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Versatility of the Endoplasmic Reticulum Protein Folding Factory

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ABSTRACT The endoplasmic reticulum (ER) is dedicated to import, folding and assembly of all proteins that travel along or reside in the secretory pathway of eukaryotic cells. Folding in the ER is special. For instance, newly synthesized proteins are *N*-glycosylated and by default form disulfide bonds in the ER, but not elsewhere in the cell. In this review, we discuss which features distinguish the ER as an efficient folding factory, how the ER monitors its output and how it disposes of folding failures.

KEYWORDS chaperones, PDI, BiP, calnexin, EDEM, ERAD

INTRODUCTION

Protein Folding & Chaperones

Genes encode proteins through mRNA intermediates, which are translated by ribosomes: the central paradigm of biology. Protein biosynthesis, however, is not the result of translation alone. To become biologically functional proteins, translated polypeptides need to reach their proper three-dimensional conformation—a process referred to as protein folding. In essence, the final conformation of proteins lies embedded in the primary sequence of the polypeptide chains (Epstein *et al.*, 1963). Energy minimization is the driving force behind protein folding. Weak short-range interactions between neighboring or adjacent residues either stabilize or destabilize structural elements. These are combined and rearranged until the polypeptide reaches a final structure, called the native state. The native state occupies a minimum of potential energy in the folding "landscape" (Bryngelson *et al.*, 1995), whereas free energy is higher for proteins that are not fully folded, the folding intermediates. Following this principle, protein folding requires neither extrinsic factors nor energy input (Anfinsen & Scheraga, 1975; Jaenicke, 1991).

Some small proteins indeed may fold efficiently on their own, but folding of many proteins is slow and inefficient, because the course toward the native state can be bumpy. Semi-stable folding intermediates can persist because of local energy minima in the folding landscape (Daggett & Fersht, 2003). *In vivo*, protein folding is assisted by chaperones and folding enzymes. They transiently associate with maturing secretory proteins to catalyze slow folding events and to prevent polypeptide chains from undergoing unproductive interactions with themselves or their environment; a process that results in protein aggregation (Ellis &

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Hemmingsen, 1989; Gething & Sambrook, 1992). Folding factors are among the most highly expressed proteins in the cell. For instance, the cytosolic chaperone Hsp90 or the endoplasmic reticulum resident chaperone BiP each account for 1.5%–3% of the soluble protein content of the cell, whereas all chaperones and folding enzymes together contribute 15%–25% (our unpublished observations). Despite the abundance of chaperones and other folding factors, protein misfolding and accumulation of protein aggregates lie at the basis of many diseases (Rutishauser & Spiess, 2002; Dobson, 2003).

Because protein folding is fundamental to life, many of the key folding machineries are conserved from bacteria to man. In contrast to prokaryotes, eukaryotes contain various organelles separated by lipid membranes. Translocation of fully folded proteins across membranes is often avoided. Instead, folding occurs in different compartments of the eukaryotic cell: cytosol, mitochondria, and chloroplasts in plants, each with its having their own folding machinery.

Origin and Function of the Endoplasmic Reticulum Protein Folding Factory

Another eukaryotic cell compartment with a distinct folding machinery is the endoplasmic reticulum (ER). Whereas mitochondria and chloroplasts seem to have arisen from endosymbiont origins, the periplasm of Gram-negative bacteria may be the ontological predecessor of the ER. A conserved protein channel conducts newly synthesized proteins into the lumen of both periplasm and ER (Keenan *et al.*, 2001) and the ER sustains disulfide bond formation, like its prokaryotic equivalent (Sevier & Kaiser, 2002). The periplasm is dedicated to processing of outer membrane proteins, and hence, the synthesis of the barrier protecting bacteria from their surroundings. Similarly, one could consider the secretory machinery of unicellular eukaryotes such as yeast to be merely a cell wall factory. The ER thus can be regarded as the intracellular equivalent of the outside world (Helenius *et al.*, 1992).

In effect, the ER is the cradle of all cell surface proteins, of proteins that are secreted, and of proteins that reside in any compartment along the exocytic and endocytic pathways. Together, these ER clients represent approximately one third of all eukaryotic proteins, as has been determined for yeast (Ghaemmaghami *et al.*, 2003). While clients of other folding machineries remain within the compartment where they fold, the ER folding machinery is unique in the sense that most of its clients leave the compartment once they are fully folded. Thus, the ER can be regarded as a folding factory that produces proteins for other compartments of the cellular endomembrane system (Figure 1).

The ER is also the major site of membrane lipid synthesis in eukaryotic cells. Vesicular transport ensures that both membrane lipids and proteins travel to the Golgi and, eventually, to other destinations in the endomembrane system. In contrast, mitochondria are autonomous organelles. They do receive membrane lipids from the ER, not via vesicular transport, but likely via direct membrane contact sites (de Kroon *et al.*, 2003). For some time, peroxisomes were also regarded as autonomous organelles, but recently it has become apparent that some peroxisomal membrane proteins first arrive in the ER membrane, where they concentrate to

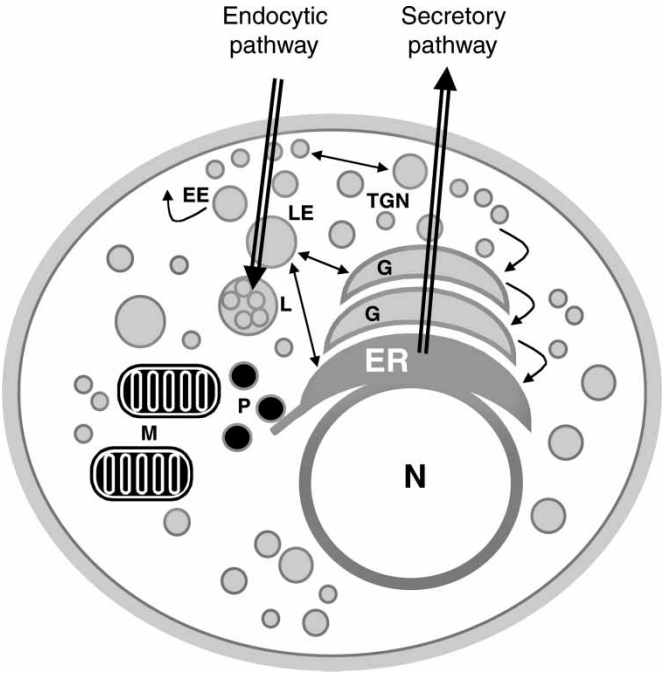

FIGURE 1 **The central role of the ER in the cellular endomembrane system. The nucleus is marked with (*N*). The ER and the nuclear envelope are depicted in dark gray. All organelles where resident proteins originate from the ER are depicted in light gray: secretory pathway compartments Golgi apparatus (*G*) and Trans Golgi Network (*TGN*), cell surface and extracellular space, and endocytic compartments early endosomes (*EE*), late endosomes (*LE*) and lysomes (*L*). Large arrows symbolize the two major transport routes. Small arrows symbolize the multitude of other transport routes that communicate between secretory and endocytic pathways. Mitochondria (*M*) and peroxisomes (*P*) are depicted in black, because they do not form part of the secretory or endocytic pathways, except that the peroxisome membrane is derived from the ER and therefore indicated as a gray line.**

distinct subdomains (Geuze *et al.*, 2003). Once these domains separate from the ER, the import machinery for peroxisomal matrix proteins is formed and the precursor organelles finally develop into fully functional peroxisomes (Tabak *et al.*, 2003).

Proteins in the cytosol and mitochondria remain under constant surveillance of the chaperones that guided their maturation. These chaperones are equally well equipped to perform maintenance on proteins showing occasional structural flaws. In contrast, secretory proteins and proteins of the exocytic and endocytic systems can no longer rely on any chaperone assistance after their exit from the ER. This may provide an explanation for the sophistication and stringency of the so-called "quality control" mechanisms in the ER: in principle, only correctly folded ER-clients can exit from the ER (Ellgaard & Helenius, 2003).

Multicellular organisms have tremendously diversified the roles of their secretory proteins. To cite two examples: intercellular communication depends on cell surface expression of a multitude of receptors for metabolites and hormones; and adhesion molecules at the cell surface largely determine the supercellular architecture of tissues. The more outward orientation of higher eukaryotes is also reflected in the genome. Compared to yeast, a larger percentage of human genes encode proteins that travel along the secretory pathway and human cell surface proteins on average have bulkier ectodomains than yeast's cell wall proteins (Lander *et al.*, 2001). Accordingly, the secretory capacity of the ER of higher eukaryotes easily outmatches the unicellular cell wall factory. Glands and the immune system thrive by virtue of specialized cells that are devoted to secretion of proteins into, for instance, blood (e.g., immunoglobulins) or the gut (e.g., pepsins). Production rates in professional secretory cells can be enormous. For example, the daily antibody output of plasma cells can equal their own mass.

Special to the ER folding factory is that client proteins acquire both *N*-linked glycans and disulfide bonds. Glycosylation and disulfide bond formation might well have developed originally as beneficial assets in creating a robust cell wall. These post-translational modifications certainly enhanced the possibilities in protein design. The majority of ER clients are glycosylated, and many cannot fold without their hydrophilic glycans, since the polypeptide alone is too hydrophobic and prone to aggregation. Likewise, most ER clients only fold correctly under oxidizing conditions. The unique protein folding conditions in the ER even may have directed evolution of ER clients. Indeed, metazoan ER clients on average have more β-sheets and fewer α-helical elements than cytosolic proteins, while a number of folds (such as the Ig fold, the EGF fold, the fibronectin type III fold, and the cadherin fold) are "over-represented" within the ER client fold repertoire (Yu Xia and Mark Gerstein, personal communication). Thus, not only is the ER a specialized folding compartment with distinct characteristics, but ER client proteins also form a league of their own in the way they fold.

ENTER THE ER

Targeting of Client Proteins into the ER Lumen

In all kingdoms of life, proteins that exit the cell, traverse the membrane via a translocation pore, the translocon (Keenan *et al.*, 2001). In eukaryotes, proteins destined for the secretory pathway are translocated across the ER membrane. ER clients are translocated either during translation (co-translational) or when translation already is completed (post-translational). While it has been studied in detail in yeast, post-translational translocation into the mammalian ER has received little attention, and its relevance remains poorly understood (Zimmermann, 1998; Rapoport *et al.*, 1999).

A stretch of ~17 to 35 hydrophobic residues at the N-terminus of ER client proteins, the signal peptide, destines them to enter the ER (Walter & Johnson, 1994; Martoglio & Dobberstein, 1998). The signal peptide binds signal recognition particle (SRP) and the ER-client protein-SRP complex in turn is recognized by the SRP receptor (SR), which is a component of the translocon complex in the ER membrane (Gilmore *et al.*, 1982a; Gilmore *et al.*, 1982b). Both SRP and the SR are GTPases that together form a catalytic chamber for two GTP molecules (Egea *et al.*, 2004; Focia *et al.*, 2004). They reciprocally stimulate each other's GTPase activity, whereupon SRP releases the signal peptide and the targeting complex disassembles (Miller *et al.*, 1993). In case of co-translational translocation, ER client proteins are synthesized by ribosomes that line up on the cytosolic side of the ER membrane. As soon as the signal peptide emerges from the ribosome, the ribosome-nascent ER client-SRP complex docks onto the translocon. As a result, the nascent protein directly enters the translocon pore (Johnson & van Waes, 1999).

The Translocon

The core of the translocon is the hetero-trimeric Sec61 complex that is composed of an α-subunit, which spans the ER membrane 10 times, and β- and γ-subunits, which are single-span proteins in most organisms (Clemons *et al.*, 2004). CryoEM studies suggested that the translocon is composed of oligomeric rings of the Sec61 protein complex (Stirling *et al.*, 1992; Hanein *et al.*, 1996). The crystal structure of the Sec61 homolog SecY from the archea *Methanococcus jannaschii* however shows that a single SecY protein complex already forms a channel that can conduct proteins entering the ER lumen (Van den Berg *et al.*, 2004). The channel has an hourglass shape: it has a central ring of hydrophobic residues at the constriction, which has a diameter of only 8 Å and funnel-shaped openings to either side of the ER membrane (Van den Berg *et al.*, 2004). At the ER lumenal side, the funnel is closed off by a short helix. This suggests that its displacement is necessary when the SecY complex is engaged in translocation (Van den Berg *et al.*, 2004). SecY was crystallized in the absence of client proteins, while the Sec61 oligomeric rings were isolated in the presence of nascent chains, which may indicate that ring (dis-)assembly still is important for on/off cycling or gating of the translocon.

Signal Peptide Cleavage and Membrane Anchoring of ER Clients

From most ER client proteins the signal peptide is cleaved off by signal peptidase even before chain termination, thereby generating a new lumenal *N*-terminus. This proteolytic cleavage occurs at the ER lumenal side (Blobel & Dobberstein, 1975). Many ER clients become soluble proteins: after signal peptide cleavage and complete passage of their C-terminus through the translocon pore, they are no longer associated with the membrane. Many other ER clients, however, remain anchored to the membrane. Proteins with a single membrane anchor in the ER membrane can be divided into five categories: tail-anchored proteins, glycosyl-phosphatidyl-inositol (GPI)-anchored proteins and type I, type II and type III membrane proteins (Figure 2).

Unlike soluble ER clients, the C-terminus of type I membrane proteins is not fully translocated. These proteins have a stretch of ~20 hydrophobic residues

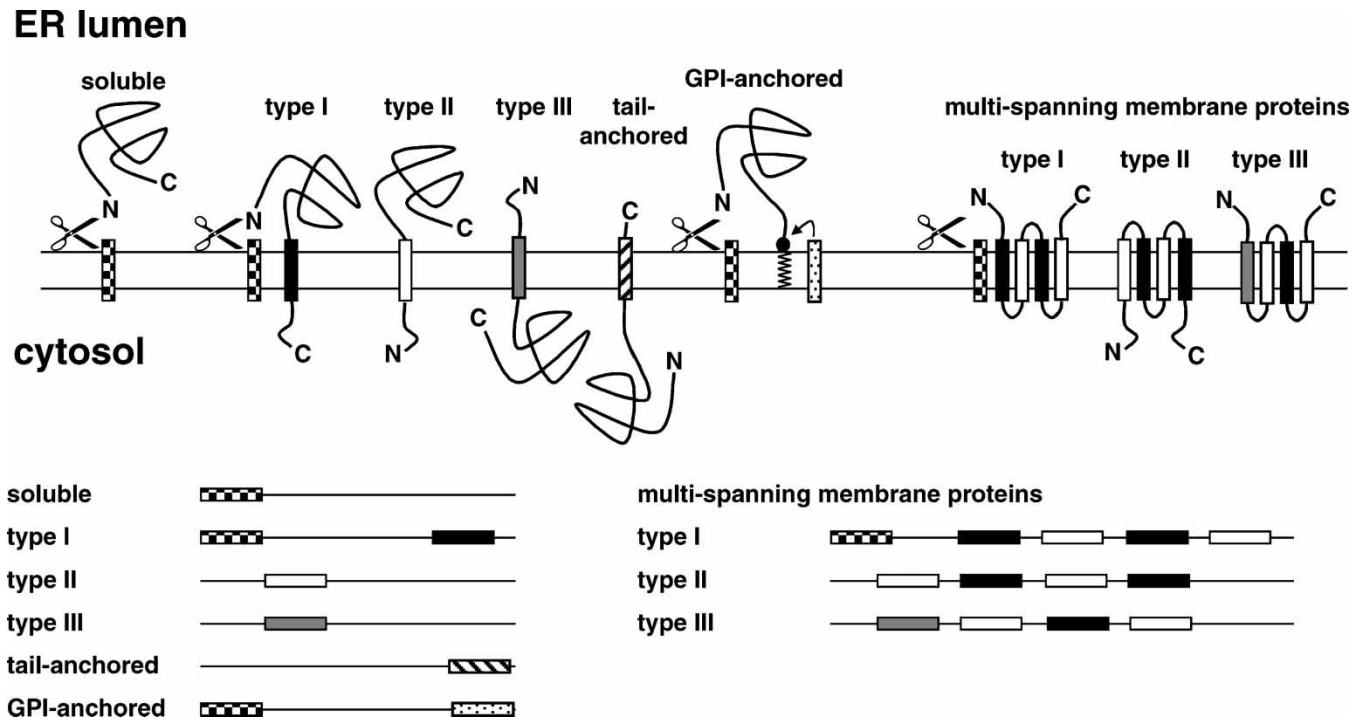

FIGURE 2 **Membrane insertion and topology. Cleavable signal peptides are indicated as chequered boxes; "stop transfer" signal as black boxes; signal anchors as white boxes; reverse signal anchors as gray boxes; tail-anchor signals as striped boxes and GPI-anchor signals as speckled boxes. N- and C-termini are indicated. Signal peptidase is indicated by pair of scissors. The arrow indicates that the GPI-anchor signal is removed from these ER-clients, while they instead are attached to the membrane with the GPI-anchor, indicated as a black circle and a zigzag-line.**

that act as stop-transfer signal for the translocon. As a result, these proteins have an N-terminal ectodomain in the ER lumen, while the stop transfer signal forms a transmembrane domain (TMD) that connects the ectodomain with the cytosolic C-terminal domain. Conversely, type II membrane proteins have the opposite orientation. Their signal peptide is not removed. Instead, it anchors these proteins in the membrane as a TMD. These so-called signal anchors can also be located internally within the polypeptide chain. Thus, type II membrane proteins have a cytosolic *N*-terminus and a C-terminal ectodomain (N_{cyt}/C_{exo}) (von Heijne & Gavel, 1988; Goder & Spiess, 2001).

