli* DnaK (182, 209), whereas

Sec63p (a Type III Hsp40) is a polytopic membrane protein in which only one ~70 amino acid lumenal segment is homologous to DnaJ (68, 213). Strains containing temperature-sensitive mutations in *KAR2* and *SEC63* accumulate preproteins at the nonpermissive temperature (210, 252), and microsomes or reconstituted proteoliposomes prepared from *kar2* and *sec63* mutant strains are defective for translocation in vitro (27, 29, 210, 214). A role for BiP during translocation into washed or reconstituted ER-derived mammalian vesicles has also been suggested (60, 178).

Two prevailing models to account for the action of the BiP-Sec63p complex have been proposed, based primarily on studies of the mechanics of posttranslational translocation into isolated mitochondria (reviewed in 24). In the Brownian ratchet model, the translocation pore is passive and the interaction of the preprotein with BiP in the ER lumen prevents retro-translocation (228). Polypeptide oscillations within the pore provided by thermal energy lead to the emergence of longer segments of the preprotein in the ER, allowing for a higher stoichiometry of BiP binding and the prevention of retro-translocation. Support for this model for translocation into the yeast ER comes from a study by Matlack et al. (149) in which reconstituted proteoliposomes lacking lumenal components but containing antibodies against pp$\alpha$F could support the translocation of pp$\alpha$F through the Sec61 complex and into vesicles. This impressive proof-of-principle and a recent mathematical modeling study (139) indicate that a ratchet is sufficient for posttranslational translocation in a highly defined in vitro system.

The second model depicts the BiP-Sec63p complex as a motor in which successive rounds of ATP binding and hydrolysis are coupled to the interaction with, and pulling and release of, a preprotein in the lumen of the ER (84). Consistent with this model, mutations in BiP that prevent an ATP-dependent conformational change and interaction with Sec63p, but not peptide binding, inhibit translocation both in vivo and in vitro in a dominant manner (152). The motor model may also be supported by the observations that mitochondrial Hsp70 cannot support pp$\alpha$F translocation into ER-derived reconstituted vesicles even though it interacts with pp$\alpha$F (25) and that mutations in the BiP-binding domain of Sec63p abrogate BiP interaction and posttranslational translocation (29, 47, 146). Thus it seems that BiP must interact with the substrate and be anchored to the inner face of the ER membrane to exert force and pull preproteins into the ER. In contrast, if BiP is simply a molecular "glue," its interaction with Sec63p may not be essential for import. However, an examination of BiP-peptide interactions in the presence or absence of Sec63p by surface plasmon resonance studies led to the intriguing conclusion that Sec63p may expand BiP's peptide-binding repertoire (160). Thus, BiP is bound near the translocation channel via its interaction with Sec63p in order to receive the preprotein, and Sec63p, in turn, signals BiP to bind to a wider spectrum of chemically diverse peptide segments. This result resolves the problem of how a single chaperone can bind either with a high enough affinity or in sufficient amounts to effect import, given that Hsp70s bind preferably to highly hydrophobic sequences (18, 70, 211) that may not be abundant in all secreted proteins.

The original identification of Hsp70 and Hsp40 in bacteria as DnaK and DnaJ—factors required for the ATP-dependent activation of the DnaB helicase at the replication origin during λ phage DNA replication (289)—suggests that the BiP-Sec63p complex may also be thought of as an energy-dependent, regulatory machine. Early support for this model came from the observation by Sanders et al. (214) that the interaction of a preprotein with Sec61p at the cytoplasmic face of the ER was compromised in a *kar2* mutant at the nonpermissive temperature. This result was confirmed using a more refined system in which the transfer of a signal sequence-containing preprotein from the integral membrane signal sequence-binding components of the translocation machinery (encoded by the *SEC62*, *SEC71*, and *SEC72* genes) to the translocation pore could be assessed in a solubilized system (147). These later studies suggested that BiP activates the Sec62p-Sec71p-Sec72p complex upon its ATP-dependent interaction with Sec63p, although data supporting an alternate theory have been presented (197). Nevertheless, the retention of BiP via Sec63p adjacent to the translocation channel may couple the recognition of a preprotein at the ER membrane, the entry of a preprotein into the channel, and its subsequent translocation into the ER.

Alternatively, the chaperones may be required to gate the pore. Using an in vitro assay in which lumenal access of a preprotein confined within the Sec61 complex could be assessed in mammalian microsomes, Johnson and colleagues showed that BiP seals the pore until the translocating polypeptide reaches a length of ~50 amino acids. Because the ability of BiP to seal the pore was ATP-dependent, and because of the recent identification of Sec63p homologues in the mammalian ER (159, 231, 249), it will be interesting to determine whether BiP-dependent gating in the mammalian ER is through a direct interaction with Sec61p, or through a Sec63p homologue.

One expectation from these models is that the chaperones should be required for both cotranslational and posttranslational translocation: Signal sequence recognition at the translocation machinery and/or gating of the channel are required regardless of how the preprotein is delivered to the ER membrane. Consistent with this hypothesis, microsomes derived from thermosensitive *kar2* and *sec63* strains were defective for the import of both a co- and posttranslationally translocated substrate (26). More recently, Stirling and colleagues selected for yeast specifically defective for the import of a cotranslationally translocated substrate and recovered mutations primarily in *SEC61* and *SEC63* (278). The authors also characterized further a group of *kar2* mutants required for co- and posttranslational translocation in vitro (26, 214) and confirmed that BiP function is necessary for both pathways in vivo. The biochemical characterization of the corresponding Sec63 and Kar2 mutant proteins should help elucidate how the lumenal chaperones might regulate and facilitate co- and posttranslational preprotein import into the ER.

As discussed in the Introduction, the action of Hsp70-Hsp40 pairs can be regulated further by other co-chaperones. Mutations in a lumenal, ~100-kDa protein with some homology to Hsp70 (known variably as Lhs1p/Cer1p/Ssi1p) are synthetically lethal with a translocation-defective allele of *KAR2*, and strains lacking

the *LHS1/CER1/SSI1* gene are unable to posttranslationally translocate some preproteins into the ER (7, 51, 89). In addition, a genetic selection to uncover factors associated with the translocation machinery in *Yarrowia lipolytica* led to the identification of Sls1p, and *Y. lipolytica* strains deleted for *SLS1* are defective for the translocation of a secreted alkaline protease (19). Both the *Y. lipolytica* Sls1 protein and *S. cerevisiae* homologue of Sls1p (known as Per100p, Sil1p, or scSls1p) interact with the ATPase domain of BiP and regulate its activity (127, 245, 250), possibly by acting as a nucleotide exchange factor for BiP (127). While deletion of *SIL1* alone does not compromise translocation (250), suggesting that Sil1p may modulate another aspect of BiP function in *S. cerevisiae*, it exacerbates the translocation defect when combined with a translocation-defective *kar2* mutant (127). These recent results point to additional chaperone modulators required for protein translocation.

## THE ENDOPLASMIC RETICULUM ENSURES PROPER FOLDING AND MATURATION

As soon as nascent chains enter the ER they face an environment dramatically different from that within the cytoplasm, but more similar to that present outside the cell. As the primary regulator of cellular $Ca^{++}$ levels (235), the ER possesses a dramatically higher concentration of free $Ca^{++}$ than found in the cytosol (1 mM in the ER compared with 100 nM in the cytosol; 39). Also, the ER is significantly more oxidizing than the cytoplasm (with a redox potential of –230 mV versus –150 mV; 114), which in turn means that disulfide bond formation is favored within the ER, whereas disulfide bonds are virtually absent in the cytoplasm (208). In fact, a hallmark of secretory proteins is the presence of disulfide bonds that in many cases are absolutely required for folding and/or activity (208). Additionally, many secretory proteins are N-glycosylated in the ER, a modification frequently required for proper folding and/or activity (104).

