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Review

The human PDI family: Versatility packed into a single fold

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Abstract

The enzymes of the protein disulfide isomerase (PDI) family are thiol–disulfide oxidoreductases of the endoplasmic reticulum (ER). They contain a CXXC active-site sequence where the two cysteines catalyze the exchange of a disulfide bond with or within substrates. The primary function of the PDIs in promoting oxidative protein folding in the ER has been extended in recent years to include roles in other processes such as ER-associated degradation (ERAD), trafficking, calcium homeostasis, antigen presentation and virus entry. Some of these functions are performed by non-catalytic members of the family that lack the active-site cysteines. Regardless of their function, all human PDIs contain at least one domain of approximately 100 amino acid residues with structural homology to thioredoxin. As we learn more about the individual proteins of the family, a complex picture is emerging that emphasizes as much their differences as their similarities, and underlines the versatility of the thioredoxin fold. Here, we primarily explore the diversity of cellular functions described for the human PDIs.

Keywords: Disulfide-bond formation; Endoplasmic reticulum; Protein disulfide isomerase; PDI; Protein folding

1. Introduction

Disulfides are covalent bonds produced by oxidation of two free thiols — in proteins they form between two cysteine residues. They serve important functions during protein folding and in stabilizing protein structures – both as intramolecular bonds and when formed intermolecularly between two protein chains – but also through their capacity to work as regulatory switches in redox signaling.

Mammalian cells produce a fascinating collection of complicated disulfide-bonded structures. Disulfides are present in a large fraction of the close to 1/3 of all human proteins that traverse the secretory pathway [1]. The synthesis and folding of these proteins take place in the ER where the environment is conducive to disulfide-bond formation — the ER lumen is relatively more oxidizing than the cytosol, and a number of enzymes catalyze the formation of native disulfides. This activity was first shown for PDI [2] that is an abundant protein of the ER and the founding member of the PDI family (the members of which we refer to as PDIs). It is an essential protein in *S. cerevisiae* [3], and the human as well as the yeast protein have been thoroughly investigated in vitro. Based on these and similar studies on other family members, we now have a quite detailed understanding of the structure and enzymatic properties of single catalytically active thioredoxin-like domains, the basic unit of PDIs. As the name implies, these domains are structural homologs of the cytosolic reductase thioredoxin.

Current work is directed at understanding the interplay between domains within the multi-domain structure found in most PDIs. For PDI itself, the domain composition has been known for years [4–6] and recently the crystal structure of the entire protein from *S. cerevisiae*, Pdi1p, was solved [7] (Fig. 1A and B). Another center of attention is the physiological function of individual proteins, including studies of their cellular redox regulation and the identification of endogenous substrates. Together, these topics also constitute the main focus of this review, where we will limit ourselves to the mammalian system and the processes that take place in the ER unless directly otherwise stated (for recent studies on the extracellular function of P5 and ERp57 see [8,9]).

2. The proteins of the human PDI family — an overview

With the increasing number of cDNA sequences deposited in the public domain in recent years, the number of known human

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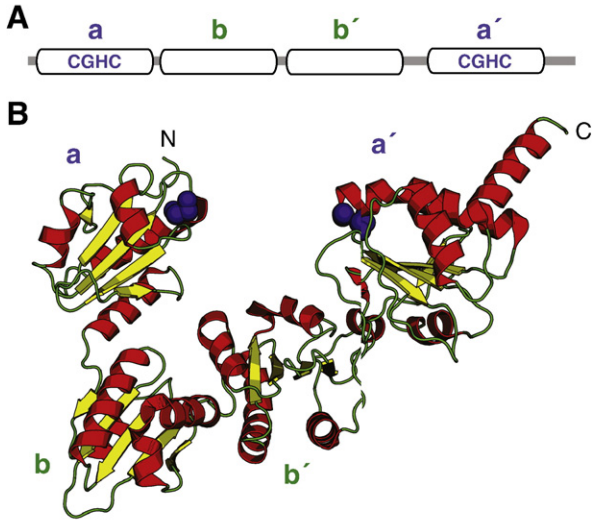

Fig. 1. Domain composition and three-dimensional structure of PDI. A, schematic overview of the PDI domain composition. Thioredoxin-like domains are shown as rounded rectangles with purple lettering denoting catalytic domains (**a** and **a′**) and green lettering non-catalytic domains (**b** and **b′**). The active-site sequences are shown. The C-terminal extension beyond the **a′** domain constitutes the prominent α-helix visible at the C-terminus in the crystal structure of Pdi1p (B). The α-helices are shown in red, β-strands in yellow and loops in green. The active-site cysteines are depicted as purple spheres.

PDIs has also quite rapidly expanded. The family comprises 19 published members that all contain a predicted signal sequence and at least one thioredoxin-like domain (Table 1)[10–12]. Although the family name implies an isomerase function, this activity has not been demonstrated experimentally for all. PDILT has been shown not to have any appreciable oxidoreductase activity [13], which probably also holds true for other PDIs with only one cysteine in the active site, such as the newly discovered Hag2 and Hag3 [14]. Moreover, ERp27 and ERp29 are non-catalytic family members. It is therefore important to stress that the grouping of proteins into the human PDI family is not based on one common function or the exact same enzymatic properties, but rather on sequence and structural similarity as well as ER localization (see below).