Type I and type II membrane proteins have in common that the protein (portion) C-terminal of the signal peptide/anchor reels into the ER lumen. The signal peptide/anchor therefore must adopt an N_{cyt}/C_{exo} orientation. Goder & Spiess recently showed in an elegant *in vivo* study that the signal anchor of a type II membrane protein enters the translocon in an N_{exo}/C_{cyt} orientation, whereupon it inverts to an N_{cyt}/C_{exo} orientation early during translocation (40–50 sec) (Goder & Spiess, 2003). This inversion is driven by electrostatic forces (Goder & Spiess, 2003), according to the positive inside rule: at the cytosolic side, residues adjacent to a TMD or a signal anchor are often positively charged (arginine and lysine), while at the lumenal side, on average, negatively charged residues flank the membrane spanning domain (von Heijne & Gavel, 1988). The translocon itself may electrostatically enforce orientation of membrane spanning domains. Several conserved charged residues in Sec61 are opposite to the positive inside rule and, when mutated, fidelity in establishing topology of membrane proteins is reduced (Goder *et al.*, 2004).

In contrast to type I and type II membrane proteins, type III membrane proteins have so-called reverse signal anchors that do not invert orientation in the membrane. Instead, they directly dictate the translocation of the *N*-terminal part of the protein. Effectively, these proteins adopt the same N_{exo}/C_{cyt} orientation, as type I membrane proteins do. Different from type I proteins, however, the *N*-terminal part of type III proteins is fully synthesized before translocation. In principle, these *N*-termini therefore can already start to fold at the cytosolic side of the membrane, which would impede their translocation. This may explain why the majority of type III proteins have only short *N*-terminal ectodomains (Higy *et al.*, 2004).

The opposite of type III membrane proteins are the tail-anchored proteins: their C-terminus is inserted into the ER membrane. While the *N*-terminal cytosolic domain forms the bulk of these proteins, only few residues protrude into the ER lumen. Since the signal for membrane insertion only emerges from the ribosome when it reaches the stop codon, the insertion of tail-anchored proteins is obligatorily post-translational. Some tail-anchored proteins, like cytochrome *b5*, do not require any assistance for their membrane integration *in vitro* (Kim *et al.*, 1997). In fact, an engineered hydrophobic C-terminal tail of 11 leucine residues followed by a single valine suffices for insertion into ER membranes *in vitro* (Whitley *et al.*, 1996). It is still under debate whether *in vivo* membrane integration of tail-anchored proteins is guided by ER protein(s) and, if so, whether the regular import machinery (Abell *et al.*, 2003) or a separate tail-anchored protein specific integration machinery then would be responsible (Kutay *et al.*, 1995).

The fifth class of ER client proteins that have a single membrane anchor comprises GPI-anchored proteins. They are targeted to enter the ER lumen by a cleavable signal peptide similar to that of soluble ER clients and type I proteins. The signal for GPI attachment consists of a moderately hydrophobic peptide of 10 to 20 residues at the extreme C-terminus. It is linked by a spacer of 10 to 12 residues to a cleavage site, the ω-site, represented by a pair of small, uncharged residues at the 0 and +2 positions. When the signal is cleaved off, the GPI anchor is transferred *en bloc* to the new carboxyl terminus of the protein in a transamidase reaction. The GPI-anchor then serves as an alternative means for membrane attachment (Udenfriend & Kodukula, 1995).

Multi-Spanning Membrane Proteins

Next to single-spanning membrane proteins, a broad category of ER clients has more than one membrane anchor. For instance, the multidrug resistance protein 1 spans the membrane 17 times (Bakos *et al.*, 1998). The most *N*-terminal membrane spanning domain can be a regular TMD, a signal anchor or a reverse signal anchor, in the same manner as type I, II or III single membrane spanning proteins. Subsequent TMDs act as stop transfer or reinsertion signals, thereby alternating orientation starting from the most *N*-terminal topogenic signal onwards (Higy *et al.*, 2004) (Figure 2).

For several multi-spanning membrane proteins, integration of the first membrane spanning domain is critical. Subsequent TMDs often are less hydrophobic. Their stabilization in the lipid bilayer can be facilitated by interactions with the first, most hydrophobic, TM segment (Heinrich & Rapoport, 2003). Interestingly, a small protein of the translocon complex, PAT-10, remains associated with only the first TMD (the reverse signal anchor) of opsin, a seven trans-membrane domain protein, until the protein is fully translated and integrated into the membrane (Meacock *et al.*, 2002). This interaction is independent of the sequence and orientation of the TMD. PAT-10 will bind any TMD as long as it is the first to emerge from the ribosome (Meacock *et al.*, 2002). PAT-10 therefore may assist the first TMD in directing topogenesis. Still, downstream TMDs also obey the positive inside rule, albeit less stringently (von Heijne, 1989), illustrating that topology is not always dictated by the orientation of the most N-terminal signal. For some multi-spanning membrane proteins the insertion of several internal TMDs is even essential for insertion of the N-terminal reverse signal anchor and, hence, for translocation of the N-terminus (Nilsson *et al.*, 2000).

Role of the Translocon Complex in Topogenesis

At a mechanistic level, insertion of multi-spanning membrane proteins is still poorly understood. TMDs can adopt an alpha helical structure already inside the ribosomal tunnel (Woolhead *et al.*, 2004) with a length of ~40 residues (Matlack & Walter, 1995). This tunnel is too narrow, however, for the formation of more complex secondary structure in nascent proteins (Jenni & Ban, 2003). Based on the crystal structure of SecY, the translocon pore likewise appears to have too small a diameter to accommodate further folding (Van den Berg *et al.*, 2004). The translocon pore therefore seems to form an extension of the ribosome tunnel, accommodating ~25 to 30 residues in addition (Matlack Walter, 1995; Kowarik *et al.*, 2002). Formation of more complex secondary structure than α-helical elements alone indeed only commences at a distance of ~64 residues from the peptidyltransferase center inside the ribosome, at least for "classic" ER clients with a cleavable signal peptide (Kowarik *et al.*, 2002).

ER clients that have a signal anchor on the other hand can start to fold almost directly after extrusion from the ribosome (Kowarik *et al.*, 2002). This suggests that the signal anchor immediately egresses from the narrow translocon pore or that the pore widens when it encounters signal anchors or TMDs. Their membrane integration indeed occurs by lateral displacement, a reaction that requires the translocon channel to open toward the lipid bilayer (Martoglio *et al.*, 1995). It remains controversial whether TMDs leave the translocon singly (Mothes *et al.*, 1997) or whether clusters of TMDs collectively partition into the lipid bilayer (Borel & Simon, 1996; Johnson & van Waes, 1999). Possibly, the manner of membrane integration differs from one multi-spanning membrane protein to the other. For TMDs that are separated by only few residues, it is clear that their membrane insertion must be co-operative, but when sufficiently distant, TMDs may depend on their own topogenic determinants to establish orientation.

For some multi-spanning membrane proteins, charged residues within TMDs may have to team up with oppositely charged residues in fellow TMDs to establish the proper topology, as was demonstrated for the voltage sensor in the K^+ channel, KAT1 (Sato *et al.*, 2003). It may therefore be important that TMDs do not simply diffuse into the lipid bilayer once they egress from the translocon pore. As part of the translocon complex, the translocating chain-association membrane protein (TRAM) indeed can prevent such diffusion (Do *et al.*, 1996). The presence of charged residues within a TMD is an important determinant for its association with TRAM (Heinrich *et al.*, 2000; Meacock *et al.*, 2002), which is in line with a role of bundling of TMDs that ultimately will be held together by salt bridges between charged residues.

Folding and Topology

Altogether, topogenesis of membrane proteins is a dynamic process. The nascent polypeptide can reorient within the translocation machinery, in order to let protein loops between TMDs "probe" whether they are on the appropriate side of the ER membrane. The final topology will be determined by interactions of TMDs and the charge distribution (positive inside rule). Perhaps even more decisively, exposure of protein loops to either the cytosol or the ER lumen will submit them to the respective organellar folding machineries. Folding starts as soon as the polypeptide emerges from the translocon (Nicola *et al.*, 1999; Chen & Helenius, 2000). Loops that are destined to become ectodomains will

encounter their natural folding environment in the ER lumen. Glycosylation, disulfide bond formation and/or interaction with ER resident folding factors can all contribute to "freeze" topology (Goder *et al.*, 1999).

CONDITIONS IN THE ER LUMEN

Chaperone Composition and Molecular Crowding Within the ER Lumen

Some of the chaperones and folding enzymes present in the cytosol or mitochondria have analogs in the ER. Still, for two important classes of chaperones, the Hsp60s and the family of small Hsps, no ER resident equivalents have been found, except that the latter family has representatives in the ER of plants (Helm *et al.*, 1993). Conversely, ER folding factors that catalyze thiol-oxidation have no analogs in cytosol or mitochondria, but they do in the bacterial periplasm. Finally, the ER contains an expanding array of folding factors with activities unique to the ER. A full list of ER resident folding factors is given in Table 1.

The fact that fully folded ER clients leave the ER implies that folding factors and the clients that receive their assistance have the ER lumen to themselves. Consequently, chaperones and folding enzymes are very concentrated inside the ER lumen, almost in the millimolar range (Stevens & Argon, 1999). The chaperones and folding enzymes interact with one another in large complexes (Meunier *et al.*, 2002) and thereby form a dense, network-like structure within the ER lumen (Tatu & Helenius, 1997). The network-like composition of the ER lumenal content may explain the absence of Hsp60 or small Hsp family members. They confine unfolded proteins to a secluded environment. The Hsp60s form active folding cages (Fenton & Horwich, 2003), whereas small Hsps may merely shield unfolded proteins from the environment (Van Montfort *et al.*, 2001). The ER folding machinery already seems to fit like a glove around the folding substrate, which could make the seclusion strategy, and hence the presence of these classes of chaperones in the ER superfluous.

ER Retention

Different from their clients, ER resident folding factors should not travel any further along the secretory pathway. The association of individual ER resident chaperones and folding enzymes to the network of

TABLE 1 **List of ER resident folding factors.**

Family	Mammals	Synonym(s)	Accession N^o	Yeast
Hsp family members etc.				
Hsp90	GRP94	gp96, endoplasmin	P08113	
Hsp70	BiP	GRP78	P20029	Kar2p
Hsp70/NEF	GRP170	ORP150, CBP-140	Q9JKR6	Lhs1p
NEF	SIL1	BAP	Q91V34	Sil1p
Hsp40	ERdj1	Mtj1	Q61712	
	ERdj2	Sec63	Q9UGP8	Sec63p
	ERdj3	HEDJ	Q9UBS4	
	ERdj4		Q9QYI6	
	ERdj5		Q8CH78	
				Scj1p
				Jem1p
PPIases				
CyP	CyP-22	Cyclophilin B	P24369	Cpr5p
FKBP	FKBP2	FKBP13	P45878	Fkb2p
	FKBP7	FKBP23	O54998	
	FKBP9	FKBP63	Q9Z247	
	FKBP10	FKBP65	Q61576	
	FKBP11	FKBP19	Q9D1M7	
	FKBP14	FKBP22	P59024	
PDI, Ero and Erv family members				
PDI	PDI	P4HB, THBP	P09103	Pdi1p
				Eug1p
				Mpd1p
				Mpd2p
				Eps1p
	ERp57	ERp61, ER-60	P27773	
	ERp72	CaBP2	P08003	
	P5	CaBP1	Q63081	
	PDIP		Q13087	
	PDIR		Q921X9	
	ERp46	PC-TRP, endoPDI	Q91W90	
	ERp18	ERp19	Q9CQU0	
	ERp29	ERp28	P57759	
	ERp44		Q9D1Q6	
	ERdj5	JPDI	Q8CH78	
	PDILT*		AAH44936	
	TMX1		Q8VBT0	
	TMX2*		NP_057043	
	TMX3*		NP_061895	
PDI/Erv	QSOX1	Quiescin	Q9DBL6	
	QSOX2	Quiescin-like 1	Q8K0M2	
Erv				Erv2p
Ero	Ero1α		Q9QY03	Ero1p
	Ero1β		Q8R2E9	
Lectins, glycan trimming enzymes etc.				
CNX/CRT	Calnexin		P35564	Cne1p
	Calreticulin		P14211	
	Calreticulin 2		Q9D9Q6	
	Calmegin		P52194	
UGGT	UGGT		Q9NYU2	
EDEM	EDEM1		Q925U4	Htm1p
	EDEM2		Q91VV3	
	EDEM3		AAH60718	
Glucosidase I	Glucosidase I		Q80UM7	Gls1p
Glucosidase II	Glucosidase II α subunit		Q8BHN3	Gls2p
	Glucosidase II β subunit		O08795	
Mannosidase I	ER α1,2-mannosidase*		Q9UKM7	Mns1p

"General" ER resident chaperones and folding enzymes are listed per family. For mammalian folding factors the SWISS-PROT or GenBank accesion number of the murine variant is given, except for the proteins indicated by an asterisk were the accesion number relates to the human variant. Direct homologs in yeast of mammalian folding factors are listed on the same row. Orthologs in yeast that belong to the same protein family, but that do not have a direct homolog in mammals are listed on separate rows. For a complete list of "private" chaperones see (Ellgaard *et al.*, 1999; Schröder & Kaufman, 2005).

fellow folding factors already set hurdles for their escape from the ER. What is more, occasional runaways are actively resorted back to the ER by means of retention or retrieval signals. Lumenal ER proteins in mammals have a KDEL (in yeast HDEL) sequence or a closely related tetrapeptide at their C-terminus that mediates ER residency (Munro & Pelham, 1987; Pelham, 1990). When KDEL-containing proteins escape from the ER, they encounter the KDEL receptor already in the *cis*-Golgi (Scheel & Pelham, 1996). The cytosolic domain of the KDEL receptor binds to coat complex I (COPI), which mediates retrograde vesicular transport back to the ER. In the ER, the KDEL-receptor releases its substrate because of the higher pH compared to the Golgi (Wilson *et al.*, 1993). Type I ER membrane proteins like calnexin contain a dilysine motif in their cytosolic tail, which mediates ER residency (Nilsson *et al.*, 1989). Type II ER membrane proteins are retained in the ER in a similar manner through a diarginine motif (Schutze *et al.*, 1994). Retrieval and retention of these ER membrane proteins is achieved by direct interaction of the positively charged motifs with COPI (Letourneur *et al.*, 1994; Teasdale & Jackson, 1996).