The ER serves as both a protein-folding compartment and a gatekeeper, guaranteeing the structural integrity of each protein before it is presented extracellularly (93, 113). As such, the ER is highly enriched in factors that promote efficient protein folding and prevent improperly folded proteins from progressing through the ER. Members of virtually all classes of chaperones, except the Hsp60/GroEL family, are found within the ER (235). The central role of the most abundant Hsc70 in the lumen, BiP, in protein folding has been well-documented (81a, 262), and yeast with reduced levels of lumenal Hsp70 activity exhibit protein folding defects (228a) and synthetic interactions with mutated alleles of genes encoding the lumenal Hsp40 chaperones and components of the oligosaccharyl transferase (179, 218, 242a). BiP most likely aids folding by preventing off-pathway intermediates from forming. Both BiP and another Hsp70 homologue in yeast, Lhs1p (Hsp170), also play an active role in the re-folding of heat-damaged secreted proteins in the lumen (120, 215).

Protein folding in the ER presents challenges not faced by the folding of proteins in other cellular compartments. For example, the chemical steps involved in disulfide bond formation and rearrangement are intrinsically slow compared to conformational rearrangements. Moreover, partial native structure can dramatically inhibit access to buried cysteines, further slowing disulfide rearrangement (264). Similarly, transmembrane domains must be inserted with correct topology into the ER membrane if a protein is to adopt its native structure. This problem is solved in part through the cotranslational insertion of transmembrane domains. However, as illustrated by the recently described maturation of aquaporin-1 (144), transmembrane domains are not always correctly oriented until after synthesis of nearly the entire polypeptide. How this reversal of topology is accomplished remains obscure. Given these particular demands on folding in the ER, there are, not surprisingly, several chaperone systems unique to the ER: general chaperones, such as calnexin and its soluble homolog calreticulin and the machinery responsible for the introduction of disulfide bonds into proteins, as well as chaperones dedicated to assisting the maturation of a single or a limited number of proteins.

## OXIDATIVE PROTEIN FOLDING IN THE ER

Since the classical protein folding studies performed by Anfinsen, it has been clear that oxidation can proceed spontaneously in an aerobic environment. However, the observation that protein folding occurs far more rapidly in vivo than in vitro suggests that protein oxidation must be catalyzed within living cells (1). Indeed, it was this observation that led to the initial identification of protein disulfide isomerase (PDI) over 30 years ago as a chaperone that catalyzed the rearrangement of disulfide bonds (85). Oxidative protein folding in vitro requires only a source of oxidizing equivalents and an enzymatic activity to rearrange disulfide bonds (71). These two activities can be supplied by an appropriate redox buffer, usually a mixture of reduced and oxidized glutathione (GSH and GSSG, respectively), and PDI. The ratio of GSH to GSSG in vivo varies between the cytoplasm and the secretory pathway, with the cytoplasm having a ratio of GSH:GSSG of ~100:1 and the secretory pathway having a ratio of ~3:1 (114).

Since glutathione ratios differ between the secretory pathway and the cytoplasm, the presence of this buffer alone was considered sufficient to maintain an environment favorable for protein oxidation. However, every disulfide bond formed in a nascent chain introduces reducing equivalents into the ER that must be disposed of to keep the environment in an oxidized state. A number of explanatory models have been proposed, including import of oxidizing equivalents from the cytoplasm, secretion of reducing equivalents, and different enzymatic activities (71, 114, 287), none entirely satisfactory.

Work from two labs using *S. cerevisiae* has led to the identification of factors that are required for proper oxidative protein folding (72, 201). Central to these studies is the fact that the redox balance of living cells can be manipulated through

treatment with the membrane-permeable reducing agent dithiothreitol (DTT) (22, 119). Whereas wild-type cells tolerate limited quantities of DTT, cells with defective oxidation machinery exhibit an increased sensitivity to this drug. Screening for mutant yeast cells either with an increased sensitivity to DTT (201) or with specific defects in secretion (72) resulted in the identification of a novel factor, termed *ER* Oxidoreductase 1 (*ERO1*). This gene is specifically required for oxidative protein folding, as mutations in *ERO1* result in a folding defect for three substrates that contain disulfide bonds, while proteins that do not depend on disulfide formation for their folding are secreted with normal kinetics (72, 201). Furthermore, *ERO1* activity determines the overall redox balance within cells: Whereas *ero1* mutation results in DTT sensitivity, overexpression of *ERO1* leads to increased resistance to DTT. The central role played by *ERO1* is underscored by the fact that homologs of the Ero1p protein can be found in all eukaryotic organisms examined (34, 72, 187, 201).

Much evidence indicates that a direct interaction between Ero1p and PDI is responsible for the introduction of disulfide bonds in ER lumenal proteins (Figure 1). First, whereas PDI is normally found in an oxidized state in vivo, mutations in *ERO1* result in the steady-state accumulation of PDI in its reduced form (73). Second, mutations in the active-site cysteines of PDI result in the isolation of mixed-disulfide complexes between Ero1p and PDI (73, 248). This complex is likely to be an intermediate formed in wild-type cells during the oxidation of PDI by Ero1p. Further support for the conservation of this oxidation machinery is supplied by the finding that mammalian homologs of Ero1p can also be isolated in mixed-disulfide cross-links with PDI (13). Even more compelling is the recent development of an in vitro oxidative folding system in which PDI is a required component for Ero1p-dependent oxidation of RNase A (248).

What about the role of glutathione in oxidation? Recent genetic and biochemical data counter the long-held belief that glutathione in the ER served as the source of oxidizing potential. Kaiser and co-workers removed glutathione from yeast using a strain lacking a gene required for its biosynthesis, *GSH1*, and found that oxidative protein folding was proficient (52a). Surprisingly, this mutation suppressed the folding defect of *ero1-1* mutant strains, suggesting that the presence of GSH places a burden on the oxidation system. Removal of this burden through the deletion of *GSH1* allows *ero1-1* mutant cells to generate enough oxidizing equivalents to grow again. In an in vitro system, the addition of oxidized glutathione was dispensable for oxidation of RNase A (248), indicating that Ero1p itself can generate oxidizing equivalents. Furthermore, the addition of reduced glutathione at concentrations up to 2 mM had no effect on the ability of Ero1p to oxidize substrates. Also, reduced glutathione was not oxidized by Ero1p in vitro unless PDI was present, suggesting that Ero1p does not oxidize glutathione and that the presence of GSSG within the secretory pathway is in fact a by-product of the reduction of secretory proteins and/or PDI. This view is consistent with the genetic experiments suggesting that the role of glutathione in the secretory pathway is actually to prevent overoxidation (52a).