Table 1 provides an overview of important traits of the human PDIs. The unifying feature in terms of sequence and structure is the thioredoxin-like domains that can be either catalytic or non-catalytic. By convention these two types of domains are called **a**- and **b**-type domains, respectively. The **a**-type domains usually contain two cysteines in a CXXC active-site motif with an intervening GH sequence being the most common in the PDIs. By sequence similarity rather than catalytic activity some domains lacking one or even two active-site cysteines are also categorized as **a**-type domains (Table 1). The **b**-type domains do not have cysteines in the active site and are therefore not redox active. Generally, the sequence similarity is much higher for **a**-type than **b**-type domains since the latter are missing a number of residues important for catalysis that are conserved among the **a** domains. Only two PDIs contain additional types of domains in the ER — ERdj5 has an N-terminal J-domain that binds and stimulates the ATPase activity of the ER chaperone BiP in vitro [15,16], and ERp29 comprises an α-helical so-called D-

Table 1
Overview of the proteins of the human PDI family listed according to size with the soluble proteins above the thick line and the transmembrane proteins below

Name	Accession	Length	ER-localization motif	Domain composition	Number of **a**-type domains	Active-site sequence	PDB accession numbers[a]
Hag 3	Q8TD06	165	QSEL[b]	**a**	1	CQYS	
ERp18	O95881	172	EDEL	**a**	1	CGHC	1sen
Hag 2	O95994	175	KTEL[b]	**a**	1	CPHS	
ERp28/29[c]	P30040	261	KEEL	**b–D**	0	n.a.	1g7e; 2c0e; 1ovn
ERp27	Q96DN0	273	KVEL	**b–b′**	0	n.a.	
ERp44	Q9BS26	406	RDEL	**a–b–b′**	1	CRFS	
ERp46[d]	Q8NBS9	432	KDEL	**a°–a–a′**	3	CGHC, CGHC, CGHC	**a°**: 2diz
P5	Q15084	440	KDEL	**a°–a–b**	2	CGHC, CGHC	**a**: 2dml; **a′**: 1x5d
ERp57	P30101	505	QEDL	**a–b–b′–a′**	2	CGHC, CGHC	**a**: 2alb; **b–b′**: 2h8l; **a′**: 2dmm
PDI	P07237	508	KDEL	**a–b–b′–a′**	2	CGHC, CGHC	**a**: 1mek; **b**: 2bjx; **a′**: 1x5c; Pdi1p: 2b5e
PDIr	Q14554	519	KEEL	**b–a°–a–a′**	3	CSMC, CGHC, CPHC	
PDIp	Q13087	525	KEEL	**a–b–b′–a′**	2	CGHC, CTHC	
PDILT	Q8N807	584	KEEL	**a–b–b′–a′**	2	SKQS, SKKC	
ERp72	P13677	645	KEEL	**a°–a–b–b′–a′**	3	CGHC, CGHC, CGHC	**a**: 2dj1; **a′**: 2dj2; **a°**: 2dj3
ERdj5	Q8IXB1	793	KDEL	**J–a″–b–a°–a–a′**	4	CSHC, CPPC, CHPC, CGPC	
TMX	Q9H3N1	280	Unknown	**a**	1	CPAC	1x5e
TMX2[c]	Q9Y320	296	KKDK	**a**	1	SNDC	2dj0
TMX4	Q9H1E5	349	RQR	**a**	1	CPSC	
TMX3	FLJ20793	454	KKKD	**a–b–b′**	1	CGHC	

The lengths include the predicted signal sequence and the grey coloring of thioredoxin-like domains denotes those for which a three-dimensional structure has been solved. The assignment of domains is based on previously published data and our own bioinformatics analysis. J: J-domain, D: D-domain.

[a]PDB accession numbers are given only for structures of metazoan proteins with the exception of the Pdi1p protein from *S. cerevisiae*.

[b]The ER localization of Hag2 and Hag3 has been confirmed for myc-tagged variants of both proteins (Ruddock, L., personal communication).

[c]The names ERp28 and ERp29 are often used interchangeably in the literature, although the human protein was originally named ERp28 and the rat protein ERp29.

[d]ERp46 is also known as EndoPDI.

[e]The ER localization for TMX2 has not been experimentally confirmed. Moreover, it is unclear whether its thioredoxin-like domain faces the ER or the cytosol.

n.a. — not applicable.

domain at the C-terminus that – at least in the fruitfly ortholog – contains a binding site for its substrate [17,18].

Most human PDIs are soluble proteins with a C-terminal KDEL-like ER-localization motif. Although ERp44 is also found in the ER, it primarily localizes to the ER-Golgi intermediate compartment (ERGIC) and *cis*-Golgi despite its C-terminal RDEL sequence [19–21]. Four PDIs are transmembrane proteins of the TMX (for "thioredoxin-related transmembrane protein") subgroup. TMX2 and TMX3 have typical C-terminal KKXX ER-localization motifs. However, no localization study has been published for TMX2. In TMX4, two arginines of a cytosolic RQR sequence play a key role in retaining the protein in the ER (Roth, D. and Ellgaard, L., unpublished data). A similar RQR sequence is also present in TMX.

3. Catalytic properties

The redox activity of the PDI enzymes is governed by the CXXC active site. When in the oxidized state, the disulfide can be transferred to the substrate to catalyze its oxidation whereby the active site itself becomes reduced. When in the reduced state, substrate disulfides can be reduced and the active site ends up in the oxidized state. These thiol–disulfide exchange reactions proceed through the formation of a transient mixed disulfide between enzyme and substrate (Fig. 2). Since reduction requires the second cysteine to resolve the mixed disulfide with the substrate, mutation of this residue leads to the accumulation of covalent complexes through an intermolecular disulfide [22,23].

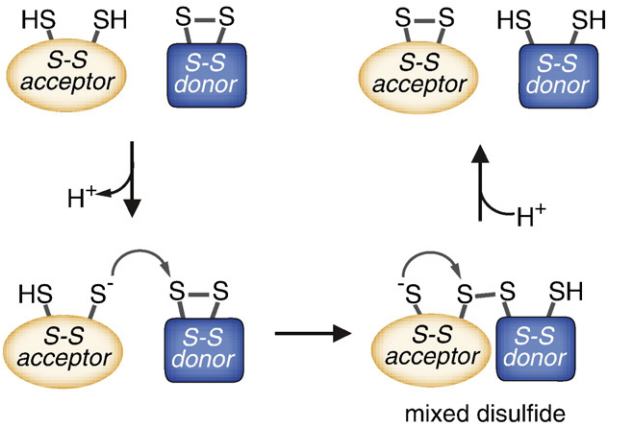

Fig. 2. Mechanism of thiol–disulfide exchange reactions. Thiol–disulfide exchange reactions between disulfide (S–S) donor and acceptor proteins occur via two cysteine residues in each protein (only depicted by the sulfur atom, S). In the first step, one cysteine in the disulfide acceptor is deprotonated to form a reactive thiolate anion, which in turn attacks the disulfide bond between the two cysteines in the disulfide donor. This leads to the formation of a transient mixed disulfide between the two proteins that is released by the attack of a thiolate anion derived from the second cysteine in the disulfide acceptor. The reaction is completed by the protonation of the resulting thiolate anion in the disulfide donor. Catalytic PDIs harboring a CXXC active-site sequence are specialized disulfide-carrier proteins that act both as disulfide-donor and -acceptor enzymes, i.e. catalyzing both cysteine oxidation and disulfide reduction in their substrates. Disulfide isomerization reactions catalyzed by PDI family members (not depicted) proceed through the same basic reaction chemistry as thiol–disulfide exchange reactions. Isomerizations involve at least one further cysteine in the substrate and typically end up with no net thiol–disulfide exchange between PDI family member and substrate [26].