Calcium

The ER lumen has a neutral pH (Kim *et al.*, 1998) and its electrolyte composition may be similar to that in the cytosol, except that the calcium concentration is significantly higher in the ER than in the cytosol (Meldolesi & Pozzan, 1998). Calcium is actively transported into the ER lumen from the cytosol by sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps. Release of calcium by ryanodine and IP_3 receptors from ER to cytosol activates signal transduction cascades that regulate diverse processes, such as membrane permeability, glycogen metabolism and muscle contraction (Brostrom & Brostrom, 2003).

Many ER resident proteins bind calcium, albeit with low affinity (Macer & Koch, 1988; Nigam *et al.*, 1994). The abundance of calcium binding proteins accounts for the large calcium storage capacity (Koch, 1990). It may be advantageous that most calcium is bound to the protein matrix in the ER. For instance, a relatively low concentration of free calcium could ease maintenance of the calcium gradient over the ER membrane by SERCA pumps. Moreover, escape of calcium via the secretory pathway is counteracted through ER retention of the proteins calcium is bound to. Despite their calcium-binding properties, the primary role of ER chaperones and folding enzymes lies in the folding process itself. They seem to bind calcium as a side-job. In contrast, calsequestrins, a class of muscle specific proteins, are dedicated calcium binders. They reside in a specialized form of ER, the sarcoplasmic reticulum (SR) and facilitate the calcium cycling process between the SR and the cytosol necessary for muscle contraction (Berchtold *et al.*, 2000).

Other calcium binding proteins in the ER lumen belong to the CREC family (CREC is an acronym of the first four identified members): Cab45 (Scherer *et al.*, 1996), reticulocalbin (Ozawa & Muramatsu, 1993), ERC-55 (Weis *et al.*, 1994), and calumenin (Yabe *et al.*, 1997). A fifth family member is crocalbin (Hseu *et al.*, 1999). The CREC family members are lumenal ER resident proteins except Cab45, which instead localizes to the Golgi (Scherer *et al.*, 1996). Similar to calmodulin, the central calcium binding protein in the cytosol, CREC family members contain six to seven EF-hand motifs. Still, the CREC proteins are too lowly abundant in the ER to provide a substantial contribution to intracellular calcium stores (Honoré & Vorum, 2000). Thus far, no role in protein folding (or any other role) has been attributed to any CREC family member either (Honoré & Vorum, 2000).

Calcium binding may be a secondary task for individual ER resident folding factors, but for the functioning of the ER as a whole, calcium is important. Calcium depletion, as provoked by thapsigargin or A23187, can interfere with the network of interactions between ER resident proteins or perturb the function of individual ER folding factors (Corbett *et al.*, 1999). Consequently, low calcium levels lead to retention, aggregation, and finally to degradation of some ER client proteins (Lodish & Kong, 1990).

SIMILARITIES BETWEEN FOLDING IN THE ER AND FOLDING IN OTHER COMPARTMENTS

Hydrophobic Interactions

Like other folding compartments, the ER lumen provides an aqueous environment to its folding clients. Consequently, it forces burial of hydrophobic residues in the interior and exposure of hydrophilic residues to the exterior of folding proteins. Stretches of hydrophobic residues will associate with hydrophobic partners,

whether proteinaceous or membrane lipids, avoiding aqueous surroundings directly upon synthesis. In that way, folding may follow a series of minor local nucleation events, initiating tertiary structure. From these primary nucleations the folding process would propagate, culminating in the native state (Daggett & Fersht, 2003).

ER Resident Hsp70 Chaperones: BiP, GRP170 & co

When hydrophobic patches of nascent proteins interact with their environment at will, they easily team up with unwanted partners. Consequences are misfolding and aggregation. BiP and its yeast equivalent, Kar2p, chaperone hydrophobic interactors in the ER in a similar fashion as their fellow Hsp70s in other folding compartments (Bukau & Horwich, 1998). BiP binds to short hydrophobic patches that are exposed in incompletely folded proteins, in misfolded proteins or in unassembled subunits of oligomers (Flynn *et al.*, 1991). Like other Hsp70s, BiP is an ATPase, illustrating the energy requirement of its chaperone activity. The ATPase catalytic site is located in the *N*-terminal domain. ATP hydrolysis induces a conformational change, which enhances the association of the C-terminal domain of BiP with its substrate. When ADP is exchanged for ATP again, BiP releases its substrate and the chaperone cycle is completed (Gething, 1999). Apart from its functioning as a classical Hsp70, BiP/Kar2p is employed in numerous ER specific roles (Hendershot, 2004) that will be discussed below.

Similar to other Hsp70s, BiP/Kar2p activity is regulated by co-chaperones in the form of J-domain containing proteins, also known as the Hsp40 family. They interact with Hsp70s to stimulate ATP hydrolysis, thus stabilizing binding of the chaperone to the folding substrate (Bukau & Horwich, 1998; Misselwitz *et al.*, 1998). The list of ER resident proteins with J-domains is growing still. At present, three have been discovered in yeast: Sec63p (Feldheim *et al.*, 1992), Scj1p (Schlenstedt *et al.*, 1995), and Jem1p (Nishikawa & Endo, 1997). Five J-domain containing proteins have been discovered in the mammalian ER. For clarity, Shen and colleagues recently proposed to (re-)name these ERdj1 to ERdj5 (ER-localized DnaJ homologues) (Shen *et al.*, 2002; Cunnea *et al.*, 2003). ERdj1, ERdj2 and ERdj3 were previously identified as Mtj1 (Brightman *et al.*, 1995), hSec63 (Skowronek *et al.*, 1999), and HEDJ, respectively (Yu *et al.*, 2000).

Employment of different ERdj proteins as co-chaperones could modulate BiP activity and diversify its function. Yeast Sec63p (Feldheim *et al.*, 1992; Misselwitz *et al.*, 1999) and both mammalian ERdj1 (Dudek *et al.*, 2002) and ERdj2 (Tyedmers *et al.*, 2000) associate with the translocon. Together with BiP, the ERdj proteins hence are likely to assist in guiding nascent proteins across the ER membrane and/or contribute to retrotranslocation of misfolded ER substrates for degradation (Plemper *et al.*, 1997). The latest mammalian Hsp40 family member, ERdj5, also resembles PDI, which provides a link between BiP activity and disulfide bond formation (Cunnea *et al.*, 2003; Hosoda *et al.*, 2003).

The ATPase cycle of BiP/Kar2p is not only stimulated by ERdjs, but also by nucleotide exchange factors. The main nucleotide exchange factor for Kar2p is Sil1p (Boisrame *et al.*, 1998; Kabani *et al.*, 2000; Tyson & Stirling, 2000) or its homolog BAP in mammals (Chung *et al.*, 2002). Surprisingly, a second nucleotide exchange factor of Kar2p is Lhs1p (Steel *et al.*, 2004), as will be discussed below. Lhs1p is the homolog of GRP170 in the mammalian ER (Baxter *et al.*, 1996; Craven *et al.*, 1996). Next to BiP/Kar2p, GRP170/Lhs1p is the second ER resident member of the Hsp70 family (Chen *et al.*, 1996; Easton *et al.*, 2000; Park *et al.*, 2003). Like BiP, GRP170 has affinity for hydrophobic peptide stretches (Spee *et al.*, 1999; Park *et al.*, 2003) and it acts as a chaperone of ER clients, such as immunoglobulin (Lin *et al.*, 1993), thyroglobulin (Kuznetsov *et al.*, 1994), and GP80, a secretory protein of renal epithelial cells (Bando *et al.*, 2000). Lhs1p also promotes release of misfolded proteins from heat shock induced aggregates and renders them substrate for re-entry into productive folding pathways (Saris *et al.*, 1997; Saris & Makarow, 1998).

The Role of ER Resident Hsp70 Chaperones in Translocation

Like their mitochondrial counterparts, the ER resident Hsp70s are important for protein translocation into the organellar lumen. A role for BiP has been suggested in sealing off the lumenal side of the translocon during synthesis of non-lumenal domains of multi–membrane spanning proteins, or when the translocon is unemployed altogether (Hamman *et al.*, 1998; Haigh & Johnson, 2002; Alder *et al.*, 2005). Based on the crystal structure of SecY, however, Sec61 itself already seems to provide a plug that would seal the translocon channel

(Van den Berg *et al.*, 2004; Clemons *et al.*, 2004). Taking into account the phylogenetic distance between *M. jannaschii* SecY and eukaryotic Sec61, one could speculate that a role for BiP in gating the translocon only has arisen later in evolution.

Both BiP/Kar2p (Sanders *et al.*, 1992; Tyedmers *et al.*, 2003) and GRP170/Lhs1p (Craven *et al.*, 1996; Dierks *et al.*, 1996; Hamilton and Flynn, 1996; Tyson & Stirling, 2000) are essential for vectorial protein translocation into the ER lumen. In fact, the two Hsp70s work closely together during translocation, as was demonstrated in yeast. When ADP-bound Kar2p is associated with an ER client that is emerging into the ER lumen, Lhs1p acts as nucleotide exchange factor of Kar2p (Steel *et al.*, 2004). As a consequence Kar2p releases the ER client, but since Kar2p stimulates ATPase activity of Lhs1p at the same time, Lhs1p associates with this ER client instead (Steel *et al.*, 2004). This handing-over mechanism ensures that at any time the ER-client is bound to an ER-resident chaperone. In that way, the two ER resident Hsp70s could act as a "molecular ratchet" on the lumenal side to prevent back-slip of the ER client into the cytosol (Matlack *et al.*, 1999). During co-translational translocation the ribosome provides forward motion as well (Jenni & Ban, 2003). The "molecular ratchet" may therefore be especially important for post-translational translocation (Rapoport *et al.*, 1999).

GRP94, the ER Resident Hsp90 Chaperone

GRP94 is the ER resident member of the family of Hsp90s. It has relatives in cytosol, mitochondria and in bacteria. GRP94, also known as endoplasmin or gp96, is one of the most abundant proteins in the ER lumen of mammalian cells (Koch *et al.*, 1986), but is lacking in yeast (Argon & Simen, 1999). GRP94 has been implicated in the folding process of many ER clients (*e.g.*, immunoglobulins (Melnick *et al.*, 1992), MHC class II (Schaiff *et al.*, 1992), thyroglobulin (Kuznetsov *et al.*, 1994), and procollagen (Ferreira *et al.*, 1994)). As its cytosolic counterpart Hsp90, GRP94 has peptide binding capacity (Nieland *et al.*, 1996; Argon & Simen, 1999), and dimerizes via its C-terminal domain (Yamada *et al.*, 2003). The cognate variant of Hsp70, Hsc70, delivers clients to Hsp90, indicating Hsp90 chaperone activity is required only late in the folding process (Smith *et al.*, 1992; Young *et al.*, 2004). In a similar manner, immunoglobulins are transferred from BiP to GRP94 (Melnick *et al.*, 1994), suggesting that the Hsp70 to Hsp90 handing-over mechanism is conserved between compartments.

It remains elusive, however, whether the chaperoning mechanism of GRP94 is similar to Hsp90. The chaperone cycle of cytosolic Hsp90 is modulated by a range of co-chaperones including p23 (Johnson *et al.*, 1994), Hip, Hop (Frydman & Hohfeld, 1997) and Aha1 (Mayer *et al.*, 2002; Lotz *et al.*, 2003). In contrast, co-chaperones of GRP94 have not yet been found. Hsp90 has low intrinsic ATPase activity, but is stimulated by Aha1 (Panaretou *et al.*, 2002). How ATP hydrolysis couples to chaperone activity of Hsp90 is unclear. GRP94 can bind ATP, albeit with significantly lower affinity than its cytosolic counterpart. Still, GRP94 seems to lack ATPase activity (Rosser *et al.*, 2004). Instead, adenosine nucleotides may act as regulatory ligands that could induce changes in conformation of GRP94, corresponding to gain or loss of its chaperone activity (Rosser & Nicchitta, 2000; Soldano *et al.*, 2003).

PPIases

Flexibility of the polypeptide backbone of proteins is an important determinant in folding kinetics. Apart from steric hindrance by side chains, the rotational freedom throughout the peptide chain is even, because residues are uniformly coupled by peptide bonds. An exception is the peptide bond between any residue and the imino acid proline. Its side chain is covalently bound to the nitrogen of the peptide bond, which limits flexibility of the peptide backbone to a *cis*- or *trans*-orientation of the proline residue. Isomerization between the two is catalyzed by the folding enzyme class of peptidyl-prolyl *cis-trans* isomerases (PPIases) (Schmid *et al.*, 1993). Like other folding compartments, the ER counts a collection of PPIases. Both yeast (Frigerio & Pelham, 1993) and mammalian ER (Hasel *et al.*, 1991; Price *et al.*, 1991; Arber *et al.*, 1992) harbor a single representative of the cyclophilin family of PPIases. An inhibitor of the cyclophilins, cyclosporin A, retards maturation of transferrin (Lodish & Kong, 1991) and of triple helix formation of collagen (Steinmann *et al.*, 1991), illustrating the relevance of this PPIase for protein folding in the ER. Six members of another family of PPIases, the FK506-binding proteins, localize to the mammalian ER (Galat, 2003): FKBP13 (Nigam *et al.*, 1993), FKBP23 (Nakamura *et al.*, 1998), FKBP60 (Shadidy *et al.*, 1999), FKBP65 (Patterson *et al.*, 2000), FKBP22 and FKBP19

(Galat, 2003). Little is known about the individual specificity of any of these PPIases.

ELEMENTS UNIQUE TO FOLDING IN THE ER I: OXIDATIVE FOLDING

Disulfide Bond Formation

Perhaps the most distinctive feature of protein folding in the ER is the abundance of disulfide bonds that must form during maturation of ER clients. The oxidative conditions in the ER parallel those in the periplasm of bacteria, but are in sharp contrast to the cytosol or the mitochondria, where disulfide bond formation is highly disfavored. The reducing environment in the cytosol is reflected by a ratio of ~1:60) (Hwang *et al.*, 1992) or even lower (Østergaard *et al.*, 2004) for oxidized (GSSG) versus reduced (GSH) glutathione, the major small thiol in the cell. This ratio is ~1:3 in the ER, corresponding to a redox potential compatible with disulfide bond formation (Hwang *et al.*, 1992). Disulfide bond formation is vital: if formation of disulfides is hampered by reducing agents such as DTT, productive protein folding in the ER comes to a halt, and the cell will die eventually (Braakman *et al.*, 1992a; Braakman *et al.*, 1992b).

Why do ER clients obtain disulfide bonds? There are several reasons. Covalent intra- and intermolecular cross-links can add stability to proteins. This may be of particular relevance for proteins that enter the secretory pathway, because, upon exit from the ER, they are no longer under surveillance of any protein folding machinery. Disulfide bond formation is also an effective way to create large oligomers, such as IgM (see below). In addition, disulfide bonds may serve as checkpoints in the folding process. The ER folding machinery can join protein strands by disulfide bonds, when cysteine residues come in close enough proximity. A 'provisional' disulfide bond restricts the flexibility of its surroundings and could set the stage for subsequent formation of native hydrogen bonds and Van der Waals interactions. As such, folding possibilities become more limited with each subsequent oxidizing step, which would give directionality to the folding process. Consistent with such a scenario, ER clients acquire DTT resistance only some time after completion of their disulfide-bonded structure (Tatu *et al.*, 1995).

Formation of disulfide bonds is a redox reaction. The coupling of two sulfhydryl groups of cysteine residues is a two-electron reaction that requires an oxidant (electron acceptor). In principle, this thiol-oxidation reaction would suffice to render substrate proteins in a disulfide-bonded state. For most ER clients, the process of disulfide bond formation is more complex however, since they often contain numerous cysteines. Like the three-dimensional structure lies embedded in the primary sequence, only a single arrangement of intra- and/or intermolecular cysteine pairs corresponds to the native folded structure. Any incorrect pairing of cysteines must therefore be unscrambled and, eventually, be replaced by correct disulfide bonds. This implies that the ER must sustain thiol-oxidation and -reduction at the same time.