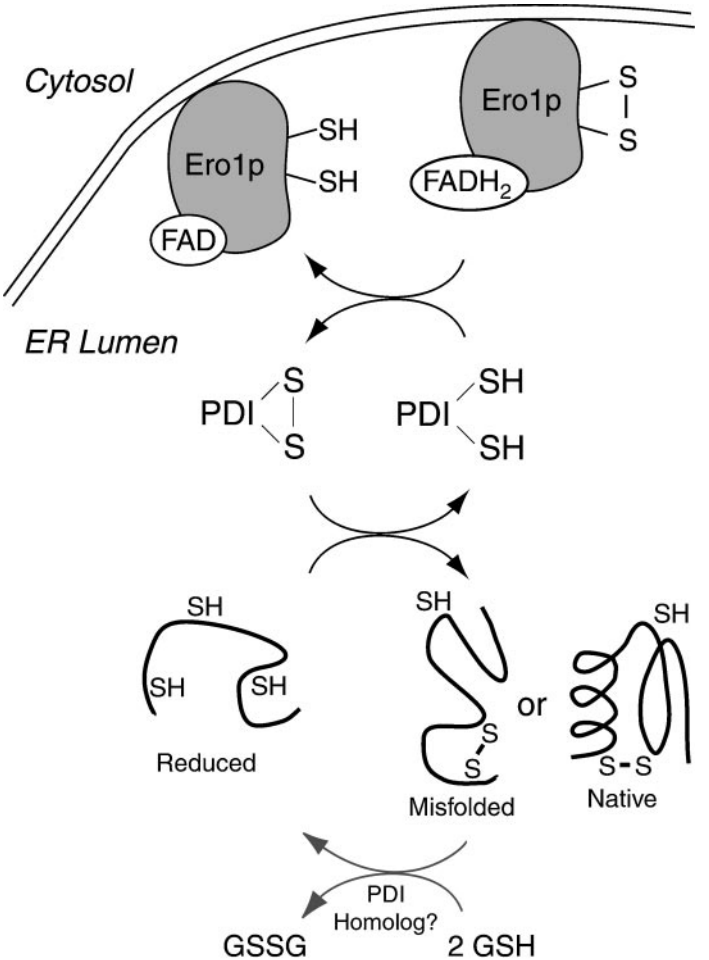


**Figure 1** Disulfide bonds are introduced into folding proteins through the actions of Ero1p and protein disulfide isomerase. Oxidizing equivalents are generated on Ero1p using FAD as a co-factor. These oxidizing equivalents are passed on to PDI, but not other PDI homologs, which is then able to oxidize substrate proteins. Ero1p cannot oxidize substrates directly, nor does it directly oxidize GSH. Instead, GSH oxidation appears to be a consequence of reduction of pre-existing disulfide bonds. See text for details.

An important outstanding question is how oxidizing equivalents are introduced into the ER. As all redox reactions involve the movement of electrons from one molecule to another, there must be a "sink" for the excess electrons generated within the ER. An oxidation system analogous to that in the ER of eukaryotic cells is found in the periplasm of *E. coli* (208). In this system, electrons are

disposed of through the respiratory chain (134), ultimately passing through either cytochrome bo oxidase or cytochrome bd oxidase to oxygen (2a). However, in contrast to the bacterial oxidation system, when components of the respiratory chain in *S. cerevisiae* are inactivated the oxidation machinery is unaffected (248). Instead, oxidative protein folding depends exquisitely on cellular levels of FAD. The significance of FAD was confirmed in vitro, as oxidation of RNase A by Ero1p required the addition of FAD.

Although FAD is sufficient for oxidation in vitro, its precise role in vivo is not clear. FAD may act as a co-factor for Ero1p. In this scenario, another protein is likely to bind Ero1p and catalyze a reaction leading to the regeneration of FAD from the $FADH_2$ that is produced during the oxidation of PDI. Alternatively, $FADH_2$ may be released, allowing Ero1p to bind a different, oxidized molecule of FAD. In either case, some mechanism must regenerate FAD within the cell. $FADH_2$ reacts very rapidly with free oxygen to generate FAD and $H_2O$, but oxidative protein folding occurs even under anaerobic conditions in a manner that is dependent on functional Ero1p (248). This suggests that another oxidizing source must be present. This has also been seen in the oxidizing environment of the bacterial periplasm, where fumarate acts as the terminal electron acceptor under anaerobic conditions (2a).

## PROTEIN QUALITY CONTROL: RETENTION

The primary step in ER quality control is retention of misfolded or misassembled proteins, and the most common mechanism for retaining misfolded proteins is through association with other proteins that are themselves normally retained (65). As the exposure of epitopes rendering a protein susceptible to recognition by molecular chaperones is a feature likely to be common to all misfolded or misassembled proteins, chaperones are particularly good candidates for retention molecules. In fact, the calnexin/calreticulin system (282), BiP (92, 113), and PDI (207) have been implicated in aspects of quality control (for review, see 65).

The processing of the core oligosaccharyl glycan in the ER plays a major role in protein quality control and exemplifies one mechanism by which misfolded proteins may be retained in the ER. Initially, a branched chain of sugars of the composition $Glc_3Man_9GlcNAc_2$ is added to asparagines within the sequence motif NX(S/T). However, proteins that fold rapidly in the ER are less prone to this modification (109), suggesting that a steric block arising from secondary structures may occlude the consensus sequence. Glycoproteins with the attached $Glc_3Man_9GlcNAc_2$ moiety undergo a rapid trimming, in which the three external glucose residues are removed sequentially through the actions of glucosidase I and II (104). A single glucose residue can be added back by UDP-glucose:glycoprotein glucosyltransferase (UGGT; 69, 191), but UGGT only recognizes misfolded proteins. The monoglycosylated glycan is recognized by the lectins calnexin and its

soluble homolog calreticulin, which retain the glycoprotein in the ER and facilitate its folding (104). Removal of the terminal glucose triggers dissociation of the calnexin/calreticulin-glycoprotein complex and the correctly folded glycoprotein can then exit the ER (102); however, misfolded proteins are recognized by UGGT and re-glycosylated, leading to re-association with the lectins and permitting another chance at folding (91). Thus, unlike modification of glycosyl groups in the Golgi, which is used to create diversity, modification of the basic glycosylation structure in the ER appears to be used primarily by the quality control machinery to distinguish folded from misfolded proteins.

Recent studies using a GFP-tagged secretory protein and fluorescence photobleaching experiments in mammalian cells provide evidence for another mechanism of retention: lack of a positive secretion signal. The glycoprotein of vesicular stomatitis virus (VSVG) is normally secreted; however, a temperature-sensitive variant of this protein (tsO45) leads to misfolding and ER retention at 40°C. By tagging this VSVG mutant with GFP and examining the kinetics of recovery after photo-bleaching, Nehls et al. observed that the retained protein is highly mobile, with a mobility equivalent to that seen with the mutant protein examined at the secretion-permissive temperature of 32°C (175). Furthermore, repetitive photo-bleaching of cells expressing the mutant VSVG at 40°C showed a gradual loss of total fluorescence, consistent with a model in which mutant VSVG cannot be exported from the ER but remains free to diffuse within it. When cells are depleted of ATP or express an ATPase-defective BiP mutant, the VSVG mutant becomes immobile. Consistent with previous suggestions from studies of the influenza hemagglutinin (HA) protein (112), these results indicate that the ER may form a dense matrix that inhibits the movement of normally mobile proteins. With VSVG, the aggregates were held together by disulfide bonds, as mobility could be restored through treatment of the cells with DTT (175).