The PDIs also catalyze the rearrangement of wrongly formed disulfides in the process called isomerization. Since non-native disulfides are often formed during folding and prevent the formation of the native structure [24], isomerization is a crucial reaction. Mechanistically, it can take place by rounds of reduction (of the non-native disulfide) and oxidation (to introduce the correct pairing of cysteines) [22,25]. Isomerization can also occur within the substrate molecule while bound to the enzyme through a mixed disulfide. When initiated from the reduced state of the enzyme the intramolecular isomerization pathway involves no thiol–disulfide exchange. In principle, the process can also be initiated from the oxidized state, in which case it can be mechanistically coupled to substrate oxidation [26].

The reactive species in thiol–disulfide exchange reactions is the thiolate anion (Fig. 2). In thiol–disulfide oxidoreductases, the N-terminal cysteine of the CXXC motif generally has a low pK_a-value that allows its deprotonation at physiological pH. The thermodynamic stability of the active-site disulfide, i.e. the redox potential, is obviously a determining factor for the redox activity performed by the enzyme. The redox potential is influenced by a variety of sequence characteristics, and the catalytic properties of single domains are modulated by these traits [10]. Still, all CXXC-containing PDIs tested so far have been able to catalyze thiol–disulfide exchange in vitro (see for instance [27–31]).

Single thioredoxin-like domains can catalyze oxidation and reduction [32–34]. For the more complex isomerization reaction, single domains (or mixtures of these) of PDI and ERp57 are poor catalysts and the entire molecules are required for full activity [32,33,35]. Moreover, PDI is a significantly better isomerase than ERp57 and P5 [30,33]. In addition to low-affinity binding sites in the **a** and **a′** domains [36], a binding site in the **b′** domain of PDI is required, but not sufficient, for efficient binding of larger peptides and misfolded proteins [37,38]. The preference of these three binding sites for hydrophobic sequences explains the broad substrate specificity for non-native proteins observed for PDI, and results in efficient and dynamic association with substrates [26]. By the affinity for partially unfolded polypeptide chains, PDI can either gain access to buried thiols or disulfides in substrate proteins and catalyze their oxidation, reduction or isomerization, or chaperone a folding intermediate by inhibiting aggregation [39–41]. The relation between substrate binding and catalytic activity is significantly less well characterized in other PDIs, with the exception of ERp57 (see Section 6.1.2).

4. Structure–function relations

The first structure solved at atomic resolution for a human PDI was that of the PDI **a** domain (PDI**a**) [42]. This structure shows a number of features characteristic for redox-active domains of the entire family and has clearly helped improve our understanding of how the PDIs function. The secondary structure elements are arranged in a β_1-α_1-β_2-α_2-β_3-α_3-β_4-β_5-α_4 fashion [43,44]. The residues of the active site are positioned at

the N-terminus of the second helix, with the first of the active-site cysteines partially surface exposed. Other key features include a hydrophobic patch found in the vicinity of, as well as a loop containing a proline in the *cis*-conformation positioned close to the active site, with both traits implicated in substrate binding (see for instance [42,45,46]).

Structures are now known for a substantial number of single redox-active domains from mammalian PDIs (Table 1). In particular, the RIKEN Structural Genomics Initiative has deposited several such (unpublished) NMR structures in the PDB database. Most structures solved are from closely related domains with the common CGHC active-site sequence. The proteins containing these domains behave similarly with respect to reaction kinetics, pH dependence and the nature of the rate-limiting step in an in vitro peptide oxidation assay [27]. Not surprisingly, examination of the structures reveals only very minor variation from the thioredoxin fold of PDI**a** and the general features known to be important for activity are conserved throughout. While these structures will be useful for molecular modeling purposes, we predict that only limited insight of general importance will be gained from solving additional three-dimensional structures of conventional redox-active thioredoxin-like domains in isolation.

By far most of the human PDIs are multi-domain proteins that contain thioredoxin-like domains of both the **a**- and **b**-types present in different arrangements (Table 1). The recent crystal structures of full-length Pdi1p [7] and ERp57**bb′** [47] have provided the first exciting information about the three-dimensional domain organization of these proteins. The structure of Pdi1p shows a U-shaped molecule with a base formed by the **b** and **b′** domains, and the arms by the two redox-active domains (Fig. 1B). Small angle X-ray scattering of full-length ERp57 showed that the overall shape of the two proteins is highly similar [47]. A continuous hydrophobic region characterizes the surface of the **bb′** fragment in Pdi1p and creates a platform for interaction with unfolded polypeptides. The surface of ERp57**bb′** contains a highly conserved positively charged patch that constitutes the binding site for the negatively charged P-domain of calnexin (CNX) and calreticulin (CRT), the two lectin chaperones that govern substrate binding of ERp57 to glycoproteins (see Section 6.1.2) [48–53]. The positively charged residues in ERp57**b′** that contact the P-domain are found in a position equivalent to the active site in redox-active **a**-type domains [47].

Overall, the **b**-type domains serve a number of functions within the multi-domain structures of PDIs. As observed for ERp57**b′**, a substrate-binding pocket in the fruitfly ortholog of ERp29 has been mapped to a position corresponding to that of the active site in **a**-type domains [54]. At least certain non-catalytic domains might therefore have evolved to bind either substrates or co-factors in a similar manner [10,11,55]. The **b**-type domains have an additional structural role in positioning the catalytic domains favorably for interaction with substrates or regulators such as the oxidase Ero1. Thus, work on Pdi1p shows that only within the context of the full-length protein are the **a** and **a′** domains recognized differentially by Ero1 with the **a′** domain being the preferred substrate [56]. Recent work shows that **b**-type domains also structurally stabilize neighboring **a**-type domains [57].