PDI, Ero and Thiol-Oxidation

Although disulfide bonds can form *in vitro* using, for instance, molecular oxygen as electron acceptor and metal ions as catalysts, in the ER lumen thiol-oxidoreductases both catalyze the reaction and serve as disulfide donor (Bulleid & Freedman, 1988). The archetypal oxidoreductase in the ER is protein disulfide isomerase (PDI). PDI has four domains with a thioredoxin fold (Kemmink *et al.*, 1997; Ferrari *et al.*, 1998; Kemmink *et al.*, 1999). Two of these, the a- and a′-domain, contain a CXXC motif that is the active site of the oxidoreductase (Vuori *et al.*, 1992; LaMantia & Lennarz, 1993). The two other thioredoxin-like domains are the b-domain and b′-domain. They lack a redox-active motif, but possess peptide-binding capacity instead (Klappa *et al.*, 1998; Pirneskoski *et al.*, 2004). This suggests that b-domains can establish a close interaction with (partly) unfolded ER substrates, allowing the a-domains to catalyze disulfide bond formation. Its peptide binding capacity could also explain why PDI can serve as a chaperone during *in vitro* folding of cytosolic proteins that lack cysteines (Wang & Tsou, 1993; Song & Wang, 1995; Yao *et al.*, 1997). At its C-terminus, PDI has an acidic c-domain with calcium binding properties (Macer & Koch, 1988).

When PDI is oxidized (*i.e.*, the two cysteines in the active site are disulfide linked), PDI can function as electron acceptor and hence as disulfide donor for client proteins that emerge in the ER lumen (Freedman *et al.*, 1994). The active site disulfides are unstable and easily disrupted by accessible free sulfhydryl groups in the nascent client protein, yielding a mixed disulfide between PDI and client instead (Huppa & Ploegh, 1998;

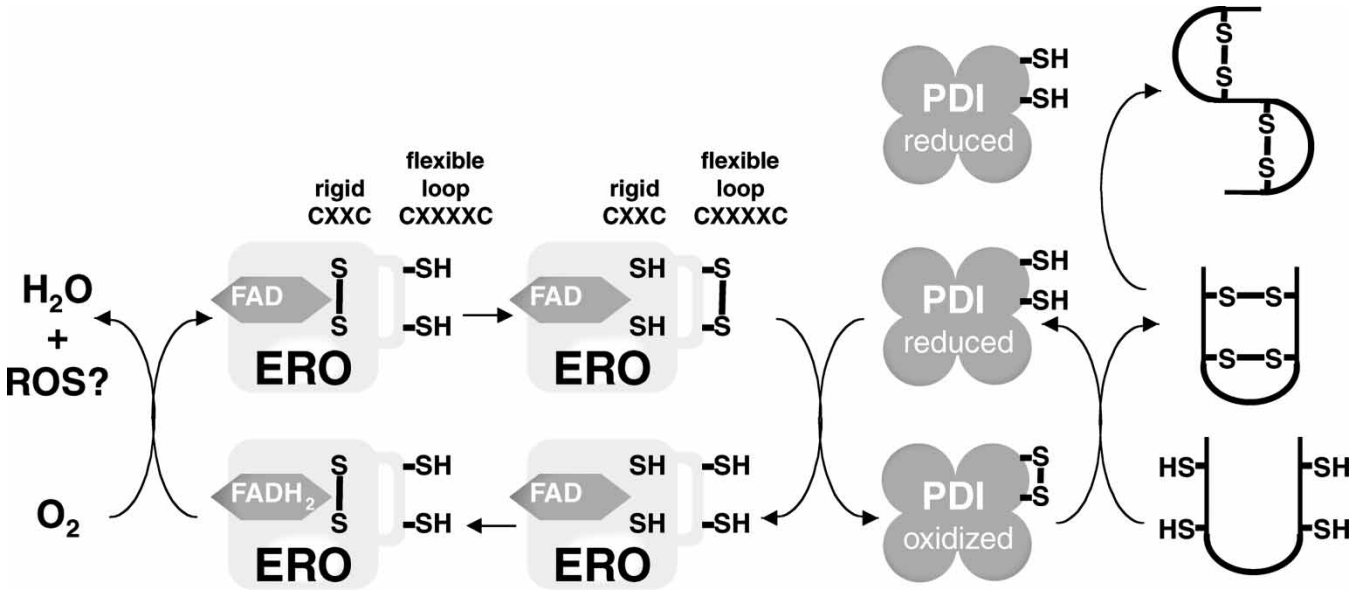

FIGURE 3 **Thiol-oxidase and disulfide isomerase mechanism of PDI and the pathway of electron/disulfide shuttling via Ero1 and FAD. Oxidized PDI functions as disulfide donor for the oxidative folding of ER client proteins (thiol-oxidation), while reduced PDI can shuffle their disulfide bonds (disulfide isomerization). For simplicity, only one of the two redox-active CXXC sites of PDI is shown. PDI is recharged by a cascade that consists of Ero1, its cofactor FAD and O_2. Electrons flow from the ER client to the CXXC active site of PDI. Next the electrons from PDI flow to the CXXXXC motif of Ero1, which is present on a flexible loop and subsequently from the CXXXXC to a "rigid" CXXC motif present in the interior of Ero1. Finally, electrons flow from the "rigid" CXXC to FAD, while O_2 serves as terminal electron acceptor. As a result, reactive oxygen species such as H_2O_2 may be generated.**

Molinari & Helenius, 1999). A second free sulfhydryl of the nascent chain can subsequently disrupt the mixed disulfide, resulting in a disulfide between two substrate protein cysteines. As a consequence, PDI is released in its reduced state (Figure 3).

Fully folded proteins exit from the ER. Consequently, a net flux of disulfide bonds is leaving the compartment that must be matched by an equal flux of electrons out of the ER. In yeast, the essential protein relay supporting this flux, and hence disulfide bond formation, involves, next to PDI, the ER oxidoreductin 1 protein (Ero1p) (Pollard *et al.*, 1998; Frand & Kaiser, 1999; Frand *et al.*, 2000; Tu & Weissman, 2004). Ero1p can recharge reduced PDI (Frand & Kaiser, 1999), whereby its cofactor flavin adenine dinucleotide (FAD) is reduced to $FADH_2$ (Tu *et al.*, 2000). Ero1p then uses molecular oxygen as terminal electron acceptor, converting $FADH_2$ into FAD, perhaps resulting in the generation of reactive oxygen species (ROS), such as H_2O_2 (Tu & Weissman, 2002; Harding *et al.*, 2003; Haynes *et al.*, 2004; Tu & Weissman, 2004) (Figure 3). Mammalian cells contain two Ero1p homologues: Ero1α (Cabibbo *et al.*, 2000) and Ero1β (Pagani *et al.*, 2000), which both can complement Ero1p deficient yeast and therefore seem to have role in oxidative folding equivalent to Ero1p. Accordingly, Ero1α directly interacts with PDI (Benham *et al.*, 2000).

Apart from the human Ero1p homologs, another FAD binding protein of the yeast ER, Erv2p (Gerber *et al.*, 2001), can also rescue yeast that lacks Ero1p, but only when overexpressed (Sevier *et al.*, 2001). Now that crystal structures of both Erv2p (Gross *et al.*, 2002) and Ero1p (Gross *et al.*, 2004) have been solved, it is clear that these proteins are not only functionally but also structurally related, despite the lack of sequence homology. They both have a rigid disulfide bonded CXXC motif adjacent to a stably bound FAD cofactor. In the Ero1p structure, a disulfide bonded CXXXXC motif is in close proximity to the CXXC on a flexible segment of the protein (Gross *et al.*, 2004). For the disulfide transfer reactions, one could therefore envision that disulfides are transferred to PDI from the rigid CXXC via the flexible CXXXXC shuttle (Figure 3).

Erv2p has a CXC motif in its C-terminus that could fulfill a similar role as the CXXXXC motif of Ero1p. While the Ero1p disulfide shuttle is intramolecular, the thiol-shuttling mechanism of Erv2p requires that it is a homodimer. The CXC of one Erv2p subunit then serves as shuttle dithiol for the CXXC of another Erv2p subunit (Gross *et al.*, 2002; Gross *et al.*, 2004). Although Erv2p is not conserved in the strict sense

between yeast and man, the mammalian ER harbors two type I proteins that have an Erv2p homologous domain (Thorpe *et al.*, 2002). The first was independently identified as quiescin (Coppock *et al.*, 1998) and as sulfhydryl oxidase (Hoober *et al.*, 1999a). The protein is therefore referred to as quiescin/sulfhydryl oxidase 1 (QSOX1) and its family member as QSOX2. As established for QSOX1 (Hoober *et al.*, 1996), QSOX proteins bind FAD and homodimerize in a similar manner as Erv2p. Moreover, QSOX proteins have a CXXC motif that aligns with that of Erv2p. Unlike Erv2p, however, QSOX proteins contain two additional CXXC motifs: one further towards their C-termini and one in their N-terminal domains. Interestingly, these N-terminal domains are homologous to the a- and a′-domain of PDI (Coppock *et al.*, 1998; Thorpe *et al.*, 2002). This suggests that QSOX disulfide shuttling is confined within a single protein, as opposed to the two-protein oxidizing relay represented by Ero1p and PDI. As was established 30 years ago, QSOX proteins indeed can introduce disulfide bonds into client proteins on their own (Chang & Morton, 1975; Janolino & Swaisgood, 1975). The electrons seem to flow from the client via the PDI-like *N*-terminal CXXC and the C-terminal CXXC, which serves an analogous role as CXC of Erv2p and CXXXXC of Ero1p, to the middle CXXC, and ultimately to FAD and O_2 (Raje & Thorpe, 2003).

Thiol-Isomerization and -Reduction

Although QSOX1 can act alone as strong thiol-oxidase of several substrates, including DTT and PDI, it does not necessarily promote folding of its clients. For instance, reduced RNase is rapidly oxidized by QSOX1 *in vitro*, but activity of the enzyme is hardly restored, because QSOX obstinately introduces disulfide bonds whether native or aberrant (Hoober *et al.*, 1999b). This example nicely illustrates that thiol-oxidation is necessary but not sufficient for native disulfide bond formation. Of equal importance is the unscrambling of erroneous disulfide bonds. Although oxidized PDI catalyzes thiol-oxidation, reduced PDI catalyzes reduction or isomerization of disulfides (Freedman *et al.*, 1994) (Figure 3). Indeed, RNase that is "randomly" oxidized by QSOX1 readily regains enzymatic activity in the presence of PDI (Hoober *et al.*, 1999b).

The *N*-terminal free cysteine in the active sites of reduced PDI can disrupt a pre-existing disulfide bond of a substrate protein, resulting in a mixed disulfide between substrate and PDI, similarly as in the oxidase reaction of PDI. The disulfide isomerization cycle is complete when this cysteine teams up with another cysteine than its former partner (Freedman *et al.*, 1994). Altogether, "genuine" thiol-isomerization entails that PDI releases its substrate only after disulfide bond rearrangement is complete. As a net result, PDI remains in a reduced state (Figure 3). Parallel to that, thiol-isomerization can be achieved via cycles of separate reduction and re-oxidation events (Schwaller *et al.*, 2003).

Ample evidence for the role of PDI in disulfide isomerization has been obtained *in vitro* (see e.g. (Weissman & Kim, 1993). In mammalian cells, protein folding also involves disulfide bond isomerization, as was demonstrated for the envelope glycoprotein of human immunodeficiency virus (HIV envelope) (Land *et al.*, 2003) and the low-density lipoprotein (LDL) receptor (Jansens *et al.*, 2002). The question remains whether PDI is responsible for these isomerization reactions or that other oxidoreductases catalyze them. Indicative for the importance of their isomerase function, however, PDI and several PDI-like proteins are predominantly present in a reduced state in the mammalian ER (Mezghrani *et al.*, 2001). In contrast, thiol-isomerization is of less importance in yeast (Solovyov *et al.*, 2004). Accordingly, only one third of PDI active sites are in a reduced state (Frand & Kaiser, 1999; Xiao *et al.*, 2004). Replacement of PDI by the a′ domain alone, which has very limited isomerase activity, hardly affects growth (Xiao *et al.*, 2004). Possibly, ER clients that are essential for yeast viability have evolved to be independent of disulfide isomerization.

The fact that a substantial fraction of PDI and its relatives are present in a reduced state suggests that, next to the oxidizing pathway, there is a reducing pathway operational in the ER. Glutathione serves as a reducing buffer that balances Ero1 activity. This small thiol can directly reduce PDI family members, as has been demonstrated for the PDI-like protein ERp57 (Jessop & Bulleid, 2004). Accordingly, reduced levels of glutathione lead to increased kinetics in ER client oxidation, because Ero1 activity is no longer antagonized. The folding efficiency does not necessarily increase, however, since non-native disulfide bonds are no longer efficiently unscrambled (Chakravarthi & Bulleid, 2004; Molteni *et al.*, 2004). Apparently, glutathione traverses the ER membrane, because enzymatic activity in the cytosol that reduces glutathione is required to sustain

efficient thiol-isomerization in the ER lumen (Jessop & Bulleid, 2004).

PDI Family

Distant family members of PDI can be found in other compartments of the cell. Thioredoxin (TRX) in the cytosol is an oxidoreductase with similar architecture as the a and a′-domain of PDI. While PDI can catalyze oxidation, reduction and isomerization of disulfide bonds, TRX is a dedicated thiol-reductase: it relieves cytosolic proteins from erroneously formed disulfide bonds by reducing them with its CXXC motif as disulfide acceptor (Holmgren, 1985). DsbA, the thiol-oxidase of the bacterial periplasm (Bardwell *et al.*, 1991), shows no obvious sequence homology with PDI or TRX, but its structure still is remarkably similar to that of TRX (Martin *et al.*, 1993), suggesting that all these oxidoreductases are phylogenetically related, albeit very distantly.

The same CXXC motif differs extensively in redox potential amongst the variety of thioredoxin family members, from −260 mV for TRX to −100 mV for DsbA. PDI has an intermediate position with a redox potential of −180 mV (Hawkins *et al.*, 1991; Lundstrom & Holmgren, 1993; Wunderlich & Glockshuber, 1993). Not only the character of the residues between the two cysteines, the surrounding residues and noncovalent interactions imposed by three-dimensional structures, but also the pH, are determinants of the redox potential of the CXXC motif (Huber-Wunderlich & Glockshuber, 1998). The context of the active site cysteines is in fact remarkably conserved among the most abundant PDI family members: APWCGHCK/Q (Ferrari & Soling, 1999). This canonical sequence apparently provides the optimal redox potential for basal disulfide bond formation and/or isomerization in the ER, whereas variations on this theme in other family members likely reflect divergence in oxidoreductase function.

In addition to PDI, yeast has three other ER resident proteins that contain thioredoxin domains with a CXXC motif: Mpd1p, Mpd2p and Eps1p (Tachikawa *et al.*, 1995; Tachikawa *et al.*, 1997; Wang & Chang, 1999). A fourth family member, Eug1p, has a very similar architecture as PDI, except that it has CXXS active sites (Tachibana & Stevens, 1992). Consequently, the active sites of Eug1p cannot bear disulfide bonds, which seems to rule out Eug1p as a disulfide donor. Eug1p may act as disulfide isomerase, although its isomerase activity is markedly lower than that of PDI (Nørgaard & Winther, 2001).

The investigator of the mammalian ER faces an even more complex array of oxidoreductases (Figure 4). Three PDI family members in the mammalian ER are structurally homologous to yeast PDI. Mammalian PDI itself was already discovered in 1963 (Goldenberger *et al.*, 1963). It is the most abundant and most extensively studied oxidoreductase in the ER. The PDI-like protein PDIp shares the abb′a′ architecture with PDI, except that the a′-domain contains a CTHC motif and that PDIp lacks the acidic c-domain. More remarkable is that PDIp expression seems restricted to the pancreas (Desilva *et al.*, 1996; Volkmer *et al.*, 1997). ERp57, which also lacks an acidic c-domain, is the third thiol-oxidoreductase with an abb′a′ structure (Urade *et al.*, 1992; Freedman *et al.*, 1994). Like PDI, it is ubiquitously expressed, but its close association with the two ER resident lectin chaperones calnexin and calreticulin (see below) distinguishes ERp57 from PDI and determines that ERp57 is specialized in glycoprotein folding (Oliver *et al.*, 1997; Zapun *et al.*, 1998; Oliver *et al.*, 1999). Slightly different in architecture is ERp72: a°abb′a′, where a° stands for a third a-like domain N-terminal of the a-domain (Mazzarella *et al.*, 1990; Ferrari & Soling, 1999). At its extreme N-terminus, ERp72 contains an acidic c-domain (Mazzarella *et al.*, 1990; Ferrari & Soling, 1999).