In their studies of the subcellular localization of VSVG by indirect immunofluorescence, Hammond & Helenius provide another suggestion for how the temperature-sensitive VSVG might be retained in the ER (92). At the nonpermissive temperature, VSVG was found throughout the ER as well as in ER-Golgi intermediate compartments. The only marker that co-localized with the VSVG protein was BiP. When BiP localization was examined in non-transfected cells or in cells expressing VSVG at the permissive temperature, BiP was seen only in the ER. This suggests that misfolded VSVG can pull BiP out of the ER, but that VSVG-BiP complexes are returned to the ER, thus keeping VSVG mutant protein from being secreted.

Given the broad spectrum of chaperone systems in the ER, how is it determined which chaperone system is selected by translocating nascent chains? Insight into this question has come from recent work by Molinari & Helenius, who observed that the E1 nascent chain of Semliki forest virus associated with BiP, while the p62 viral nascent chains bound to calnexin (161). If interaction with calnexin was blocked, both proteins immunoprecipitated with BiP, suggesting that the lack of

interaction between p62 and BiP was not due to a lack of a suitable BiP binding site. This hypothesis was confirmed by examining interactions between BiP and HA, which contains numerous glycosylation sites near its N terminus. Consistent with glycosylation being the determining factor, the native HA protein did not bind BiP; however, when the most N-terminal glycosylation sites were mutated, strong interactions with BiP could be detected. Thus, the presence of N-terminal glycosylation sites seems to direct nascent chains to the calnexin system, while the absence of such sites directs nascent chains to BiP.

One of the first recognized needs for a quality control system is in the retention of misassembled protein complexes; polypeptides that normally associate with other polypeptides cannot progress through the secretory pathway until they are bound to their partners (113). A molecular basis for this observation is suggested by recent studies that analyzed the trafficking of subunits of a mammalian ATP-sensitive potassium channel (281). This channel is composed of four regulatory subunits (known as SUR1/2A/2B) and four potassium ion channel subunits (known as Kir6.1/2), and proper surface expression of the channel requires the co-expression of all eight subunits. Sequence analysis combined with mutagenesis identified a sequence, RXR, whose presence blocks the trafficking of channel subunits to the cell surface and retains them in the ER (281). The similarity to KKXX motifs, whose presence at the C terminus of transmembrane proteins of the secretory pathway leads to ER retention (117), suggests that a similar mechanism may prevent the trafficking of proteins containing either motif.

However, unlike KKXX motifs, the use of an RXR motif as a retrieval mechanism has been documented only in proteins whose final location is not the ER (281). Importantly, the RXR motif functions only when present in unassembled or partially unassembled complexes (281), suggesting that the RXR motif is buried in the assembled complexes. Thus, the masking of RXR-containing sequences in the folded or mature state of a protein appears to be the factor that allows the RXR motif to act in a quality control mechanism, rather than a constitutive retention mechanism. These results do not differentiate between a retention mechanism and a retrieval mechanism. A more general role for RXR motifs is suggested by the observation of several such sequences in the cytosolic loops of CFTR, the transmembrane conductance-regulator that is responsible for all inherited cases of cystic fibrosis. When these sequences are altered in a $\Delta$F508 CFTR mutant, which is ordinarily retained in the ER, the protein can be secreted (38).

## PROTEIN QUALITY CONTROL: ER ASSOCIATED PROTEIN DEGRADATION (ERAD)

Because unfolded proteins cannot always achieve their native state in the ER, eukaryotic cells have evolved a constitutively active quality control system to rid the ER of misfolded proteins. This process, termed ER-associated protein degradation (ERAD; 154), involves three key steps: (*a*) recognition of the aberrant

polypeptide; (*b*) export of soluble proteins to the cytoplasm back through the translocation pore ("retrotranslocation"); and (*c*) degradation by the proteasome. The conformational diversity of proteins entering the ER and the requirement for ERAD to selectively degrade only misfolded or regulated proteins suggests that this process is complex. However, biochemical and genetic studies together are elucidating the cellular players and mechanisms in this elaborate safety net.

The prevailing evidence suggests that many of the same molecular chaperones involved in folding proteins in the ER are also involved in the removal of ERAD substrates. Aberrant secretory proteins may have exposed structural motifs (221, 230, 271) or hydrophobic patches that could prolong chaperone interactions and trigger their destruction. Consistent with this model, the chaperones BiP (30, 198), calnexin (154), and protein disulfide isomerase (PDI; 82) are required for the degradation of some ERAD substrates in yeast. In addition, two lumenal Hsp40 homologs in yeast, Scj1p and Jem1p, interact with BiP (179, 218) and help prevent the aggregation of misfolded proteins prior to their retrotranslocation (180). Biochemical studies indicate that mammalian calnexin prevents the aggregation of unfolded (and unglycosylated) proteins in solution (115), an activity that also promotes protein folding in vitro in conjunction with yeast BiP and the Sec63p J domain (D. Williams, personal communication). Aggregation of ERAD substrates prior to their export would preclude their transit through the translocation channel.

Is there a signal that distinguishes between slowly folding proteins and those that are terminally misfolded? Recent studies indicate that competition between enzymes that attach or remove sugar moieties may function as a timer for the folding of individual glycoproteins in the mammalian ER (65, 140). As discussed above, the trimming of glucose residues on the branched $Glc_3Man_9GlcNAc_2$ oligosaccharide triggers dissociation of the calnexin/calreticulin-glycoprotein complex so that the correctly folded glycoprotein can exit the ER (102). After prolonged retention of a misfolded protein in the ER, the trimming of mannose residues may divert the protein from the calnexin-catalyzed folding pathway into the degradation pathway, which may or may not be dependent on further interactions with calnexin (2, 42, 44, 57, 140, 244, 274). Consistent with this model, degradation of a yeast ERAD substrate, a mutated form of the vacuolar-targeted carboxypeptidase Y (CPY*), depends upon glycosylation and requires the mannosidase I-generated $Man_7GlcNAc_2$ moiety (118, 133), but there is limited evidence for a calreticulin or calnexin binding cycle in *S. cerevisiae*. Instead, factors like the recently identified $\alpha$-mannosidase-like protein, Mnl1p, may identify glycoproteins containing $Man_7GlcNAc_2$ linkages as ERAD substrates (172). In contrast, *Schizosaccharomyces pombe* calnexin is essential and more homologous to calnexin in higher eukaryotes (121, 189), and glucosyltransferase activity is required for *S. pombe* viability under stress conditions (66). If the calnexin/calreticulin cycle is a general feature of ERAD, an as yet undiscovered lectin must target glycoproteins containing trimmed mannoses for ERAD. However, not all ERAD substrates

are glycosylated, suggesting the existence of multiple mechanisms for identifying terminally misfolded proteins.

The export of soluble ERAD substrates occurs by retrotranslocation (or dislocation) through the Sec61p translocation pore. The strongest evidence supporting this hypothesis is the stabilization of yeast ERAD substrates in vitro (195) and in vivo (198, 285) in *sec61* mutant microsomes or cells, respectively. Despite using the same channel and requiring BiP, the isolation of ERAD-specific mutations in *KAR2* (BiP; 30) and *SEC61* (273, 285) suggests that translocation and retrotranslocation are mechanistically distinct. As mentioned above, Sec63p is an Hsp40 homolog that cooperates with BiP in the import of proteins into the ER, but it appears to play a less prominent role in ERAD (180, 195, 198). Conversely, Scj1p and Jem1p are required for ERAD but not for translocation (180). In addition, because signal sequences are cleaved concomitant with translocation, there must be a different mechanism for targeting ERAD substrates to the lumenal face of the Sec61 pore. Several studies suggest that BiP may deliver misfolded proteins to the Sec61 channel (30, 132, 221, 230) and perhaps gate the pore to regulate opposing traffic (90a, 200). Römisch and coworkers also propose a role for PDI in targeting one ERAD substrate to BiP at the translocation pore (82), and Norgaard et al. (181a) report that expression of any one of four other PDI homologues restores ERAD activity in yeast lacking PDI.