5. Redox regulation of the human PDIs

The tripeptide glutathione constitutes the primary cellular redox buffer. With a cysteine in the middle position it can exist in a reduced (GSH) and a dimeric oxidized (GSSG) form. Many enzymatic studies on PDIs have been performed in vitro using glutathione redox buffers made up to mimic the redox conditions of the ER lumen. These are relatively more oxidizing than in the cytosol [58], but the physiologically relevant value of the $[GSH]^2$:[GSSG] relation is not known. Furthermore, PDIs function in the context of the ER environment, and the conditions in the ER are vastly different from any test tube solution. For instance, the in vivo redox state of a given PDI is not only determined by its redox potential and the glutathione redox buffer in the ER, but is also modulated by the presence of oxidases, potential reductases and substrates, their availability and the kinetics of reaction.

A major pathway for the generation of disulfide bonds in the ER of human cells involves the FAD-binding oxidases Ero1α and Ero1β [59,60]. Work on the better characterized ortholog in *S. cerevisiae*, Ero1p, shows that it uses the oxidative equivalents from the conversion of molecular oxygen to hydrogen peroxide to specifically oxidize a subset of yeast PDIs in a thiol–disulfide exchange reaction [61–63]. It is reasonable to assume that the human Ero1 enzymes fulfill the same function since their overexpression significantly increases the fraction of oxidized PDI [64,65] (Appenzeller-Herzog, C. and Ellgaard, L., unpublished data). Therefore, the oxidized fractions of ~34% of PDI**a** and ~31% of PDI**a′** in endogenous PDI at steady-state [66] likely result in part from the activity of Ero1.

Given the high intracellular glutathione concentration [67] and the many in vitro studies showing efficient thiol–disulfide exchange between PDIs and glutathione (see for instance [32,68–71]), it is not surprising that the redox state of PDIs also depends on the $[GSH]^2$:[GSSG] relation in the ER. In particular, a reductive pathway has been uncovered, where GSH from the cytosol crosses the ER membrane by facilitated diffusion [72–75]. Consequently, the active sites of PDIs are reduced by GSH, a reaction that – while also competing with protein thiols in folding substrates – generates GSSG and hence contributes to the formation of the glutathione redox buffer in the ER (Fig. 3).

The in vivo redox state of ERp57 and other PDIs has been published to be in the reduced state [74]. More recently, it has been observed that TMX3 [28,66] and several other family members including ERp57 [76] (Appenzeller-Herzog, C., Roth, D. and Ellgaard, L., unpublished data) are partially oxidized in vivo. Although, as pointed out above, a redox equilibrium between PDIs and the glutathione redox buffer in the ER cannot be assumed, the moderate redox potentials measured for the PDIs investigated to date of around −160 mV are consistent with the occurrence of their active sites in both the reduced and oxidized state in the ER [28,33,68,77]. Concerning a possible influence of Ero1, co-immunoprecipitation of PDIs with Ero1α has failed to detect any homolog of PDI in complex with Ero1α,

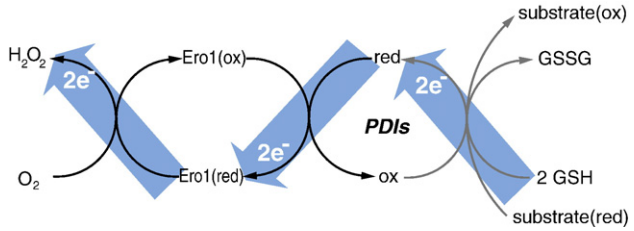

Fig. 3. Graphical overview of electron flow pathways to and from PDIs. The redox state of a PDI given by the ratio of oxidized (ox) and reduced (red) active sites is governed by opposing thiol–disulfide exchange reactions. While reduced PDIs can be oxidized by oxidized Ero1 that ultimately transfers the electrons ($2e^-$) onto molecular oxygen (O_2) (black arrows), oxidized PDIs can react with GSH or reduced substrates (grey arrows). The electron fluxes associated with the formation of GSSG and oxidized substrates (blue arrows) lead to the stoichiometric generation of hydrogen peroxide (H_2O_2). For simplicity, reduction of substrates by (reduced) PDIs is omitted from the scheme, and only the net flow of electrons from nascent polypeptide substrates onto PDIs is depicted.

while PDI itself was readily co-immunoprecipitated [64]. However, since mixed disulfides are very difficult to trap, this negative result does not exclude the possibility that family members other than PDI are also subject to Ero1-mediated oxidation. Indeed, indirect evidence points to ERp57 as a potential substrate for Ero1, since Ero1p oxidizes ERp57 in vitro [56], CXXS active-site mutants of ERp57 trap Ero1α in a mixed-disulfide complex [78], and overexpression of ERp57 in tissue-culture cells helps retain overexpressed Ero1α in the cell as is the case for PDI [79].

As indicated in Fig. 3, uncontrolled active-site oxidation by Ero1 followed by reduction via GSH would set up a futile cycle resulting in the generation of hydrogen peroxide and GSSG in the ER [80]. Therefore, in analogy to the situation in yeast [81], it is conceivable that under hyperoxidizing conditions a feedback mechanism negatively regulates Ero1 also in mammalian cells. Overall, Ero1 and GSH (or reduced substrates) play opposing roles in the control of the redox state of PDIs (Fig. 3), whereas the extent of Ero1-mediated oxidation of particular PDIs remains to be determined.