The PDI-like protein, ERp46, has three a-domains, like ERp72, but it lacks b-domains altogether (Knoblach *et al.*, 2003). It is highly expressed in endothelial cells suffering from hypoxia (Sullivan *et al.*, 2003) and in activated B-lymphocytes (Wrammert *et al.*, 2004; our unpublished observations). Another PDI-like protein that contains three TRX domains is P5 (Chaudhuri *et al.*, 1992; Fullekrug *et al.*, 1994; Hayano & Kikuchi, 1995a), yet only the first two are a-domains, while the third is a b-domain. At its C-terminus P5 has an acidic c-domain (Ferrari & Soling, 1999). Interestingly, the alfalfa P5 homolog contains a distinct d-domain instead of the b- and c-domains (Ferrari & Soling, 1999). For a change, the d-domain has no TRX fold but is a fully alpha-helical structure (Liepinsh *et al.*, 2001). Alfalfa P5 can rescue the PDI knockout yeast despite the lack of a b-domain (Ferrari & Soling, 1999), which suggests that b-domains are not essential for overall disulfide bond formation. Alternatively, the d-domain has a similar role as the b-domain and therefore can compensate for its loss. The b- and d-domains are combined in the oddest

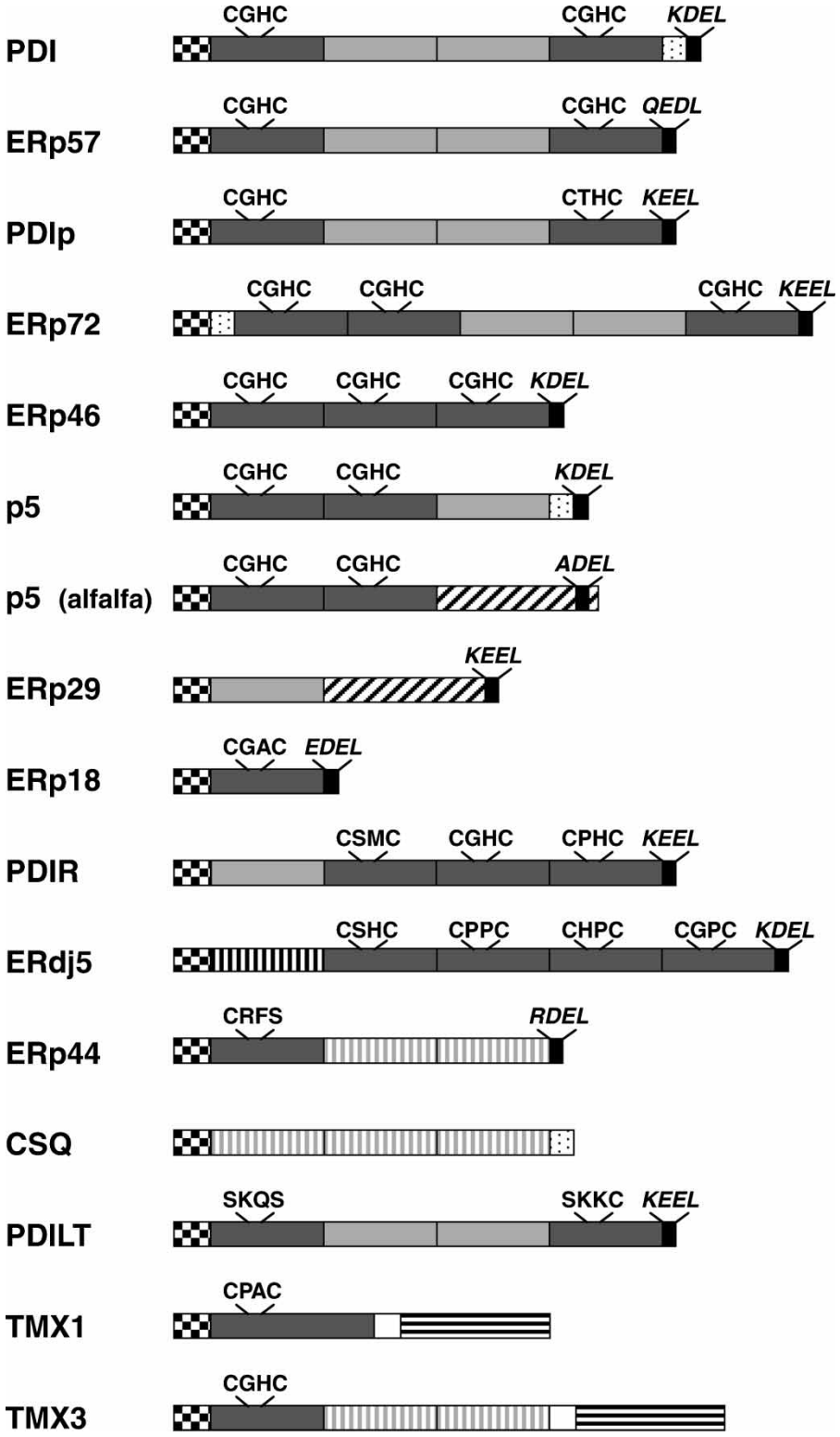

FIGURE 4 **Domain structure of PDI family members. Signal peptides are indicated as chequered boxes; a-like domains are depicted as dark gray boxes and the CXXC(-like) motifs are indicated; inactive b-like domains as light gray boxes; calsequestrin (CSQ)-like thioredoxin domains are shown as striped gray boxes, acidic c-domains as speckled boxes; d-domains as diagonally striped boxes. The J-domain of ERdj5 as a vertically striped box and C-terminal KDEL(-like) peptides that mediate ER residency in black and the tetrapeptide sequence in *italic*. Of the three TMX family members only TMX1 and 3 are shown, since topology of TMX2 is uncertain. Their TMDs are indicated as white boxes and their cytosolic domains as horizontally striped boxes.**

family member, ERp29 (Demmer *et al.*, 1997; Ferrari *et al.*, 1998; Mkrtchian *et al.*, 1998). ERp29 has an *N*-terminal domain that is equally homologous to the a-like and b-like domains within the PDI-like family (Ferrari *et al.*, 1998; Liepinsh *et al.*, 2001). The lack of a CXXC motif, however, disqualifies this domain as an a-domain and ERp29 as an oxidoreductase. Whereas ERp29 has a single b-domain, the smallest PDI family member ERp18 has a single a-domain, but lacks b-like domains. Having a CGAC active site, ERp18 likely diverges in its oxidoreductase function from PDI (Alanen *et al.*, 2003). This protein indeed cannot rescue the PDI knockout yeast (Knoblach *et al.*, 2003).

Four PDI family members contain active sites that deviate considerably from the canonical CGHC in PDI. The first is PDIR, a poorly studied cousin of PDI with three consecutive a-domains, preceded by a single b-domain. Of the three a-domain active sites CSMC/CGHC/CPHC only the middle one obeys the canonical sequence (Hayano & Kikuchi, 1995b). Interestingly, PDIR has increased isomerase activity, when all redox-active sites are mutagenized to obey the canonical CGHC sequence (Horibe *et al.*, 2004). Conversely, when PDIR contained only CSMC redox-active sites, isomerase activity decreased (Horibe *et al.*, 2004). The second PDI family member with atypical redox-active sites is ERdj5. This protein has four a-domains with CSHC/CPPC/CHPC/CGPC active sites (Cunnea *et al.*, 2003; Hosoda *et al.*, 2003). Moreover, ERdj5 is a hybrid of two protein families. Its N-terminus harbors a J-domain and therefore ERdj5 is a co-chaperone of BiP (Cunnea *et al.*, 2003; Hosoda *et al.*, 2003).

Aside from PDI, only two other PDI family member have been found to associate with Ero1α, the first being ERp44 (Anelli *et al.*, 2002). Like ERp43, ERp44 is highly upregulated during B cell differentiation (van Anken *et al.*, 2003). ERp44 contains one a-domain at its *N*-terminus, while the remainder shows homology to calsequestrin proteins (Anelli *et al.*, 2002). The three-dimensional structure of calsequestrin reveals that it consists of three domains with a thioredoxin fold (Wang *et al.*, 1998). Consequently, the ERp44 structure must closely resemble that of its distant family members, despite low sequence homology of its C-terminal domains with any other PDI-like protein. The a-domain of ERp44 differs remarkably from those in other PDI-like proteins: instead of the standard CGHC, ERp44 contains a CRFS motif. The lack of a second cysteine excludes a role for ERp44 as an oxidase, similar as for Eug1p in yeast. Instead, ERp44 seems to contribute to the folding process as a dedicated 'thiol-retentor.' As such, it is responsible for the ER localization of Ero1α, which lacks ER retention signals itself. In addition, ERp44 may contribute to thiol-mediated retention as part of quality control in the folding process (see below) (Anelli *et al.*, 2003).

The third Ero1α interacting PDI family and the fourth with non-canonical active sites is a PDI like protein that is specifically expressed in the testis (PDILT) (van Lith *et al.*, 2005). PDILT has an abb′a′ domain structure similar to PDI, but its active sites are very different: SKQS and SKKC. The single cysteine in the a′ active site forms intermolecular disulfide bonds with Ero1α as well as with unknown proteins in the ER of testis derived cells. How these PDILT complexes relate to oxidative folding remains an open question (van Lith *et al.*, 2005).

Aside from the 12 soluble PDI family members described above, the ER contains a number of PDI family members that are membrane anchored. The first, transmembrane TRX-related protein (TMX1), is a type I membrane protein that contains a single a-like domain with a CPAC active site in its ectodomain that supports thiol-isomerization *in vitro* (Matsuo *et al.*, 2001; Matsuo *et al.*, 2004). Except for its unusual SNDC active site, the second PDI related protein with a membrane anchor, TMX2, has, to date, been poorly characterized (Meng *et al.*, 2003). It is even not entirely clear whether the active site actually faces the ER lumen or the cytosol. A third membrane bound PDI family member is TMX3. Like TMX1, TMX3 is a type I membrane protein with a single a-like domain at the *N*-terminus of its ectodomain, while its active site follows the canonical CGHC sequence. The remainder of the ectodomain is homologous to CSQ. TMX3 can catalyze thiol-oxidation *in vitro*, although not as efficiently as PDI (Haugstetter *et al.*, 2005).

Altogether, the list of PDI-like proteins that have been implicated in oxidative folding in the mammalian ER has been growing steadily and likely will continue to do so for years to come. Several as yet uncharacterized proteins present in mouse and human genomes show resemblance to PDI (Ellgaard & Ruddock, 2005) (our unpublished observations). Also non-PDI–related ER resident proteins may prove to be thiol-oxidoreductases. The challenge is to elucidate how the various thiol-oxidoreductases divide the workload in oxidative folding.

ELEMENTS UNIQUE TO FOLDING IN THE ER II: GLYCOPROTEIN FOLDING

N-Linked Glycosylation

Next to disulfide bond formation, a second post-translational modification distinguishes ER-clients: the co-translational addition of *N*-glycans. *N*-glycosylation is restricted to the ER, although the ER is not the only compartment where glycosylation takes place. Further along the exocytic pathway, in the Golgi, *O*-linked glycans are added to secretory proteins and a variety of monosaccharide units are added to some *N*-glycan cores (Kornfeld & Kornfeld, 1985). Glycosylation in the ER is unique in the sense that *N*-glycans have diverse roles contributing to the folding process. Glycosylation inhibitors such as tunicamycin (Lehle & Tanner, 1976) provoke misfolding and aggregation of many ER clients (Leavitt *et al.*, 1977). Thus, *N*-glycans are indispensable for the folding process: on a local scale, they influence the conformation of the peptide backbone; and on a global scale, they counteract the overall hydrophobicity of the polypeptide chain. Moreover *N*-glycans are employed by the quality control mechanisms of the ER to monitor the folding status of glycosylated ER client proteins (see below).

Core glycans are synthesized in a cascade of enzymatic reactions coupling monosaccharide units to lipid dolichol moieties. First, a $Man_5GlcNAc_2$ precursor, linked to dolichol by two phosphates is constructed at the cytosolic leaflet of the ER membrane. Next, this precursor translocated to the lumenal side, presumably by a putative flipase, and is subsequently elongated to $Glc_3Man_9GlcNAc_2$-PP-dolichol at the ER lumenal leaflet (Abeijon & Hirschberg, 1992; Helenius & Aebi, 2002). From the dolichol precursors glycans are transferred onto nascent chains at the side-chains of asparagine residues within the context NXS/T (where X stands for any residue except proline) (Marshall, 1972; Kornfeld & Kornfeld, 1985) (Figure 5A). The addition of *N*-glycans is mediated by oligosaccharyltransferase, which is part of the translocon complex (Johnson & van Waes, 1999). Accordingly, for the majority of *N*-glycosylation sites the coupling reaction already occurs as soon as the nascent chain emerges from the translocon pore, at a distance of only 12 to 14 residues from the ER membrane (Nilsson & von Heijne, 1993), which corresponds to ≈65 residues from the petidyltransferase center inside the ribosome (Mingarro *et al.*, 2000). Since disulfide bond formation starts equally early, close proximity of cysteines and glycosylation sites can lead to competition between disulfide bond formation and glycosylation (Allen *et al.*, 1995).

As soon as an *N*-glycan is added to a folding protein, glucosidase I will remove the terminal glucose moiety. Next, glucosidase II removes the second glucose moiety and, eventually, the third glucose (Atkinson & Lee, 1984; Kornfeld & Kornfeld, 1985) (Figure 5A). Also the mannose moieties of the *N*-glycan are trimmed in the ER. A single mannose from the middle branch of the *N*-glycan can be removed by α1,2-mannosidase (Tremblay & Herscovics, 1999) (Figure 5A). Other mannose trimming events in the ER are less well characterized, but can generate glycoforms with as few as three mannoses (Trombetta & Parodi, 2003).

Calnexin and Calreticulin, ER Resident Lectin Chaperones

After removal of the first two glucoses from their *N*-glycans, ER client proteins become substrate for a chaperone family of lectins (i.e., proteins that associate with carbohydrate structures). These lectin chaperones exclusively recognize monoglucosylated *N*-glycans (Peterson *et al.*, 1995; van Leeuwen & Kearse, 1996). Yeast harbors a single lectin chaperone, Cne1p (Parlati *et al.*, 1995), which has received little attention. The mammalian ER counts two very well characterized lectin chaperones that are expressed in all cells: calnexin (CNX) and calreticulin (CRT) and two testis-specific lectins, calmegin (CMG) (Watanabe *et al.*, 1994) and CRT2 (Persson *et al.*, 2002). CNX and CRT are homologous proteins with similar structure and function, except that CNX is membrane-anchored, whereas CRT is a lumenal protein (Ou *et al.*, 1993; Peterson *et al.*, 1995; Trombetta & Helenius, 1998). The relevance of the membrane anchor of CNX remains to be elucidated, although there is some evidence that, in addition to its lectin chaperone role, CNX might assist the folding process via interactions of its transmembrane domain with those of maturing substrates (Margolese *et al.*, 1993; Cannon & Cresswell, 2001; Swanton *et al.*, 2003). The two lectin chaperones have no equivalents in other cellular compartments and their chaperoning mechanism is unique to the ER. Association and dissociation with folding substrates is ATP-independent (Trombetta & Helenius, 1998), which suggests that the contribution of the lectins to the folding process is energy-independent.