The mechanism(s) governing export and degradation of transmembrane proteins from the ER may be distinct from that controlling the ERAD of soluble proteins. Membrane proteins, like soluble proteins, might exit the ER through the Sec61 channel because they can be co-immunoprecipitated with a component of the mammalian ER translocation channel (9, 58, 268) or are stabilized in *sec61* mutant yeast (199, 285). However, some transmembrane proteins may be directly extracted by the proteasome (150, 255a) or attacked by other proteases (67, 78, 143, 165, 168, 269, 272). The proteasome might also "shave" the cytoplasmic portions of integral ER membrane proteins, as Jentsch and colleagues have recently reported that the proteasome may be able to clip polypeptide "loops" (111). The resulting transmembrane domains might be unstable and spontaneously dissociate to the cytoplasm or could be cleaved further (263). In addition, BiP is not required to degrade four known transmembrane ERAD substrates, whereas the cytosolic hsc70 Ssa1p is necessary to degrade the integral membrane proteins Ste6p*, CFTR and Vph1p (105, 199, 283; S. Michaelis, personal communication) (see Table 1). In contrast, Ssa1p is dispensable and BiP is required for the ERAD of three soluble proteins, P$\alpha$F, carboxypeptidase Y (CPY*), and mammalian $\alpha$-1 protease (A1PiZ) in yeast (Table 1). We have suggested that Ssa1p may be required to prevent aggregation of the large cytoplasmic domains in these transmembrane proteins (283). Consistent with this hypothesis, the degradation of Sec61-2p, which contains significantly fewer amino acids in the cytoplasm than the other transmembrane ERAD substrates described above, is only modestly affected in *ssa1* mutant cells (180).

**TABLE 1** ERAD requirements for substrates in yeast[a]

| Soluble | | | Integral membrane | | |
|---|---|---|---|---|---|
| **Substrate** | **Required** | **Dispensable** | **Substrate** | **Required** | **Dispensable** |
| P$\alpha$F | BiP | Ssa1p | Ste6p* | Ssa1p | BiP |
| | PDI | Ubiquitination | | Ubc6/7p | |
| | Sec61p | Sec63p | | | |
| | Cne1p | Scj1p | CFTR | Ssa1p | BiP |
| | Scj1p/Jem1p | Cer1p/Lhs1p/ Ssi1p | | Ubc6/7p | Cne1p |
| | | Hsp90 | | | |
| | | Ssh1p | Vph1p | Ssa1p | BiP |
| | | Eug1p | | | |
| | | | Hmg2p | Ubc7p | Der1p |
| CPY* | BiP | Ssa1p | | Hrd1p/Der3p | Ubc6p |
| | Png1p | Cne1p | | | |
| | Der1p | PDI | Sec61p | Der3p/Hrd1p | Scj1p/Jem1p |
| | Hsp90 | | | Ubc6/7p | |
| | Der3p/Hrd1p | | | Cue1p | |
| | Hrd3p | | | | |
| | Sec61p | | Pdr5p* | Hrd3p | BiP |
| | Cue1p | | | Ubc6/7p | Der1p |
| | Pmr1p | | | Der3p/Hrd1p | |
| | Sec63p | | | Sec61p | |
| | Mns1p | | | | |
| | Scj1p/Jem1p | | | | |
| | Ubc6/7p | | | | |
| A1PiZ | BiP | Ssa1p | | | |

[a]Updated from Brodsky & McCracken (28a).

Multiple studies indicated that ERAD substrates are degraded in the cytoplasm by the proteasome (16, 94, 107, 122, 184, 204, 232, 265, 268). This complex proteolytic machine consists of a catalytic 20S cylindrical core particle and two copies of the 19S (PA700) regulatory particle that "caps" the 20S subunit (6). Ubiquitination is necessary for proteasomal processing of most (17, 95, 107, 122, 142, 260, 285), but not all ERAD substrates (156, 265, 279). Two ubiquitin-conjugating enzymes, Ubc6p and Ubc7p (16, 17, 107, 232), and a ubiquitin ligase, Hrd1p/Der3p (8, 55, 79), reside at the yeast ER membrane and are required to degrade many ERAD substrates (see Table 1). In addition to targeting substrates to the proteasome, ubiquitination is also required for the retrotranslocation of some proteins (16, 20, 58). Likewise, Mayer and colleagues (150) and Plemper et al. (199) were unable to detect cytosolic, ubiquitinated forms of ERAD substrates in yeast proteasome mutants. The proteasome may provide the energy, via its six resident ATPases, to directly extract ERAD proteins concomitantly with their ubiquitination (150, 255a).

Before interacting with the proteasome, ERAD substrates are de-glycosylated and de-ubiquitinated and must be unfolded to fit through a small aperture at the tip of the catalytic core of the proteasome (239, 268; reviewed in 253). The 19S subunit of the proteasome is a molecular chaperone, capable of binding and preventing the aggregation of unfolded proteins (23, 236). In addition, other cytosolic molecular chaperones including Ssa1p (see above) and Hsp90 (77, 88, 116, 222) may maintain ERAD substrates in an aggregation-free state for attack by the proteasome or help deliver substrates to the proteasome, as these chaperones have been found associated with the yeast 19S subunit (251). In mammalian cells, a putative E3 ubiquitin ligase, CHIP, could mediate the delivery of misfolded proteins from these cytosolic chaperones to the proteasome (4, 46, 157). Likewise, the mammalian nucleotide exchange factor BAG-1 may help target hsc70-bound substrates to the proteasome (108, 145).

The elucidation of the ERAD pathway provided a model for how several toxins are able to transit from the ER to the cytoplasm (101). These toxins, which include ricin, pertussis toxin, Shiga toxin, *Pseudomonas* exotoxin A, cholera toxin, and yeast killer toxins, enter host cells through the endocytic pathway and ultimately reside in the ER by virtue of harboring ER retrieval sequences at their C termini. Once in the ER, they are exported to the cytoplasm via the Sec61 channel (64, 220, 229, 266). Consistent with the ERAD machinery being required for toxin action, yeast containing mutations in the genes encoding BiP and calnexin exhibit increased resistance to the K28 killer toxin (64), and prior to export, PDI is required for toxin unfolding (246). Inhibition of proteasome activity sensitizes both yeast and mammalian cells to toxins (229, 266), suggesting that a fraction of the retro-translocated toxin is recognized as an ERAD substrate. However, most of the toxin may escape degradation because the proteins are lysine-poor (101), thus minimizing their probability of being ubiquitinated.