6. Discrete and redundant functions in protein maturation

The relative ease of recombinantly expressing the human PDIs and performing assays of thiol–disulfide exchange activity has promoted their in vitro characterization. In contrast, the cellular characterization is technically more challenging and not nearly as advanced. Functional information has been obtained by various co-immunoprecipitation tactics, secretion or folding studies under conditions of knockout or knockdown of single PDIs, and – more indirectly – by complementing the lethal *ΔPDI1* mutation in yeast with human PDI genes. Problems associated with the in vivo investigation of PDIs include the transient nature of the mixed-disulfide complexes with substrates that makes it hard to identify the latter and at least a certain redundancy among PDIs. Still, data on the oxidative folding of specific substrates by the PDIs are now emerging more rapidly as the field starts to address these aspects more directly (Section 6.1). In addition, we will cover the more specialized functions that certain PDIs fulfill in maturation and ER retention (Section 6.2).

6.1. Oxidative folding pathways

6.1.1. PDI

The importance of PDI in oxidative folding was originally illustrated by the finding that reconstitution of microsomes depleted of luminal proteins with purified PDI reverses their defect in co-translational disulfide-bond formation [82]. Still, in comparison to the wealth of information on the structure and enzymatic properties of PDI, we know very little about its involvement in specific protein maturation pathways in vivo. This reflects the special difficulties in investigating PDI. Since the protein is likely essential as is the case in yeast [3,83], knockout cell lines do not exist, while RNAi studies are complicated by the cytotoxic effects of PDI silencing on the one hand [84] and the high abundance and long half-life of the protein on the other [85,86]. Moreover, owing to its broad substrate specificity PDI is expected to bind so many proteins in the ER that a cross-linking/co-immunoprecipitation strategy to identify in vivo substrates is difficult to perform. Apart from the procollagens, where PDI plays well-studied specific roles during maturation (see Section 6.2.1), only few examples of bona fide PDI substrates are known. Nevertheless, a number of potential substrates have been identified by cross-linking followed by immunoprecipitation using substrate-specific antibodies (for examples see [87–91]), and transient disulfide-bonded complexes between PDI and Semliki Forest virus glycoprotein E1 representing co- and post-translational folding intermediates have been trapped [92]. Moreover, PDI participates in the oxidative folding of thyroglobulin [93] (see Section 6.1.4).

6.1.2. ERp57

The specificity of substrate binding by ERp57, the closest homolog of PDI, is one of the best-resolved issues in the functional characterization of the PDI family [94]. As a result of its interaction with the two related, ER lectin chaperones CNX and CRT [50–52,94](see also Section 4) ERp57 acts on glycosylated substrates [92,95]. Pharmacological interference with substrate binding by CNX and CRT (e.g. by castanospermine, an inhibitor of glycan trimming) also potently prevents the interaction of ERp57 with glycoproteins [92,95].

ERp57 is a multifunctional oxidoreductase that readily catalyzes dithiol oxidation, disulfide isomerization, and reduction in vitro [33,34,96]. Functional data suggest that it catalyzes all of these reactions in the ER as well. A potential role in oxidation comes from the finding that early oxidative folding of Major Histocompatibility Compex (MHC) class I heavy chain (HC) [97] and CD1d [98] is delayed upon inactivation of ERp57 by treatment with siRNA or castanospermine, respectively. More specifically, both studies reported impaired formation of only one of the two disulfides present in mature HC and CD1d, while the other disulfide was formed more rapidly and in an ERp57-independent way. In HC, the ERp57-dependent

disulfide was mapped to the α_3 immunoglobulin domain [97]. The same domain and the same disulfide are strictly conserved in CD1d pointing to a conserved specific engagement of ERp57 during oxidative folding of α_3 domains.

A role of ERp57 in thiol oxidation is also supported by its presence in mixed-disulfide intermediates at early time points of substrate folding. Very much like thyroglobulin [93] (Section 6.1.4), the Semliki Forest virus glycoprotein p62 is engaged co-translationally into mixed-disulfide complexes with ERp57 [92,99]. Likewise, cross-linking of in vitro-translated tyrosinase in semi-permeabilized melanocytes has revealed an early role of ERp57 (but not PDI) that was concomitant with disulfide-bond formation in the nascent polypeptide chain [100]. Taken together, it is fair to assume that ERp57 operates directly in substrate oxidation in vivo.

The in vivo function of ERp57 as a disulfide reductant has recently been highlighted by the preparative isolation of mixed-disulfide binding partners of overexpressed, tagged ERp57 [78]. Efficient co-immunoprecipitation was achieved by mutating the C-terminal cysteines of both active sites in ERp57. Active sites mutated in this manner can only trap substrates for reduction [26]. Still, 26 potential glycoprotein substrates were identified [78]. Due to the detection limits of the experiment, this does probably not cover the full spectrum of reduction substrates for ERp57. The finding that some proteins were also trapped in cells treated with castanospermine may reflect a non-physiological reactivity of the mutated ERp57 or point to a glycan-independent substrate recognition mode of ERp57. Interestingly, the trapped proteins showed a statistical over-representation of small, disulfide-rich domains with a low level of secondary structure that might be especially prone to formation of non-native disulfide bonds during folding. Two of the identified interactors, clusterin and β1 integrin, were further shown to require ERp57 for efficient oxidative folding with evidence found in both cases for a role of ERp57 in reducing incorrect disulfide bonds [78].

Posttranslational reshuffling of disulfides by ERp57 has been demonstrated for three viral proteins, although ERp57 was essential for oxidative folding of only one of these, influenza virus hemagglutinin [99]. Furthermore, the role of ERp57 in protecting cells against ER stress-induced apoptosis elicited by misfolded proteins [101,102] most probably reflects its activity to resolve non-native, deleterious disulfide bonds.