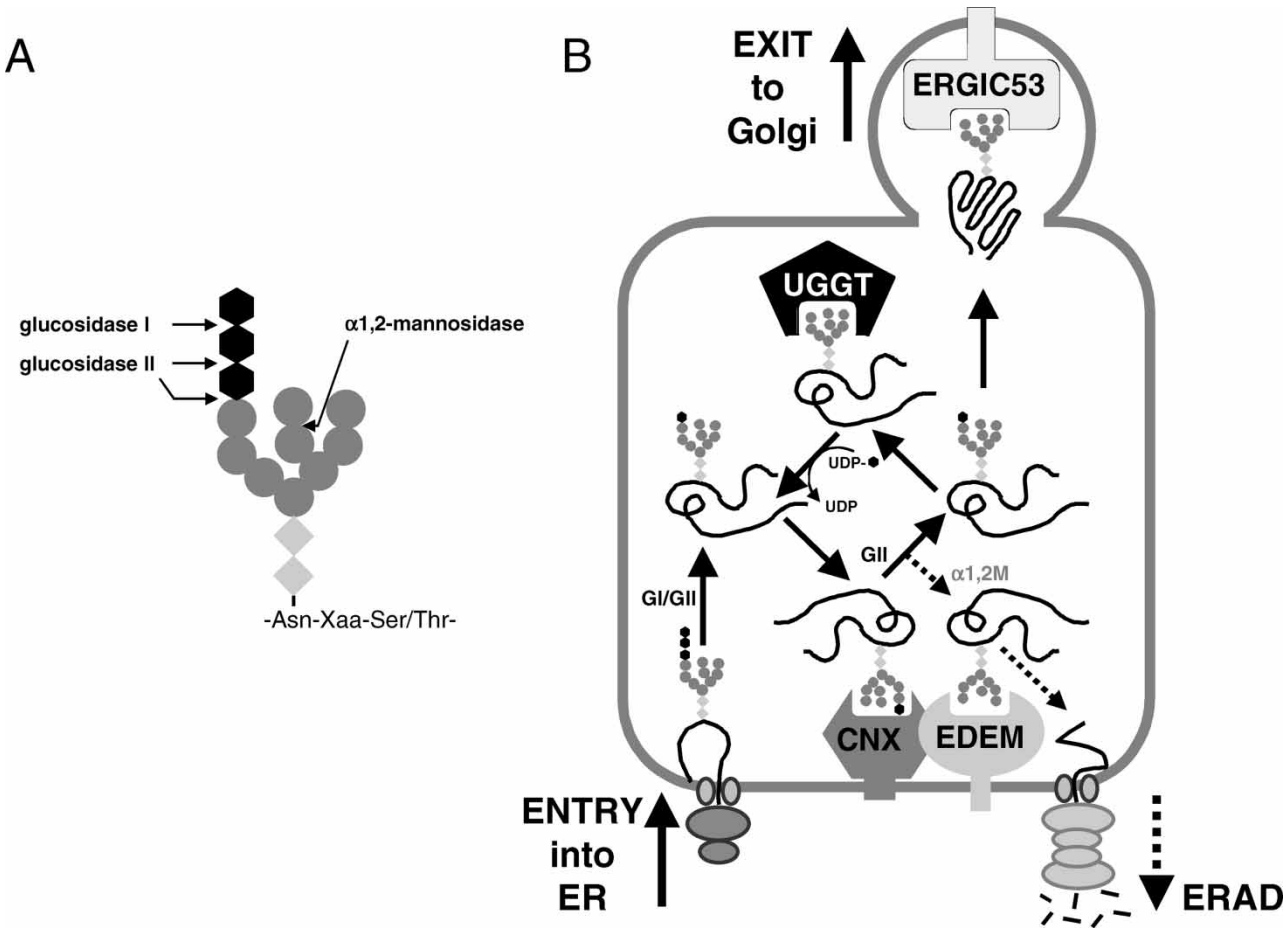

FIGURE 5 ***N*-glycans and folding of ER clients. A) Structure of the glycan as it is attached to ER clients. The glycan tree that is added to the polypeptide chain of nascent ER clients at the consensus sequence NXS/T (where X stands for any residue except proline) is a 14mer of monosaccharide units. Glucoses are depicted as black hexagons, mannoses as dark gray circles and N-acetyl glucosamines as light gray diamonds. Positions where glucosidases I & II and α1,2-mannosidase trim the glycan are indicated. B) *N*-glycans as signals for "(mis-)foldedness." *N*-linked glycans are added to nascent ER clients. Two glucose monosaccharide units (black hexagons) are removed from the glycan chain by glucosidase I (GI) and glucosidase II (GII) before CNX or CRT will associate to the ER client. (For simplicity only CNX is shown (*dark gray*). Next, glucosidase II (GII) will also remove the last remaining glucose. UGGT (black) "examines" the "foldedness" of the ER client. UGGT places a glucose it derives from UDP-glucose back onto the glycan of the ER client if the ER client is not fully folded. In that way, CNX and CRT can associate again for an additional folding round. UGGT no longer recognizes the ER client if it is fully folded, which implies the ER client is no longer substrate for the CNX/CRT cycle. They are recognized by ERGIC53 (*very light gray*) instead, which guides them to the *cis*-Golgi. Misfolded ER clients on the other hand are substrate to α1,2-mannosidase (α1,2M), which removes mannose monosaccharide units (*dark gray circle*) from the glycan chain of the ER client protein. For simplicity, only the removal of a single mannose residue is shown. Demannosylated (misfolded) ER clients are handed over from CNX to the mannose lectin EDEM (*light gray*). Association of ER client proteins with EDEM seals their fate: they are targeted for ERAD and, consequently retro-translocated to the cytosol where they are degraded by the proteasome.**

The primary role of lectin chaperones may be to keep folding intermediates in a folding competent state. Such a role is also served by many other chaperones (e.g., the small Hsps in the cytosol) but in contrast to folding substrates outside the ER, maturing secretory proteins most often are glycosylated. Glycans may sterically hinder association of classical peptide binding folding factors, disqualifying them as the sole appropriate chaperones of glycoproteins. The lectins CNX and CRT seem to have filled the vacancy. Through association to (multiple) bulky lectins, the folding substrate can no longer freely interact with itself or its surroundings, which prevents unproductive folding. Indeed, inhibitors of CNX/CRT association, such as castanospermin, provoke misfolding and aggregation of many nascent secretory proteins (Elbein, 1991). The notion that lectin association is primarily necessary to keep the substrate globally in an 'open' conformation, explains why the exact number and positions of glycans are often poorly conserved among closely related glycoproteins. Indeed, HIV envelope tolerates extensive reassortment of glycosylation positions (Fenouillet *et al.*, 1994).

The lectin chaperones also contribute to oxidative folding, albeit indirectly. Either protein forms a tandem with the oxidoreductase ERp57. One can envision that the lectins transiently fix folding intermediates in a conformation necessary for the formation of certain disulfide bonds. ERp57, as a close companion of the lectins, could then fulfill the role of catalyst (Molinari & Helenius, 1999). The architecture of the ERp57-lectin complex is quite unusual; it provides a cavity for its clients. Analogous to the folding cage of Hsp60, the cavity itself already could be beneficial for the folding process. Both lectins have an arm-like loop-domain which extends ≈110 Å (CRT) or ∼140 Å (CNX) from the lectin domains (Ellgaard *et al.*, 2001; Schrag *et al.*, 2001). The tip of the arm-like domains associates with the b′-domain of ERp57 (Frickel *et al.*, 2002; Russell *et al.*, 2004). Apparently, the lectins recruit ER-clients for ERp57. Still, association of ERp57 with ER-clients may prolong even after their dissociation from CNX or CRT (Frenkel *et al.*, 2004). It therefore cannot be ruled out that the b-domains of ERp57 have a role in client binding once the lectins have dissociated.

VARIATION IN FOLDING PATHWAYS AND MACHINERY

The way in which lectins deliver ER clients to ERp57 is but one example of handing-over mechanisms in the ER. One could indeed regard the ER folding factory as an assembly line of ER resident chaperones and other folding factors sequentially performing specialized tasks during the maturation process of ER substrates. As mentioned earlier, release of Kar2p from nascent proteins that emerge in the ER lumen of yeast, is accompanied by their association with Lhs1p (Steel *et al.*, 2004). Other examples are ER client specific: VSV G is passed on from BiP to CNX (Hammond & Helenius, 1994), while thyroglobulin associates with these chaperones in reverse order (Kim & Arvan, 1995). Immunoglobulin heavy chain, like VSV G, first associates with BiP, but afterward needs assistance from GRP94 (Melnick *et al.*, 1994). As the divergence in (sequence of) employment of chaperones by these ER-clients already indicates, there is no universal folding strategy that would apply to every single ER client. Instead, they all fold in different ways, employing distinct subsets of chaperones, dependent on size, hydrophobicity, number and position of *N*-glycans or disulfide bonds and oligomerization requirements. Even timing of signal peptide cleavage may differ and topogenesis of multi-spanning membrane proteins may not be uniform. These characteristics inherently determine to what extent the maturing ER client imposes difficulties on the folding machinery and explains why there is variety in speed and efficiency of folding and assembly processes amongst different clients of the folding machinery in the ER.

Variation in Signal Peptide Cleavage and Membrane Anchoring

Tampering with the signal peptide cleavage site is known to affect proper folding of several proteins, for instance VSV G (Shaw *et al.*, 1988). This suggests that the timing and site of signal sequence cleavage is important for folding. Compared with other secretory proteins, the signal peptide of HIV Envelope is cleaved off very late (Li *et al.*, 1994; Li *et al.*, 1996). This made it experimentally feasible to demonstrate that indeed folding of Envelope needs to proceed to some extent before its signal peptide can be removed (Land *et al.*, 2003).

The example of HIV envelope demonstrates that the signal peptide can act as a transient membrane anchor. In the case of the prion protein (PrP), the signal peptide serves as a permanent signal anchor for some molecules, while from other molecules it is removed (Stewart *et al.*, 2001). In fact, PrP can adopt several different topologies. The most common: "topoform" of PrP is exclusively linked to the membrane via a GPI-anchor (Lehmann & Harris, 1995). However, PrP contains a mildly hydrophobic domain that may act as a TMD for some molecules. Strikingly, when it is integrated into the membrane, it does so in either orientation, giving rise to so-called ^{Ntm}PrP and ^{Ctm}PrP variants (Hegde *et al.*, 1998). In addition, a fraction of PrP accumulates in the cytosol, because its signal peptide does not efficiently target PrP for translocation into the ER lumen (Rane *et al.*, 2004). This so-called cyPrP variant is neurotoxic and can assume the misfolded PrP^{Sc} conformation. CyPrP likely represents the "topoform" that is responsible for the onset of prion disease (Ma & Lindquist, 2002; Ma *et al.*, 2002).

Variety in topogenesis may be a more general phenomenon. The adenovirus E3-6.7K protein can insert into the membrane as a type II or a type III protein, while another topoform is fully translocated (Moise *et al.*, 2004). It is tempting to speculate that these topoforms each have distinct functions. Alternatively, the

co-existence of various "topoforms" merely reflects inefficiency in topogenesis. Mature aquaporin 1 (AQP1) has six TMDs. They alternate topology from the first signal anchor TMD onwards. Early after synthesis, however, the second TMD of the majority of AQP1 molecules does not integrate into the membrane, while the third TMD spans the membrane in the "wrong" orientation. This topoform is selectively degraded, while the few AQP1 molecules that established correct topology mature and exit the ER (Buck & Skach, 2005).

Variation in Glycosylation and Lectin Employment

CNX and/or CRT involvement is dependent on the number and position of the *N*-glycans of the ER substrate. The LDL receptor contains two *N*-glycans, but neither CNX nor CRT is required for its maturation (Jansens, Pena & Braakman, manuscript in preparation). In contrast, the single glycan on the Semliki Forest virus (SFV) E1 protein still allows an interaction with either CNX or CRT (Molinari & Helenius, 1999). VSV G, which contains two glycans, is different: it binds *in vivo* to CNX but not to CRT (Hammond & Helenius, 1994). In an *in vitro* binding assay, however, CRT can associate with VSV G (Peterson & Helenius, 1999), suggesting that merely its membrane attachment determines that CNX gets priority in the intact ER. Proteins that are more heavily glycosylated, such as influenza A virus hemagglutinin (HA) (Hebert *et al.*, 1997) and HIV envelope protein (Otteken *et al.*, 1996), associate with both CNX and CRT. The lectins can bind simultaneously to individual folding HA molecules, showing specificity for particular glycans (Hebert *et al.*, 1997; Daniels *et al.*, 2003). Lectin association in general and preference for either CNX or CRT apparently is dependent on the topology of the *N*-glycan in the folding protein.

CRT deficiency is lethal *in utero*, because development of the heart is impaired (Mesaeli *et al.*, 1999). CNX deficiency is not lethal, but $cnx^{-/-}$ mice suffer from severe motor disorders or early postnatal death (Denzel *et al.*, 2002). Although both lectins have an important role during development, $cnx^{-/-}$ and $crt^{-/-}$ cell lines are viable. Depletion of CRT accelerates maturation of several glycoproteins with only a slight decrease in folding efficiency, whereas folding of many glycoproteins is unaffected in CNX depleted cells. A notable exception is Influenza HA, which is critically dependent on CNX to leave the ER as a properly folded trimer. Only depletion of both CNX and CRT leads to a dramatic loss in folding efficiency and stringency of quality control (see below) (Molinari *et al.*, 2004). Altogether, the two lectins have largely overlapping function, but each is also individually a key component of the ER folding factory.

Variation in Disulfide Bond Formation and Oxidoreductase Employment

Compared to the simple dichotomy in lectin chaperones, the oxidoreductases form a broad spectrum. Still, oxidative folding in yeast must predominantly be the concern of PDI alone. Whereas deletion of PDI is lethal (Farquhar *et al.*, 1991; LaMantia & Lennarz, 1993), deletion of all four PDI-related proteins combined does not render yeast more sensitive to DTT and folding of the model substrate carboxypeptidase Y is unaffected (Nørgaard *et al.*, 2001). This raises the question what role these proteins might serve in addition to PDI. Several possibilities can be envisaged. They may be involved in oxidative folding under specific (stress) circumstances or they could serve a specific clientele of substrate proteins. Partner proteins of PDI-like proteins may guide them to exert specific tasks, as the lectin chaperones recruit ERp57 to glycoproteins. Alternatively, PDI family members may have another role than disulfide donor or isomerase, for instance reductase of proteins that are targeted back to the cytosol for degradation (Fassio & Sitia, 2002). Eps1p indeed seems to fulfill such a role (Wang & Chang, 2003) (see below).

For the various oxidoreductases in mammals only very few data exist that indicate they have specificity for the catalysis of individual disulfide bonds in particular. The two SFV glycoproteins p62 and E1 offer an example of differential substrate specificity of oxidoreductases. Whereas SFV p62 forms mixed disulfides with both ERp57 and PDI, SFV E1 only does so with PDI. ERp72, however, failed to form mixed disulfides with either of the two (Molinari & Helenius, 1999). Oxidative refolding of α_1-antitrypsin is efficiently catalyzed by PDIR, but far less so by P5 or PDI (Horibe *et al.*, 2004). Likewise, other ER clients may differ in their dependence on particular oxidoreductases to catalyze formation of distinct disulfide bonds. Also, oxidation and isomerization events may require assistance from specialized sets of oxidoreductases. The LDL receptor first oxidizes to species that have higher mobility in SDS-PAGE than the mature form that exits the ER (Jansens

et al., 2002). Therefore, the oxidation and isomerization processes are at least separated in time and possibly also with respect to which oxidoreductases are needed at the different stages.

Variation in Kinetics and Efficiency of Folding and Assembly

Extensive disulfide bond isomerization is also characteristic for HIV envelope folding (Land *et al*., 2003). This may in part explain why its folding process is very slow in comparison with for instance influenza HA (Braakman *et al*., 1991). Although slow, folding of Envelope occurs with high yield and does not involve aggregation (Land *et al*., 2003). Although folding of both procollagen and thyroglobulin is productive, it involves formation of aggregates as obligatory intermediates (Kim *et al*., 1993; Kellokumpu *et al*., 1997). Procollagen is also remarkable because its maturation requires extensive hydroxylation of proline residues in the ER (Uitto & Prockop, 1974). In the maturation process of IgM, not the folding of its subunits, but rather their oligomerization seems to form the bottleneck. Heavy chains must homodimerize and each heavy chain must heterodimerize with a light chain at the same time to form IgM-'monomers' that are in fact heterotetramers. Five 'monomers' subsequently pentamerize to form, together with a single J-chain, mature oligomeric IgM, which eventually consists of 21 subunits and contains ≈100 disulfide bonds (Reddy & Corley, 1998).