Additional components of the ERAD machinery have been identified in three independent yeast genetic screens. Stabilization of hydroxymethylglutaryl-coenzyme A reductase (HMG-R) in mutant yeast strains (94) led to the discovery of Hrd1p (also known as Der3p, see below) and Hrd3p, which form a stoichiometric complex spanning the ER membrane (79) and preferentially ubiquitinate misfolded proteins (8). Also identified in this screen was Hrd2p, a component of the 19S regulatory subunit of the proteasome. A screen by Wolf and coworkers for mutants in which CPY* is stabilized uncovered three *DER* genes, (see above; 133). Der1p is an integral ER membrane protein of unknown function (133) and *DER2* and *DER3* encode for Ubc7p and Hrd1p, respectively, factors involved in ERAD (20, 133). Finally, mutants that accumulate a heterologously expressed variant of the mammalian ERAD substrate, Alpha-1 protease inhibitor (AlPiZ; 204), have identified seven complementation groups that may represent novel genes involved in ERAD (155). Combining the continued analysis of these and other genes required for ERAD with powerful biochemical tools will ensure a finer dissection of the ERAD pathway.

## THE UNFOLDED PROTEIN RESPONSE (UPR)

A primary mechanism by which eukaryotic cells counteract the accumulation of misfolded proteins within the lumen of the ER is the unfolded protein response (UPR). This response was initially recognized in mammalian cells by the induction of a specific set of proteins in response to glucose starvation, which results in protein misfolding through the under-glycosylation of nascent polypeptides (39). The proteins induced through this treatment were designated GRPs as a consequence of their glucose regulation (e.g., GRP78 was the original name given to BiP), and consisted largely of molecular chaperones. Other treatments were soon discovered that increased the transcription of the same set of genes, including tunicamycin (an inhibitor of N-linked glycosylation), DTT, and calcium-ionophores. However, other general stress conditions, including heat shock, do not induce the expression of the same set of genes. This stereotyped response to ER-specific folding stressors is shared among all eukaryotic cells.

Rapid progress in detailing the mechanism of UPR activation became possible with the discovery of this response in *S. cerevisiae*. A promoter element, termed the UPRE, was found upstream of UPR targets in *S. cerevisiae* (164) and was subsequently used to begin genetically defining the signaling pathway between the ER and nucleus that is responsible for activation of UPR target gene expression (48, 163). The first screens identified molecules at the extreme ends of the signaling pathway (Figure 2). The signal originates in the lumen of the endoplasmic reticulum with the activation of the transmembrane serine/threonine kinase Ire1p (48, 163). When unfolded proteins begin to accumulate in the ER, the Ire1p kinase dimerizes and is autophosphorylated in *trans* (224). At the other end of the signaling pathway lies Hac1p, a member of the bZIP family of transcription factors (49, 162). Both factors are absolutely required for UPR induction, as deletion of either gene results in a strain unable to increase the expression of known UPR targets in response to ER folding stress.

The discovery of the pathway linking Ire1p and Hac1p awaited the convergence of a number of different observations. First, *HAC1* mRNA migrates differently when isolated from UPR-induced or noninduced cells (49, 162). Second, Hac1p can only be detected in cells under conditions that induce the UPR (49, 162). Finally, another genetic screen implicated *RLG1*, a tRNA ligase, in induction of the UPR (226). When combined with the observation that Ire1p contains a domain with homology to nucleases (48, 163), a model emerged in which Ire1p becomes an active nuclease when unfolded proteins accumulate within the ER. Ire1p then cleaves the transcribed *HAC1* message (termed $HAC1^u$) at specific locations near the 3′ end, removing a nonconventional intron (130, 227). The alternative splicing of the *HAC1* mRNA is completed through the action of Rlg1p, which ligates the alternative exon to the *HAC1* message, forming a new message designated $HAC1^i$ (226, 227). Only the protein encoded by the alternatively spliced message accumulates in cells. This reaction has since been reconstituted in vitro using only Ire1p, *HAC1* mRNA, and Rlg1p (86).

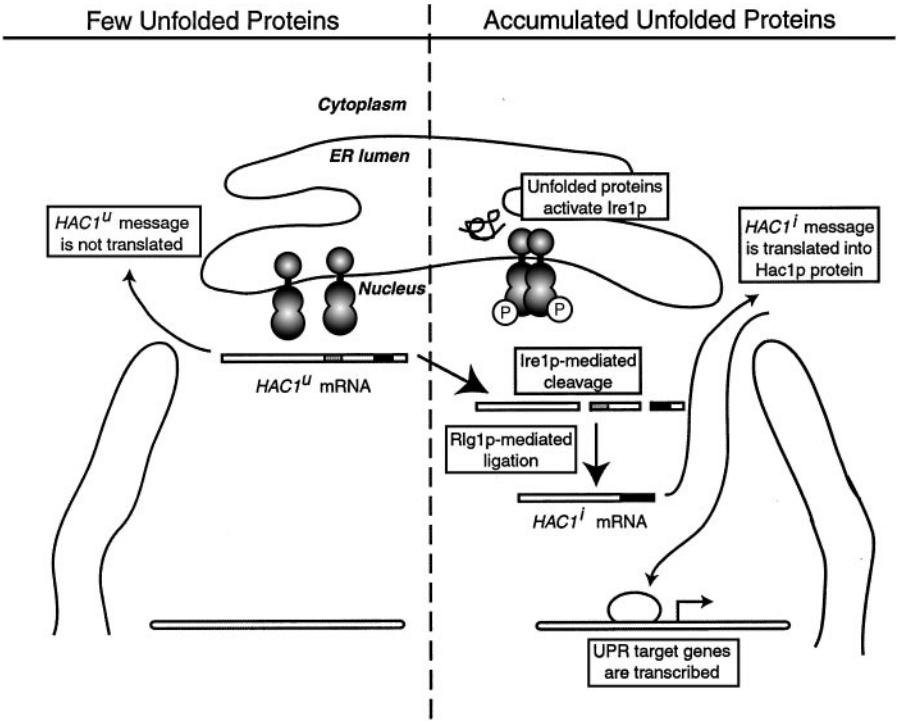


**Figure 2** A schematic model of the unfolded protein response pathway as defined in the yeast *S. cerevisiae*.Upon the accumulation of unfolded proteins within the ER, Ire1p becomes activated through dimerization. This initiates an alternative splicing event, ultimately producing the *HAC1*$^{i}$ mRNA. Only the *HAC1*$^{i}$ message is efficiently translated. The Hac1p protein enters the nucleus, binds to promoter elements upstream of the UPR target genes, and activates their transcription. Although Ire1p is depicted here with its endonuclease domain localized to the nucleus, this has not been demonstrated experimentally. See text for details (modified from 223a).

One model to explain how cells might sense unfolded proteins predicts that BiP binds to the Ire1p lumenal domain during normal growth, preventing the dimerization of Ire1p molecules. As unfolded proteins accumulate in the ER, increasing amounts of BiP are recruited from Ire1p. Eventually, Ire1p dimerizes, initiating the UPR signaling pathway. This model was recently tested by Ron and co-workers (15), who detected a physical association between Ire1p and BiP in extracts from a rat pancreas–derived cell line under normal growth conditions; under conditions of UPR induction, a physical interaction between Ire1p and BiP was absent. A similar mechanism is also likely to exist in the yeast ER (186).