6.1.3. Other PDI family members

Data on the function of other PDI family members in oxidative folding are quite limited. ERp46 is highly expressed in endothelia and essential for the secretion of at least three endothelial cell survival factors during hypoxia as demonstrated by knockdown experiments [103]. Consistent with a specific function in protecting cells against hypoxic insult, ERp46 is up-regulated during hypoxia in cultured endothelial cells and primarily expressed in microareas of known or putative hypoxia in the body [103]. ERp72 has been detected in complex with various substrate proteins [89,91,104–108]. Although it comprises a **b′** domain that resembles the CNX/CRT-binding domain in ERp57 [10], the enzyme does not work in concert with these lectin chaperones in vivo [99], nor does it bind to model peptides in vitro [31]. Together with PDI, P5 and other ER folding factors it is part of a large complex that can be chemically cross-linked in living cells [109] and may represent an ER chaperone network. Notably, ERp72 often targets folding-impaired proteins, which results in ER retention of the bound substrates [104,106–108] (see also Section 7). The postmeiotic male germ cell-specific PDILT co-immunoprecipitates with its lectin partner calmegin, but the substrates of this novel, tissue-specific chaperone complex remain to be identified [13]. Overexpression of TMX protects cells from brefeldin A-induced cell death by an unknown mechanism that depends on an intact active site in the enzyme [110]. In addition to these examples, the expression of PDIp that binds to various secretory proteins during their translocation into the ER [111] is enriched in the pancreas [112,113] and under certain stress conditions in the brain [114], indicating tissue-specific functions of this enzyme. Finally, an interaction between ERp27 and the **b′** domain of ERp57 has been detected but its potential influence on substrate recognition and binding of CNX and CRT in vivo remains unknown [115].

6.1.4. The role of PDIs during biosynthesis and secretion of thyroglobulin

As mentioned above, certain substrates interact with more than one PDI. Thyroglobulin, the precursor protein for thyroid hormone synthesis, is a 660 kDa secretory glycoprotein with a slow maturation rate and a textbook example of the cooperation between different PDIs during folding of the same substrate. PDI and ERp57 engage in mixed disulfides with thyroglobulin simultaneously and co-translationally, and their release correlates with the dissociation of thyroglobulin from other ER chaperones. This indicates that PDI and ERp57 perform initial cysteine oxidation as well as isomerization of pre-existing disulfides. Interestingly, abrogation of the recruitment of ERp57 by castanospermine leads to inefficient and defective thyroglobulin folding that cannot be compensated by PDI although more of the disulfide-linked PDI adducts are trapped under these conditions [93]. This implies that certain glycosylated folding modules in thyroglobulin specifically require the assistance of ERp57 in cooperation with CNX or CRT. In addition to PDI and ERp57, chemical cross-linking has shown an interaction between ERp72 and thyroglobulin [105].

Efficient secretion of thyroglobulin depends on yet another member of the PDI family, the dimeric and non-catalytic ERp29 [116,117]. Rather than being involved in the folding process itself, ERp29 is thought to act as an escort protein [118] during ER exit of folded thyroglobulin. Studies on the *Drosophila* ortholog of ERp29 and a *Drosophila* cargo protein point to a similar mechanism of action [119,120].

6.2. Special cases of protein folding and ER retention

6.2.1. Procollagen folding, assembly and quality control

The fibrillar collagens are secreted as procollagen trimers that contain N- and C-terminal globular domains (N- and C-propeptides) in addition to the central triple helix-forming domain. For trimer formation, the C-propeptide domains of three procollagen chains fold individually, and then interact to initiate

the zipper-like winding up of the helical region. The assembly of procollagens in the ER is a complex process accompanied by a number of post-translational modifications [121], most prominently, the hydroxylation of proline residues.

PDI plays a vital role in many aspects of procollagen assembly. Firstly, it is thought to be involved in the catalysis of both intrachain and interchain disulfide bonds within and between the C-propeptide domains [122,123]. Secondly, as the non-catalytic β subunit of the enzyme prolyl 4-hydroxylase, PDI is essential for proline hydroxylation [124]. Thirdly, PDI binds to monomeric C-propeptides [125], an interaction that is not due to mixed disulfides but is governed by the peptide-binding properties of PDI [126]. While this association may be important for the recruitment of PDI to catalyze the formation of disulfide-linked C-propeptide trimers, it clearly results in ER retention of monomeric C-propeptides [126]. Thus, as a chaperone for the C-propeptide, PDI is likely to contribute to quality control retention of incompletely processed full-length procollagen. Likewise, the chaperone function of PDI also causes some degree of ER retention of overexpressed Ero1α [79].

6.2.2. Peptide loading onto MHC class I

MHC class I molecules present intracellularly derived antigenic peptides on the cell surface for immune surveillance by CD8-positive T cells. For the loading of these peptides in the ER, correctly folded and disulfide-bonded HC (see Section 6.1.2) associates with β_2-microglobulin (β_2M) to generate a heterodimer that is incorporated into the peptide-loading complex (PLC). This multisubunit assembly is composed of the HC/β_2M dimer, the transporter associated with antigen processing, the transmembrane protein tapasin, CRT and ERp57 [127]. The PLC is stabilized by multiple affinities between its components including the lectin interaction between CRT and the glycan in HC [128], the interaction between ERp57 and CRT, and a stable interchain disulfide bond between the **a** domain active site in ERp57 and cysteine 95 in tapasin [129]. ERp57 therefore plays a structural role in the PLC. More recently, also PDI has been reported as a component of the PLC [84].

A principal function of the PLC is to promote the selection of high-affinity peptide-MHC class I complexes. During this process, redox regulation of the disulfide bond in the α_2 domain of HC, which is in close proximity to the peptide-binding groove [130], is thought to play a key role [84,131–133]. Both PDI and ERp57 have been trapped in transient mixed disulfides with α_2 cysteines [84,131] and are required for the presentation of high-affinity peptides on the cell surface [84,134].

A role of ERp57 in reducing the α_2 disulfide bond has been demonstrated in vitro and in vivo [131,135]. In cells that lack functional tapasin, mixed-disulfide species between HC and ERp57 become more prominent, and more HC is found in the α_2-reduced form [131]. Because tapasin deficiency also leads to the release of HC from the PLC [131], the data suggest that in wild-type cells ERp57 reduces primarily HC that has transiently dissociated from the PLC. Similarly, only free, peptide-emptied HC is susceptible to reduction by recombinant ERp57 in vitro [135]. Therefore, ERp57 may function independently of its structural role in the PLC to specifically recognize and reduce the α_2 disulfide bond in suboptimally loaded, unstable MHC class I molecules after their release from the PLC.

PDI may play the opposing role to ERp57 and re-oxidize HC, since more α_2-reduced HC is observed upon shRNA-mediated depletion of PDI [84]. Moreover, depletion of peptides from the ER also leads to more α_2-reduced HC [84]. Because, in addition, an intact peptide-binding site in the **b′** domain of PDI is required for maintaining the oxidized state of HC and for the selection of high-affinity peptides [84], it is possible that PDI integrates the two processes of peptide loading and α_2 re-oxidation within the PLC.