Specialized ERs and Specialized ER Resident Proteins

Compared to the impressive numbers of different proteins they assist in folding, the regular set of ER chaperones seem rather limited, especially considering that even this limited team shows redundancy. If unfolded proteins fail to be recognized by one chaperone, other classes of chaperones may take over. For instance, when CNX and CRT are prevented from binding, BiP can chaperone the folding of HA instead (Zhang *et al*., 1997). Early glycosylation after appearance in the ER lumen (close to the N-terminus in type I proteins) determines preference for lectin chaperones over BiP (Molinari & Helenius, 2000).

Despite redundancy in the chaperone machinery, folding kinetics of the same ER client may differ in various cell types. For instance, Envelope folds slightly more quickly in SupT1 cells, which are natural host cells for HIV infection, than in CHO or HeLa cells (Das *et al*., 1999; Land *et al*., 2003). More striking are the differences in folding efficiency for CFTR. In several cell lines, only one quarter of synthesized CFTR molecules mature, whereas the remainder does not fold correctly and is degraded (Ward & Kopito, 1994; Kopito, 1999). Conversely, in epithelial cells, CFTR is not degraded but efficiently matures (Varga *et al*., 2004). Differences in folding kinetics can also reflect variations in the folding capacity of the ER at large. Notably, professional secretory cells, such as plasma cells or acinar cells from glands, have well developed ER cisternae to accommodate bulk folding load.

Productive folding of ER-clients however is not only dependent on the versatility of the ER on a quantitative, but also on a qualitative level. For some ER-clients the regular set of ER folding factors is not sufficient; they require special treatment in the form of a dedicated chaperone: various LDL receptor family members have an exclusive need for RAP (Bu *et al*., 1995; Bu, 2001) and some for Mesd (Hsieh *et al*., 2003), procollagens are the only proteins requiring assistance from Hsp47 (Nagata *et al*., 1988; Hendershot & Bulleid, 2000; Nagai *et al*., 2000), and MHC class I is dependent on its "private" chaperone tapasin to incorporate potentially antigenic nonapeptides and thus, to form a stable ternary complex with peptide and β2 microglobulin (Sadasivan *et al*., 1996). Some folding factors are even tissue-specific, such as the PDI family members PDIp in pancreas (Desilva *et al*., 1996) or PDILT in the testis (van Lith *et al*., 2004). Another example is the CNX/CRT family member CMG, which is exclusively expressed in male meiotic germ cells (Watanabe *et al*., 1994). CMG is essential for sperm fertility, because it is required for assembly of the fertilin α- and β-subunit into the mature hetero-dimer (Ikawa *et al*., 1997; Ikawa *et al*., 2001).

QUALITY CONTROL

ER resident chaperones and folding enzymes not only assist the folding process. They also submit the maturation of ER clients to a general quality control (QC) (Hurtley *et al*., 1989). As a rule, ER substrates that have not attained their proper tertiary or quaternary structure are retained in the ER (Ellgaard & Helenius, 2003). QC standards are based on "foldedness" of ER clients, which on average seems to suffice as criterion to guarantee their functionality once they arrive at their

destination. Incompletely folded or misfolded client proteins interact with the network of resident ER chaperones. As such, immature client proteins are retained in the ER and their further traveling along the secretory pathway is counteracted. The stringency of ER retention is dependent on the client protein and the organism. Most if not all ER chaperones seem to contribute to QC. For instance, BiP was originally discovered as binding to immature and orphan antibody subunits (Haas & Wabl, 1983). In many monomeric subunits the surface area that will form the interface between monomers in the oligomer is hydrophobic and hence allows BiP binding. Although BiP association goes through cycles of binding and release, it is sufficient to keep the majority of unassembled light chains in the ER (with the exception of secreted homodimers of some light chain isotypes (Milstein, 1965; Leitzgen *et al.*, 1997). In a similar fashion other hydrophobic patch binding chaperones such as GRP170 and GRP94 may contribute to QC.

Thiol-Mediated Retention

Analogous to exposed hydrophobic patches, exposed sulfhydryl groups provide a second target for QC. Unpaired cysteines allow formation of mixed disulfides between members of the PDI family and their clientele. For many ER clients it is difficult to distinguish whether prolonged association with thiol-oxidoreductases alone would prevent their untimely exit from the ER, since other chaperones most often operate on these substrates simultaneously when disulfide bonds are formed and shuffled. So-called thiol-mediated retention certainly plays an important role in preventing immature exit of unassembled subunits of acetylcholinesterase (Kerem *et al.*, 1993) and IgM (Sitia *et al.*, 1990). PDI, ERp72 and possibly many more oxidoreductases form mixed disulfide bonds with the single cysteine in the tailpiece of the IgM heavy chains until they form interchain disulfide bonds with other IgM "monomers" in the mature IgM pentamer (Reddy *et al.*, 1996). The recently discovered PDI-family member ERp44 may even impersonate a dedicated "thiol-retentor" (Anelli *et al.*, 2003).

The CNX/CRT Cycle

Monoglucosylated glycans provide a third sign that broadcasts immaturity of ER client proteins (Ellgaard & Helenius, 2003; Trombetta & Parodi, 2003; Helenius & Aebi, 2004). The lectin chaperones bind to their monoglucosylated substrates until Glucosidase II removes the remaining glucose moiety (Hammond *et al.*, 1994). After removal of the last glucose from the *N*-glycan, UDP-glucose:glycoprotein glucosyltransferase (UGGT) acts as folding sensor. UGGT probes the folded state of the substrate by interacting with both the glycan structure and peptide backbone of the folding substrate (Sousa *et al.*, 1992), whereby UGGT shows preference for hydrophobic patches of residues close to the glycosylation site (Taylor *et al.*, 2003). Minor local deviations from the native state already determine that the ER substrate fails according to the strict UGGT standards (Ritter & Helenius, 2000; Ellgaard & Helenius, 2003). If so, UGGT reglucosylates the *N*-glycan (Trombetta & Parodi, 1992; Parodi, 2000), thereby allowing the incompletely folded substrate to (re-)associate with CNX and/or CRT for an additional folding cycle. As a consequence, the substrate cannot leave the ER yet. The cycles of deglucosylation and reglucosylation continue as long as the ER client has not fully folded (Figure 5B). Yeast lacks UGGT activity and hence a lectin/UGGT cycle (Parodi, 1999). This suggests that the lectin/UGGT mechanism of QC has been acquired more recently in evolution.

Export from the ER

When ER clients meet all QC standards, they can exit from the ER in COPII coated vesicles. In yeast, cargo vesicles bud from the entire ER (Rossanese *et al.*, 1999). In mammalian cells, however, ER export is confined to distinct exit sites, referred to as transitional ER (Palade, 1975). While many ER clients may be aspecifically incorporated into COPII vesicles, representing bulk flow from ER to Golgi (Wieland *et al.*, 1987), several other ER clients require dedicated export receptor proteins to mediate their exit from the ER. For instance, glycoproteins are recruited to COPII vesicles by the mannose specific lectin ERGIC53 in mammalian cells (Appenzeller *et al.*, 1999) (Figure 5B). In yeast, the membrane protein Erv29 determines that the soluble ER clients prepro-α factor and carboxypeptidase Y are packaged and sent to the Golgi (Belden & Barlowe, 2001; Otte & Barlowe, 2004), whereas Emp24p serves as export receptor of GPI-anchored ER clients, like Gas1p (Muniz *et al.*, 2000, 2001). Export of membrane spanning ER clients can also be mediated by signals on the cytosolic side of the membrane. A di-acidic motif, DXE (where X represents any residue), in their cytoplasmic domain(s)

can serve as export signal (Nishimura & Balch, 1997), because it is recognized by the COPII component Sec24 (Votsmeier & Gallwitz, 2001; Miller *et al.*, 2002, 2003). Other cytosolic ER export motifs, $LXX^{L}/_{M}E$ and YNNSNPF, in SNARE proteins confer association with Sec24 and subsequent vesicular trafficking in a similar manner as the di-acidic motif (Mossessova *et al.*, 2003).

Question is why export motifs are only recognized as such when the ER client has fully matured. Next to the signs of immaturity as negative determinants for QC, "unmasking" of export motifs may represent a positive determinant for QC. For example, di-acidic export motifs in the cytosolic domains of CFTR may be buried while the protein still folds, but become accessible to Sec24 once CFTR is correctly folded (Wang *et al.*, 2004). The opposite seems to be true for the ATP-sensitive K+ channel, which in its mature form consists of four α- and four β-subunits. The monomeric subunits have RXR ER retention motifs that only become "masked" when the subunits correctly assemble into the mature hetero-octamer (Zerangue *et al.*, 1999). Still, it is difficult to envision that 'masking' of retention motifs and/or 'unmasking' of export motifs governs exit of every ER client. For instance, VSV G has its di-acidic export motif at the tip of a short cytoplasmic tail (Nishimura & Balch, 1997), which makes it unlikely to be masked during folding. Altogether, it remains unclear how ER-clients depending on their folding status are recruited to or excluded from ER exit sites.

ER-ASSOCIATED DEGRADATION

Some ER-clients misfold beyond rescue and will never pass QC standards. This implies that QC must be coupled to efficient proteolytic systems to allow clearance of misfolded proteins that otherwise would jam the ER lumen. Many misfolded ER substrates and unassembled subunits of oligomeric proteins are indeed degraded (Klausner & Sitia, 1990), but not inside the ER lumen. Instead, misfolded ER substrates that are destined for degradation are retro-translocated (dislocated) to the cytosol (Hiller *et al.*, 1996; Werner *et al.*, 1996; Wiertz *et al.*, 1996; Kopito, 1997). In principle, only ER clients that are "off pathway" are dislocated. An interesting exception is the disposal of "on pathway" ER clients in cytomegalovirus (CMV)-infected cells. The CMV proteins US2 and US11 'hijack' the retro-translocation machinery: they selectively dislocate MHC class I heavy chains in order to evade from the host's antigen presentation for immune surveillance (Furman & Ploegh, 2002).

Once ER clients are dislocated to the cytosol, they become subject to ubiquitination and subsequent proteasomal degradation, in the same manner as ill-fated cytosolic proteins (Tsai *et al.*, 2002). Altogether, this process is referred to as ER associated degradation (ERAD). In coping with the great variety of its substrates, the ERAD machinery to some extent mirrors the folding machinery. Dependent on topology, glycosylation and oxidation status of substrates, different ERAD components are required.

ERAD of Glycoproteins

An important signal for folding failure is the mannosylation status of ER client glycans. Whereas removal of glucose moieties from *N*-glycans is used as signal for correct folding and exit to the Golgi, mannose trimming may destine misfolded proteins for ERAD instead (Cabral *et al.*, 2001) (Figure 5B). Accordingly, the mannosidase inhibitor kifunensin delays degradation of ERAD substrates (de Virgilio *et al.*, 1999). In yeast, removal of a single mannose from the middle branch of the *N*-glycan by α1,2-mannosidase is sufficient to render ER clients substrate for degradation (Jakob *et al.*, 1998; Helenius & Aebi, 2001). The ill-fated ER clients are recognized by the mannosidase-like protein Htm1p/Mnl1p as soon as they bear $Man_8GlcNAc_2$ glycoforms (Jakob *et al.*, 2001; Nakatsukasa *et al.*, 2001). Htm1p/Mnl1p therefore is thought to be a lectin (Braakman, 2001). The mammalian ER harbors three homologs of Htm1p/Mnl1p, EDEM1, 2, and 3 (Hosokawa *et al.*, 2001; Mast *et al.*, 2005; Olivari *et al.*, 2004). As established for EDEM1 (Hosokawa *et al.*, 2001; Molinari *et al.*, 2003) and EDEM2 (Mast *et al.*, 2005; Olivari *et al.*, 2004), they serve as acceptor of misfolded glycoproteins similarly as Htm1p/Mnl1p. Unlike in yeast, preparation of substrates for ERAD in mammals involves extensive mannose trimming before dislocation into the cytosol (Frenkel *et al.*, 2003). This difference may reflect the presence of UGGT in the mammalian ER lumen, while it is lacking from yeast. To divert terminally misfolded proteins from the CNX/CRT cycle, their reglucosylation by UGGT must be avoided. Indeed, extensive mannose trimming renders ERAD-candidates poor substrates for reglucosylation by UGGT (Parodi, 2000).

Special to mammalian ERAD is also that EDEM1 interacts with CNX, which indicates that misfolded glycoproteins are directly handed over from a "folding lectin" (CNX) to a "degradation lectin" (EDEM1) (Oda *et al.*, 2003) (Figure 5B). It remains to be elucidated, however, by what mechanism EDEM family members transfer ERAD substrates to the dislocation pore or retro-translocon. Another important question is how ER-resident mannosidases selectively act on ER clients that are 'overdue'. It has been postulated that, inherently to the relative indolence of the ER resident mannosidases (Mancini *et al.*, 2003), mannose trimming could act as a timer for prolonged ER residency and hence for inability of ER clients to reach their native state (Helenius & Aebi, 2001). Such a mechanism however is difficult to reconcile with on the one hand the great variety in folding kinetics among different ER clients, and on the other hand the fact that several glycoproteins, such as GRP94, have the ER as their permanent residence.

Role of BiP and PDI in ERAD

As lectins, the EDEM variants likely have a role exclusive for ERAD of misfolded glycoproteins. How then are unglycosylated ERAD substrates directed to the dislocation pore? This question has received little attention. In yeast, ERAD of a mutant prepro-α factor from which all glycosylation sites were deleted (Δgpαf) is critically dependent on both PDI (Gillece *et al.*, 1999), Kar2p (Brodsky *et al.*, 1999) and its J-domain containing co-chaperones Jem1p and/or Scj1p (Nishikawa *et al.*, 2001). Still, the same set of ER resident proteins are involved in ERAD of the glycoprotein CPY* (Plemper *et al.*, 1997; Nishikawa *et al.*, 2001), indicating that their role is not exclusive for unglycosylated ERAD substrates. In mammalian cells, like in yeast, PDI cooperates with BiP in the ERAD machinery: together they direct the pancreatic isoform of β-secretase BACE457 for dislocation (Molinari *et al.*, 2002). Thus, in parallel to EDEM, BiP and PDI have a role in ERAD targeting. While the EDEM proteins may be dedicated to the ERAD pathway, BiP and PDI however participate in productive folding as well. It remains unclear how BiP and PDI "decide" when they no longer should treat ER clients as *bona fide* folding intermediates but as terminal folding failures instead.

Remarkably, yeast PDI can recognize ERAD-fated Δgpαf even though this protein has no cysteines. In fact, association of PDI to Δgpαf is critically dependent on the b′-domain of PDI alone (Gillece *et al.*, 1999). In analogy, the yeast PDI-like protein Eps1 is essential for efficient elimination via ERAD of a mutant of plasma membrane [H+]ATPase, despite its lack of disulfide bonds (Wang & Chang, 2003). Still, there is a clear advantage to involve PDI or its relatives in targeting misfolded proteins for dislocation. Functioning as thiol-reductases, PDI family members can relieve oxidized ERAD substrates from their disulfide bonds, as has been demonstrated for cholera toxin (CT) (Tsai *et al.*, 2001). CT enters the mammalian cell via endocytosis, retrograde transport and subsequent disclocation from the ER lumen into the cytosol, where it obstructs ribosomal function. In the process, the disulfide bonds of CT are reduced. Essential for this thiol-reduction are both PDI and Ero1α (Tsai *et al.*, 2001; Tsai & Rapoport, 2002). Thiol-reduction of CT is no exception. Ricin, a toxin that follows a similar retrograde transport route as CT, also requires PDI mediated thiol-reduction before its dislocation (Spooner *et al.*, 2004). Likewise, interchain disulfide bonds between Ig-μ heavy chains and light chains are disentangled prior to dislocation of ERAD-fated heavy chains (Fagioli *et al.*, 2001). It is plausible that other PDI-family members can play a similar role. For instance, ERp57 has been implicated in the reduction of MHC class I molecules that are dislocated to the cytosol for degradation (Antoniou *et al.*, 2002).