The pathway leading to UPR activation in mammalian cells is more complex than that in *S. cerevisiae* and has been less clearly defined. A number of groups have identified Ire1p homologs in higher eukaryotes, including Ire1$\alpha$ (identified in humans) (243), Ire1$\beta$ (identified in mouse cells) (258), and PERK (98, 225). Whereas both Ire1$\alpha$ and Ire1$\beta$ show homology to Ire1p throughout their entire lengths, PERK is homologous to Ire1p only in its ER lumenal domain and has a kinase domain more like that of eIF2$\alpha$ than that of Ire1p. Consistent with these data, Ire1$\alpha$, Ire1$\beta$, and PERK respond to the same inducers, but diverge in the downstream signaling events that they mediate. Whereas Ire1$\alpha$ and Ire1$\beta$ induce the expression of BiP and CHOP (another UPR target), PERK responds to the accumulation of unfolded proteins by phosphorylating eIF2$\alpha$, leading to a decrease in translation (98).

In contrast to the situation in *S. cerevisiae*, activation of the mammalian UPR appears to involve a proteolysis step at the level of Ire1 activation. Upon stimulation of the UPR, both Ire1$\alpha$ and Ire1$\beta$ are cleaved from the membrane, and the newly released, soluble form redistributes to the nucleus (181). This redistribution seems to depend on the activity of presinilin-1 (PS1), as cells lacking PS1 activity are unable to produce the soluble form of Ire1. In addition, in at least some cell lines, lack of PS1 decreases the level of UPR induction as measured by BiP expression (129, 181). However, although two groups have observed a role for PS1, a third report finds no effect of PS1 on UPR activation (216). As the conditions used in these experiments are not identical, the full significance of PS1 in UPR activation will await future experiments.

Proteolysis has also been implicated in the activation of at least one transcription factor responsible for the ER stress response in metazoan cells. ATF6, a Type-II transmembrane protein, is cleaved into two fragments in response to treatments that lead to the accumulation of misfolded proteins, and the released cytosolic domain translocates into the nucleus and induces the transcription of several chaperones (31, 100). Goldstein and co-workers subsequently demonstrated that S1P and S2P, the proteases responsible for cleavage of the sterol-starvation transcription factors of the SREBP family, are necessary for cleavage of ATF6 and for a normal ER stress response (276). However, unlike the SREBP targets of S1P and S2P, sterols do not affect activation of gene expression through ATF6.

At this point, the relationship between the ATF6 and IRE1$\alpha$/IRE1$\beta$ pathways is unclear. Data from Kaufman and co-workers suggest that ATF6 activation lies downstream of Ire1$\alpha$ activation and that the response to ER stress begins with activation of Ire1$\alpha$ (259). However, ATF6 does not appear to be alternatively spliced under conditions of ER stress (277). As both Ire1$\alpha$ and Ire1$\beta$ show homology to the nuclease domain of *S. cerevisiae IRE1* (243, 258), another transcription factor in mammalian cells, yet to be identified, may be activated in the same fashion as *HAC1* in *S. cerevisiae*. Indeed, both Ire1$\alpha$ and Ire1$\beta$ can cleave yeast *HAC1* mRNA in vitro (181, 243).

## INTERACTION BETWEEN THE UPR AND ERAD

Although the initial characterization of the UPR suggested that its targets would be limited to chaperones and factors required to maintain ER homeostasis, such as lipid biosynthesis (50), a growing body of evidence now suggests that the UPR regulates many aspects of secretory pathway function. By taking advantage of the genetic requirements of UPR activation in *S. cerevisiae* and oligonucleotide microarray technology, a list has been compiled detailing the breadth of the transcriptional output of the UPR (37, 245). From this analysis, nearly 400 genes were identified as UPR targets, of which 208 were of known or inferred function. Of these 208 genes, approximately half play roles in the secretory pathway. Thus, genes encoding chaperones that exist entirely outside the secretory pathway in yeast, such as Hsp104, Hsp60, and Hsp90, were not identified as UPR targets. Of chaperone families with members found in every cellular compartment, only those genes encoding ER-localized chaperones were identified as UPR targets. For example, of the 15 DnaJ homologs encoded in the yeast genome, only the three homologs encoding ER-localized DnaJ homologs are induced by the UPR. The functional categorization of UPR targets is depicted in Figure 3.

The UPR activates the expression of genes encoding proteins acting throughout the secretory pathway and spanning virtually all activities. How these UPR-induced targets improve the state of folding within the ER is unclear. The various activities induced by the UPR may well act in concert to reduce the lumenal concentration of misfolded protein, by either directly refolding proteins or removing them from the ER. This "fix or clear" model suggests that all activities required for folding are up-regulated, such that chaperones bind to misfolded species, prevent aggregation, and promote folding, while glycosylation enzymes assist in the folding of proteins that require carbohydrate modification to attain their proper conformation. Consistent with this suggestion, mutations that compromise either addition of GPI anchors or protein glycosylation are lethal in the absence of UPR function (177). Moreover, UPR induction in mammalian cells accelerates synthesis of the dolichol-oligosaccharides employed in asparagine-linked glycosylation (62).

In addition to up-regulating factors that directly promote folding, UPR activation may also induce factors to clear misfolded proteins from the ER. The induction of specific COPII or coatomer components might facilitate the packaging of cargo proteins into anterograde vesicles, or simply increase the overall capacity of anterograde transport. This increase in anterograde transport might catalyze the passage of misfolded species to the vacuole for degradation (110, 137), consistent with the observation that several genes involved in vacuolar targeting are also UPR targets, or the retrieval of ERAD substrates from the Golgi that must be returned to the ER for degradation (D. Ng, personal communication). Similarly, induction of lipid synthesis may lead to an increase in the volume of the ER, diluting the concentration of unfolded proteins. Finally, the induction of ERAD components directly enhances the clearance of misfolded proteins from the ER.