What mechanism then could be involved in the re-recruitment of reduced HCs into the PLC? By means of a cell-free system, the disulfide-linked conjugate between ERp57 and tapasin has recently been shown to efficiently assemble all the components of the PLC [136]. The conjugate-mediated assembly of the PLC was also required for optimal peptide loading and selection. However, since the assembly assay was performed with whole cell lysates, the data do not exclude PDI from the process of peptide delivery.

6.2.3. ERp44

ERp44 has been isolated as a disulfide-linked interactor of Ero1α [137]. An unusual feature of ERp44 is the CXXS active-site sequence that contributes to stabilize mixed-disulfide complexes with Ero1α and other proteins [138]. Whereas PDI binds to the active site of Ero1α, the single active-site cysteine of ERp44 forms mixed disulfides with different exposed free cysteines in Ero1α [138]. The best recognized role of ERp44 in protein maturation is to act in thiol-mediated retention of nascent proteins and overexpressed Ero1α in the early secretory pathway [21,138]. Whether ER retention of endogenous Ero1α also depends on ERp44 has not yet been addressed. Furthermore, the new finding that ERp44 interacts with the transmembrane ER-exit receptor ERGIC-53 that promotes its steady-state localization to the ERGIC [19] suggests that rather than true retention in the ER, ERGIC-to-ER retrieval of ERp44-substrate complexes may be the primary mechanism of action. Thus, recognition by ERp44 of incompletely folded proteins may add to the list of quality control mechanisms in the ERGIC [139].

ERp44 is up-regulated during adipocyte [21] and B cell differentiation [19]. In both cases, differentiation coincides with secretion of a disulfide-linked multimeric protein — adiponectin and secretory immunoglobulin M (IgM). Prior to secretion, the controlled intracellular retention of both proteins by ERp44 is required for faithful assembly. In the case of IgM, ERp44 cooperates with ERGIC-53 [19], while the secretion of higher-order adiponectin complexes is regulated by the interplay between ERp44 and Ero1α [21]. Ero1α is a preferred interaction partner of ERp44 and can competitively release other proteins from ERp44 [21,138].

7. The function of PDIs in retrotranslocation to the cytosol

An important function of the ER is to recognize terminally misfolded proteins and target them for retrotranslocation to the cytosol where they are polyubiquitinated and degraded by the proteasome. This process is termed ER-associated degradation (ERAD). Initial evidence for a role of PDIs in ERAD came from

mutational studies of Pdi1p. Because a catalytically inactive mutant, but not peptide-binding site-deleted Pdi1p could fully support retrotranslocation of a cysteine-free model substrate [140], this function arises from the chaperone activity of Pdi1p. Similarly, the chaperone activity of human PDI is used for dislocation of BACE457 from the ER [141], and in the unfolding and dissociation of cholera toxin subunits before retrotranslocation [142]. However, based on in vitro experiments, the oxidoreductase function of PDI does not seem to be important in cholera toxin disassembly [142]. Thus, a glutathione buffer mimicking ER conditions is capable of reducing the disulfide bond between the A1 and A2 fragments of the toxin – a prerequisite for retrotranslocation of the A1 fragment – and PDI addition gives only a slight rate enhancement of the process [143]. Further experiments suggested that only reduced PDI is able to bind cholera toxin, while oxidized PDI adopts a more open conformation and shows no unfolding activity towards the toxin [142]. Although this mechanism does not generally portray a chaperone cycle of PDI [144], the finding that Ero1α mediates the release of the toxin A1 chain from PDI [145] supported the notion that PDI acts as a redox-driven chaperone in retrotranslocation of cholera toxin. Conversely, oxidized PDI has recently been found to be required and sufficient for efficient retrotranslocation of a model ERAD substrate devoid of cysteines [146]. In analogy to cholera toxin, however, the substrate was preferentially bound by reduced PDI.

While PDI-mediated unfolding makes cholera toxin A-chain competent for retrotranslocation, ERp72 stabilizes the compact fold of the toxin and antagonizes its dislocation to the cytosol [147]. These activities are not restricted to cholera toxin, since depletion of ERp72 increases the overall accumulation of polyubiquitinated proteins in the cell when the proteasome is inhibited, while depletion of PDI shows the opposite effect. Likewise, a variant of thyroglobulin that is an ERAD substrate was stabilized under conditions of PDI knockdown, but degraded faster in ERp72-depleted cells [147]. Another secretion-incompetent thyroglobulin mutant pathologically accumulates in the ER while being engaged in both disulfide-linked interactions with ERp72 and, predominantly, non-covalent interactions with PDI [106]. The misfolded mutant displays more cysteine thiols and shows enhanced binding to ERp72 when compared to wild-type thyroglobulin. Collectively, the data suggest that mixed-disulfide interactions with ERp72 may actively interfere with PDI-facilitated disposal of misfolded ER proteins.

Nonenveloped viruses exploit cellular pathways for entry into the host cell and transport to the ER lumen, where membrane penetration occurs to shuttle the viral DNA across the ER membrane [148]. The structural rearrangements in the disulfide-rich viral capsids that are a prerequisite for genome release in part take place in the ER where they are promoted by PDIs. In the case of mouse polyoma virus, ERp29 plays a key role by triggering a conformational change in the VP1 capsid protein that leads to increased hydrophobicity of the viral particle [149]. PDI is also required for infection and possibly acts downstream of ERp29 to reduce or isomerize an interchain disulfide ring that interconnects the subunits in the VP1 pentamer [150]. Consistently, since one cysteine of these interchain-forming disulfides is not conserved in the related Simian virus 40 (SV40), this virus shows a decreased requirement for PDI during infection [150]. Instead, the VP1 subunits in SV40 are extensively cross-linked by a different set of interchain disulfide bonds not found in polyoma virus. Very recently, ERp57-catalyzed rearrangement of interchain to intrachain disulfide bonds in VP1 of the SV40 envelope was shown to be required for infection [151]. Remarkably, ERp57 appeared to act on the virus in a CNX/CRT-independent way. Whether subsequent ER membrane penetration of nonenveloped viruses is directly performed by viral capsid proteins [149,152,153], involves the ERAD pathway [151,154] or both, is currently unclear.