If reduction of disulfide bonds is a necessary step in preparing ERAD candidates for dislocation, one could consider that also their unfolding is a prerequisite. There is indeed evidence that CT unfolds before retro-translocation with assistance of PDI (Tsai *et al.*, 2001) and possibly BiP (Winkeler *et al.*, 2003). Still, unfolding prior to dislocation does not seem to be obligatory for all ERAD substrates, as is evident from the dislocation of MHC class I heavy chain proteins fused with either a dihydrofolate reductase domain (DHFR), which attains a tightly folded conformation when bound to trimetrexate (TMX) or green fluorescent protein (GFP) (Fiebiger *et al.*, 2002; Tirosh *et al.*, 2003). In the presence of proteasomal inhibitors, both fluorescent GFP (Fiebiger *et al.*, 2002) and, TMX-bound DHFR (Tirosh *et al.*, 2003) accumulate in the cytosol upon US11-stimulated retro-translocation of the MHC class I heavy chain. Thus, both fusion proteins seem to traverse the ER membrane back to the cytosol in a folded state.

The Retro-Translocon

The question whether ERAD candidates can maintain a (partly) folded conformation depends on the nature of the dislocon. Several lines of evidence suggest that the dislocon shares with the translocon the same channel composed of the sec61 protein complex (Wiertz *et al.*, 1996; Pilon *et al.*, 1997; Plemper *et al.*, 1997). The constriction of the protein translocation channel that has been proposed based on the crystal structure of SecY (Van den Berg *et al.*, 2004) seems too narrow to sustain dislocation of fully folded proteins. Its diameter is even difficult to reconcile with the fact that ERAD substrates are dislocated in a glycosylated state (Hiller *et al.*, 1996; Wiertz *et al.*, 1996). Interestingly, a four membrane spanning protein, Derlin-1, is essential for retro-translocation of MHC class I heavy chain, when it is targeted for ERAD by US11 (Lilley & Ploegh, 2004; Ye *et al.*, 2004). The groups of Rapoport and Ploegh therefore proposed that Derlin-1 forms the dislocon instead of Sec61 (Lilley & Ploegh, 2004; Ye *et al.*, 2004). Alternatively, Derlin-1 interacts with Sec61 and influences its pore size during retro-translocation. In yeast, not only Der1p, the yeast homolog of Derlin-1, (Knop *et al.*, 1996), but also Sec61 (Huyer *et al.*, 2004b) is required for dislocation of CPY*. In either case, Der in yeast and Derlin-1 and/or its family members Derlin-2 and -3 in mammalian cells (Lilley & Ploegh, 2004) could facilitate that the dislocon pore is wider than the translocon pore to accommodate traversing ERAD substrates that are "bulky."

ERAD and Topology

For membrane spanning proteins it is important to distinguish between ERAD substrates that have a folding defect in their ER lumenal or in their cytosolic domains. ERAD is indeed different for substrates with a lumenal lesion (ERAD-L) versus those with a cytosolic lesion (ERAD-C) both in kinetics and with respect to what machinery is required. It appears that only ERAD-L substrates require lumenal ERAD components such as Kar2p and Htm1p and subsequently Der1p for their dislocation (Vashist & Ng, 2004). Conversely, ERAD-C substrates, such as the 12-spanning membrane protein a-factor transporter that has a mutation in one of its cytosolic domains (Ste6-166p) require neither Der1p (Vashist & Ng, 2004) nor Sec61p (Huyer *et al.*, 2004b) for retro-translocation. It is currently unclear how the lumenal domains of ERAD-C substrates are dislocated prior to degradation. A membrane anchored version of CPY* (CT*) seems to form an exception to the "lesion site rule." Its retro-translocation is Der1p independent (Taxis *et al.*, 2003), although the mutation in the lumenal CPY* module would predict that this mutant is an ERAD-L substrate. A possible explanation for this discrepancy is that CPY* was fused to a singular TMD of a multi-spanning membrane protein. Perhaps, the "orphan" TMD is not an appropriate membrane anchor but instead mediates recognition of CT* by the ERAD-C pathway.

Ubiquitination and Proteasomal Degradation of ERAD Substrates

Ubiquitination targets proteins for proteasomal degradation. Ubiquitin moieties are added to lysine residues on the polypeptide chain by ubiquitin conjugating E2 enzymes in conjunction with E3 ubiquitin ligases. In yeast, the principal E2 enzymes involved in ERAD are Ubc6p, a tail-anchored ER membrane protein (Sommer & Jentsch, 1993), Ubc7p, which is anchored to the ER membrane through association with the trans-membrane protein Cue1p (Biederer *et al.*, 1997), and Ubc1p (Friedlander *et al.*, 2000; Jarosch *et al.*, 2002). ERAD dedicated E3 ligases in yeast are Hrd1p, also known as Der3p, (Bordallo *et al.*, 1998; Bays *et al.*, 2001) and Doa10p (Swanson *et al.*, 2001). Although the two E3 enzymes may have some overlapping specificity (Gnann *et al.*, 2004), Doa10p is predominantly required for ERAD-C but not for ERAD-L and, *vice versa*, Hrd1p is mostly necessary for ERAD-L (Vashist & Ng, 2004). A cofactor of Hrd1p is the membrane spanning protein Hrd3p. The lumenal domain of Hrd3p associates with ERAD-L substrates and it thereby "senses" their imminent dislocation. Subsequently, Hrd3p activates the ubiquitin ligase activity of Hrd1p with its cytosolic domain (Gardner *et al.*, 2000). Most of the yeast ubquitination machinery has been conserved in mammals (Kaneko & Nomura, 2003; Kostova & Wolf, 2003), except that a mammalian homolog of Doa10p still has to be identified.

Two glycoprotein specific ubiquitin ligases, the SCF(Fbs1) and SCF(Fbs2) complex have been identified in mammals (Yoshida *et al.*, 2002; Yoshida *et al.*, 2003), but not in yeast. Conserved from yeast to man is the deglycosylation of ERAD substrates by *N*-glycanase prior to proteasomal degradation (Hirsch *et al.*, 2003). As soon as ERAD substrates are ubiquitinated and

deglycosylated they can be recognized and degraded by the proteasome, but most ERAD substrates first need to be extracted from the membrane or dislocon by the AAA-ATPase p97 (Cdc48p in yeast) and its cofactors Ufd1 and Npl4 (Ye *et al.*, 2001; Jarosch *et al.*, 2002; Ye *et al.*, 2003). Dsk2p and Rad23p seem to hand over ERAD substrates from the Cdc48/Ufd1/Npl4 complex to the proteasome (Medicherla *et al.*, 2004). On the one hand Dsk2p and Rad23p can bind ubiquitinated ERAD substrates with a ubiquitin association domain (Rao & Sastry, 2002), while on the other hand, they can recruit the proteasome with a ubiquitin-like domain (Hartmann-Petersen *et al.*, 2003).

Although Dsk2p and Rad23p may have largely overlapping function (Medicherla *et al.*, 2004), Rad23p and its mammalian homolog HR23B interact with *N*-glycanase (Suzuki *et al.*, 2001; Katiyar *et al.*, 2004), suggesting Rad23p/HR23B to be glycoprotein specific. This interaction is but one example of the tight links between retro-translocation, ubiquitination, deglycosylation and proteasomal degradation. In mammalian cells, p97 interacts with both Derlin-1 (Ye *et al.*, 2004) and the E3 enzyme gp78 (Zhong *et al.*, 2004), which, next to mammalian HRD1, represents a second mammalian Hrd1p-like protein (Kikkert *et al.*, 2004). Altogether, the coordinated efforts of the ERAD machinery ensure that substrates cannot escape into the cytosolic environment. Poly-ubiquitinated ERAD substrates are only detectable in the cytosol when *DSK2* and *RAD23* are deleted in yeast (Medicherla *et al.*, 2004) and glycosylated ERAD substrates only appear in the mammalian cytosol when *N*-glycanase expression levels are reduced (Blom *et al.*, 2004).

ERAD Backup Mechanisms

At basal conditions, the ER folding and ERAD machineries jointly ensure that numbers of unfolded or misfolded conformers of ER clients remain within bounds. The regular ER folding and ERAD machineries no longer suffice in case the folding load increases or when productive folding is hampered because of adverse conditions, for example glucose starvation, hypoxia or overexpression of mutant ER clients that never reach the native state. Any of these conditions invoke the unfolded protein response (UPR) pathways. A detailed description of the intricate sensing and response mechanisms of the UPR is beyond the scope of this review. In brief, "sensors" detect folding difficulties inside the ER lumen and transduce the "bad news" in several ways. One UPR pathway leads to phosphorylation of the translation initiation factor to reduce further influx of client proteins into the ER lumen. Other UPR pathways set off enhanced transcription of UPR target genes. UPR targets include ER chaperones, folding enzymes and components of the membrane biosynthesis machinery to enlarge the ER and hence its folding capacity. Other UPR targets are the ERAD components, needed to relieve the ER folding factory, and hence the cell, from its (misfolded) burden. Eventually, the UPR may induce pro-apoptotic programs when folding problems in the ER no longer can be overcome (Travers *et al.*, 2000; Patil & Walter, 2001; Schröder & Kaufman, 2005).

ERAD and the UPR pathways are intimately linked, especially when the ER faces accumulation of misfolded load. When CPY* is expressed at low levels in *HRD1* and *DER1* deletion strains, it is still degraded, albeit with low efficiency. Conversely, when CPY* is overexpressed, its degradation kinetics are unaffected by the absence of Hrd1p (Haynes *et al.*, 2002). Instead, overexpressed CPY* is stabilized by a block in ER to Golgi transport (Caldwell *et al.*, 2001; Vashist *et al.*, 2001; Haynes *et al.*, 2002; Taxis *et al.*, 2002). Apparently, the HRD/DER pathway can be saturated and ERAD-L substrates can "escape" to the Golgi, as a result. Nevertheless, these "runaways" are targeted for ERAD by the HRD/DER independent pathway (HIP) (Haynes *et al.*, 2002). As an alternative to Hrd1p, the E3 enzyme Rsp5, in conjunction with its preferred E2 enzyme partners, Ubc4p or Ubc5p, ubiquitinate these ERAD substrates (Haynes *et al.*, 2002). The HIP pathway functions downstream of the UPR pathways (Haynes *et al.*, 2002). The HIP pathway therefore represents an ERAD backup mechanism that is only invoked when misfolded proteins accumulate in the ER lumen. It is not clear how ERAD substrates of the HIP pathways are dislocated (Haynes *et al.*, 2002), but they seem to be retrieved from the Golgi back to the ER prior to dislocation (Vashist *et al.*, 2001).

The ERAD-C substrate Ste6-166p does not travel to the Golgi even when overexpressed (Vashist *et al.*, 2001). Instead, Ste6p mutants, as well as CFTR, are sorted to distinct substructures of the yeast ER that Huyer *et al.* proposed to refer to as ER-associated compartments (ERACs) (Huyer *et al.*, 2004a). Formation of ERACs does not interfere with overall folding or trafficking. Therefore, ERACs seem to represent "holding sites" of

mutant ERAD-C substrates (Huyer *et al*., 2004a). Such 'holding sites' are reminiscent of, for instance, Russell bodies in plasma cells, where aggregated Ig molecules condense (Kopito & Sitia, 2000) or the so-called QC compartment, where unassembled asialoglycoprotein receptor H2a subunits or MHC class I heavy chains accumulate (Kamhi-Nesher *et al.*, 2001). Another example is the deposition in distinct ER regions of mutant forms of α_1-antitrypsin that in patients cause chronic hepatitis or hepatocellular carcinoma (Teckman & Perlmutter, 2000). Despite the ER dilation, the α_1-antitrypsin mutants are finally degraded, in part via "classical" proteasome-dependent ERAD (Teckman *et al.*, 2001), but also via autophagy (Teckman & Perlmutter, 2000).

Perhaps, the ER "holding" strategy combined with eventual autophagy is more common in mammalian cells than in yeast. For example, CPY* degradation in mammalian cells is only in part mediated via the proteasome (Mancini *et al*., 2003). Present knowledge of ERAD is largely based on misfolded proteins that have a relatively high turnover. Still, many misfolded proteins are retained in the ER for prolonged periods, possibly because the QC surveillance fails to mark them as ERAD candidates. Alternatively, they are too bulky to be dislocated in a folded state, while their (partial) dismantling inside the ER lumen may come at too high a cost. In either case, autophagy may serve as backup to "classical" proteasomal ERAD.

PERSPECTIVES

In conclusion, the ER is an impressively versatile protein folding factory, taking into account the great variety in client proteins it fosters. Quality standards are high; the ER folding factory faithfully delivers only correctly folded proteins, while it retains folding intermediates. In case the combined efforts of ER resident chaperones and folding enzymes are in vain, folding failures are dealt with by sophisticated clearance mechanisms.

Many key questions still deserve attention. How exactly does the ER discriminate between mature and immature clients or between *bona fide* folding intermediates and "lost causes"? Next to UGGT, which are the (mis-) folding 'sensors' that grant clients pardon to leave the ER, sentence them for additional folding rounds or ultimately to death by the proteasome? Another important challenge is to characterize the clientele of individual ER chaperones and folding enzymes. The identification of 10% of the bacterial proteome as substrates of GroEL (Houry *et al.*, 1999) and an initial characterization of the clientele of DsbA in bacteria (Kadokura *et al.*, 2004) serve as examples. Each ER client may preferentially employ only a particular subset of folding factors, but the number of model ER clients studied so far is too limited to predict the match between individual clients and the chaperones and folding enzymes they require. As the examples of client/tissue specific chaperones illustrate, adaptations in the team of ER resident folding factors may suit particular folding requirements of individual (sets of) clients. The question is whether we can likewise design a "tailor-made" ER folding factory for medically or economically relevant ER clients, such as antibodies, that currently give poor yields in heterologous expression systems.

Several congenital defects are associated with misfolding and retention in the ER of a particular ER client, the so-called ER storage diseases (ERSDs) (Rutishauser & Spiess, 2002). Misfolding of the CFTR protein and its retention in the ER, for example, causes cystic fibrosis (Lukacs *et al.*, 1994; Ward *et al.*, 1995) and LDL receptor folding defects lie at the basis of familial hypercholesterolemia (Hobbs *et al.*, 1992). Other well known ERSDs are congenital goiter and hyperthyroidism caused by mutations in thyroglobulin (Medeiros-Neto *et al.*, 1996) and emphysema and/or destructive lung or liver disease that arise when folding of α_1-antitrypsin is impaired (Perlmutter, 1996). In many cases, the affected protein displays only a minor folding lesion. As a possible therapeutic intervention, local conformational aberrations may be "splinted" by peptides or other small compounds, thereby stabilizing the protein. An example of such a "pharmacological chaperone" strategy is the rescue of V2 vasopressin receptor mutants, which cause nephrogenic diabetes insipidus, by membrane-permeable antagonists (Morello *et al.*, 2000).

The opposite, preventing folding of certain ER clients in particular, is of interest too, for instance as antiviral strategy. A successful example is the employment of glucosidase inhibitors. They interfere with the replication of Hepatitis B virus (Mehta *et al.*, 1998) and bovine viral diarrhea virus, a pestivirus model for hepatitis C virus (Zitzmann *et al.*, 1999). Minor deviations in glycosylation pattern may not be tolerated in the stringent lattice of the virion (Rudd & Dwek, 1997), which could explain why low doses of glucosidase inhibitors have a specific antiviral effect, while overall folding in the ER is not disturbed. Accordingly, the glucosidase inhibitor strategy fails with viruses that do

not have a strictly symmetric virus particle architecture, such as Newcastle disease virus (McGinnes & Morrison, 1998). Perhaps, "pharmacological antichaperones" can be identified that solely prevent the folding of particular viral glycoproteins or selectively target them for ERAD. Similarly, such specific protein "shipwrecking" could offer an avenue for therapeutic intervention against cancer (Ulloa-Aguirre *et al.*, 2004).

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