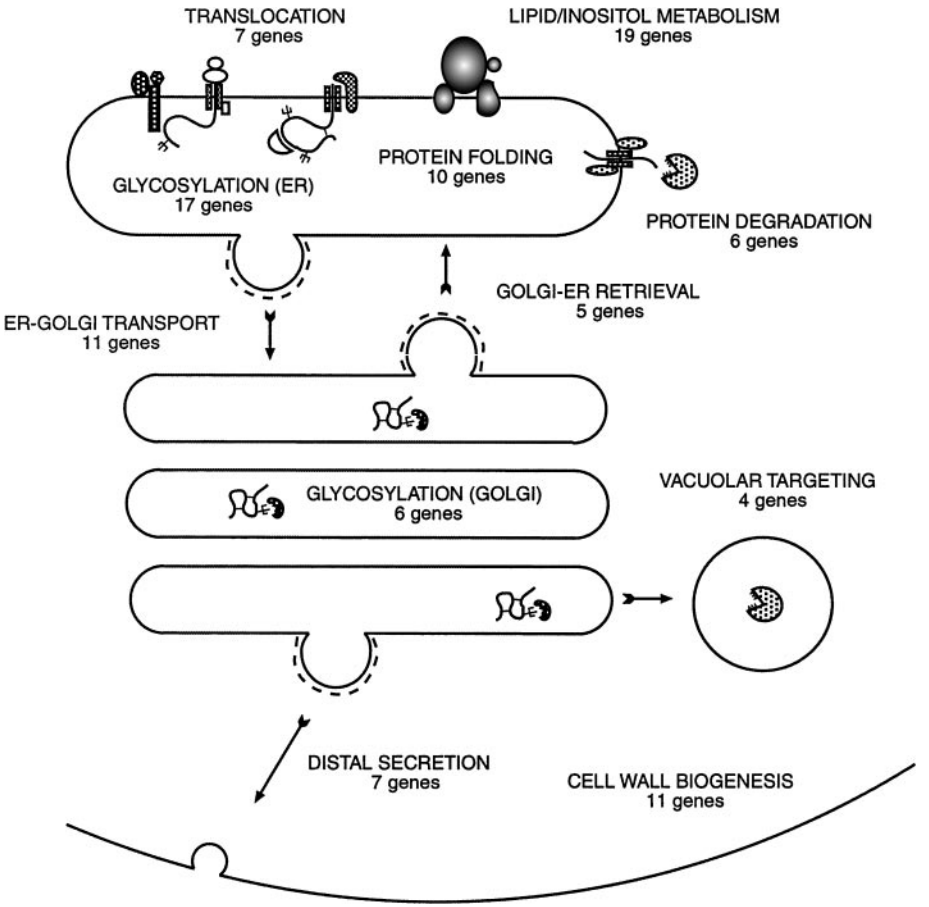


**Figure 3** Many aspects of secretory pathway function are transcriptionally induced by the UPR. A schematic diagram of the secretory pathway is shown. The number of genes whose function is either known or can be inferred from homology to characterized genes is indicated underneath each functional category (reproduced from 245).

The link between the UPR and ERAD suggests the existence of a previously unrecognized connection between two pathways that deal with the consequences of misfolded proteins. Mechanistic studies from a number of research groups have now confirmed the physiological significance of these findings. First, efficient ERAD requires an intact UPR. In particular, deletion of *IRE1* decreased the ERAD of CPY* (245) and MHC class I heavy chain (H-2K$^b$) in yeast (37). Second, loss of ERAD function leads to chronic UPR induction. Mutants defective for CPY* degradation show a small but significant induction of the UPR (75, 133, 245). Alleles of *SEC61* with specific defects in ERAD, as well as deletions of several

other ERAD components, also caused constitutive UPR induction (285). Thus, the chronic accumulation of misfolded proteins in the ER appears to be a general consequence of loss of ERAD. Third, simultaneous loss of ERAD and UPR function greatly decreases cell viability. For example, Ng and co-workers conducted a screen to identify genomic mutations that are synthetically lethal with loss of the UPR (177). A large panel of mutants were isolated and were then further classified based on functional analysis. Analysis of the rate of CPY* degradation indicated that one third of the identified mutants have defects in ERAD. The functional significance of this genetic interaction is emphasized by the synthetic lethality between mutations of genes in the UPR pathway and in a number of components required for ERAD [*SON1*, *UBC1*, *UBC7*, *HRD1*, *HRD3*, and *DER1* (75, 177, 245)] that act at multiple steps in the ERAD pathway.

In sum, these findings suggest that protein folding in the ER is inefficient, and the removal of misfolded proteins is an essential process performed together by the UPR and ERAD machineries. In the absence of the UPR, ERAD deals with the consequences of protein misfolding by retro-translocating these species to the cytoplasm where they are degraded. Conversely, in the absence of ERAD, the UPR deals with the consequences of protein misfolding by activating the expression of factors involved in protein folding, anterograde vesicular transport, or an alternate site of degradation, such as the vacuole. Thus, the UPR and ERAD systems provide partially overlapping functions in the same essential process: the removal of misfolded proteins from the ER.

## SUBSTRATE-SPECIFIC CHAPERONES

Several genes identified in yeast are required for the biogenesis of specific secreted proteins. Although the genes do not encode classical chaperones, and in many cases their specific functions are unknown, they apparently evolved to facilitate the folding or quality control of selected secreted substrates. This class of protein was first recognized genetically through the identification of a mutant strain of yeast that showed defects in amino acid uptake. The gene implicated in this study, identified as *SHR3*, was found to be ER-localized, and resulted in retention of amino acid permeases in the ER (141). Several substrate-specific chaperones have subsequently been identified in *D. melanogaster*, *S. cerevisiae*, *C. elegans*, and mammalian cells, all of which are ER-localized and required for proper secretion of only one or a subset of proteins (reviewed in 65).

In most cases, the level at which the proteins act is not clear; they may be required for folding or secretion, or they may act as a specific quality control mechanism. For example, Naik & Jones (171) screened for mutants defective for the processing of the vacuolar-targeted proteinase B (Prb1p) and isolated a gene encoding an ER-localized, integral membrane protein named Pbn1p. Two-hybrid analysis indicated that Pbn1p interacts with the Prb1p pro-peptide. *PBN1* is essential, unlike the gene encoding its substrate (*PRB1*), suggesting that Pbn1p

may play a role in the biogenesis of essential factors and/or that it is required for ER homeostasis. Interestingly, Prb1p becomes an ERAD substrate when Pbn1p function is ablated.

In contrast, in at least two other cases, homology between the substrate-specific chaperone and a class of general chaperones implies that the required activity is indeed related to protein folding. One example of a substrate-specific chaperone with homology to a specific chaperone class is a PDI homolog (*EPS1*) whose activity was identified in studies of the yeast plasma membrane ATPase, encoded by the *PMA1* gene. A dominant mutation was described that prevented the ER export of both the mutant and wild-type forms of the Pma1 protein from the ER, which became ERAD substrates. Wang & Chang (256) screened for suppressors of the dominant phenotype and uncovered the nonessential gene, *EPS1*. Wild-type and mutant Pma1p are stabilized in cells deleted for *EPS1* because they are no longer retained in the ER, suggesting that Eps1p is a quality control gatekeeper in the ER, preventing the secretion of misfolded Pma1p.

The second example involves the PPIase homolog *ninaA* that was identified in studies of rhodopsin folding in *D. melanogaster* (45, 233). *ninaA* mutant flies show a greatly reduced level of rhodopsin in the outer photoreceptor cells. Upon closer examination, it was observed that only the Rh1 and Rh2 rhodopsins were affected by the lack of *ninaA* activity, while the Rh3 rhodopsin was not (233). Cloning of *ninaA* revealed that it was a transmembrane protein and highly homologous to vertebrate cyclophilin. Like other substrate-specific chaperones, mutation of *ninaA* results in the accumulation of its substrates in the ER, although rhodopsin was also found in vesicles distributed throughout the cytoplasm (45).

Although little is known about the substrate-specific chaperones, they are clearly a growing class of proteins with important roles in the maturation of a wide variety of secretory proteins. These proteins provide a unique insight into the particular demands on protein folding in the secretory pathway. More generally, an understanding of substrate-specific chaperones might reveal previously overlooked aspects of protein folding in the ER.

## SUMMARY

By virtue of their endogenous biochemical properties and their promiscuity, chaperones have adapted as critical factors in the eukaryotic secretory pathway. Not only do molecular chaperones act as central players in each of the processes discussed in this review, but an individual chaperone may play critical roles in several of these processes, sometimes simultaneously. This has complicated genetic analyses of chaperone action in the cell. However, the isolation of mutants that are specifically defective for a single process, and/or the use of strong, conditionally acting mutants, has permitted a better molecular dissection of chaperone action during protein secretion. Equally powerful has been the use of in vitro assays in which the functions of wild-type and mutant chaperones can be ascertained

in defined systems. The continued development of biochemical assays that measure unique aspects of secretory pathway function, combined with the isolation and construction of new mutants, should permit researchers to define further how chaperones can exert their pleiotropic effects. In addition, a relatively recent, but rapidly progressing field is the solution of chaperone structures using biophysical techniques. Such undertakings will further catalyze biochemical and genetic experiments.

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