8. Additional functions of PDIs in the ER

A number of functions of PDI in the ER that are not directly related to protein maturation or retrotranslocation are known. In analogy to its structural role in prolyl 4-hydroxylase, PDI forms the β subunit of the microsomal triacylglycerol transfer protein where it functions to keep the α subunit in a catalytically active, non-aggregated conformation [155,156]. PDI also binds to thyroid hormone and estradiol. The hormone-binding sites are distinct from the peptide-binding site in PDI and owing to their high abundance these complexes may function as cellular high-capacity, low-affinity hormone reservoirs [157].

Evidence exists that PDI, as a potential link to the thiol–disulfide system for oxidative protein folding, participates in other ER redox systems. The first involves vitamin K epoxide reductase (VKOR), a transmembrane protein of the ER membrane that catalyzes the regeneration of vitamin K1 from vitamin K1 2,3-epoxide. Vitamin K1 is an essential, redox co-factor for the enzymatic carboxylation of glutamic acid residues. The activity of VKOR is governed by a CIVC motif [158] predicted to be embedded in the last transmembrane helix of the enzyme [159]. Recently, it has been proposed that PDI functions as a reductant of the active site in VKOR [160]. At present though, it is unclear how PDI would gain access to the presumably membrane-buried active site in VKOR. Secondly, PDI can reduce dihydroascorbate (the oxidized, membrane-permeable form of vitamin C) in vitro [161], and it has been proposed that this activity plays a crucial role in the accumulation of ascorbate in the ER [162]. Thirdly, recent work revealed a regulatory role for PDI in NAD(P)H oxidase-catalyzed production of reactive oxygen species in vascular smooth muscle cells [163]. NAD(P)H oxidases are membrane proteins at least a subfraction of which resides in the ER with the active site exposed in the cytosol. Thus, redox-sensitive regulation of these enzymes by PDI via a luminally oriented binding site might contribute to the integration of the ER redox state with cytosolic redox signaling.

Another intriguing function of certain PDIs is related to homeostasis of the ER luminal calcium ion concentration ($[Ca^{2+}]_{ER}$). Ca^{2+} pumps and channels in the ER membrane are responsible for regulating $[Ca^{2+}]_{ER}$. In most tissues these functions are carried out by sarco(endo)plasmic reticulum Ca^{2+} ATPase isoform 2b (SERCA2b) and by inositol 1,4,5-trisphosphate receptor type I (IP_3R1). Both proteins are subject

to regulation by a PDI family member interacting with an ER luminal domain [164,165].

Ectopic expression of ERp57 in *Xenopus* oocytes potently inhibits SERCA2b pump activity [165]. The interaction of ERp57 with the pump is mediated by CRT binding to the C-terminus of SERCA2b, which likely occurs in a $[Ca^{2+}]_{ER}$-dependent fashion. So, when $[Ca^{2+}]_{ER}$ is high, active Ca^{2+} import is stalled by the recruitment of CRT – which itself binds Ca^{2+} – and ERp57 to SERCA2b thus providing a direct feedback loop (Fig. 4). Conversely, the regulation of IP_3R1-mediated Ca^{2+} leakage out of the ER is achieved by ERp44 that physically blocks the release channel only at low $[Ca^{2+}]_{ER}$ (Fig. 4)[164]. Notably, ERp44 does not bind IP_3R1 through its active site, but via the C-terminal region.

The luminal loops of both SERCA2b (L4) and of IP_3R1 (L3V) that interact with ERp57 and ERp44, respectively, comprise a conserved pair of cysteines (Fig. 4). It is plausible that ER calcium homeostasis is controlled by a regulatory redox switch mechanism involving these two cysteine pairs, although

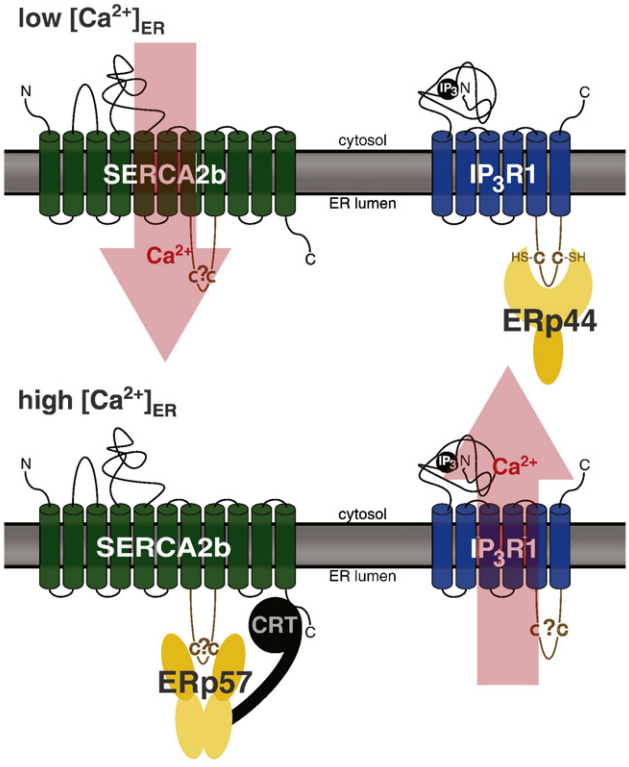

Fig. 4. The roles of ERp57 and ERp44 in the regulation of $[Ca^{2+}]_{ER}$. Ca^{2+} homeostasis in the ER is modulated by the interplay of the two multipass ER transmembrane proteins SERCA2b and IP_3R1 that pump Ca^{2+} from the cytosol into the ER lumen and release Ca^{2+} from the ER lumen into the cytosol upon stimulation with inositol 1,4,5-trisphosphate (IP_3), respectively (red arrows). When $[Ca^{2+}]_{ER}$ is low, ERp44 associates with a luminal loop in IP_3R1, which requires two loop cysteines in the thiol state (C-SH), and inhibits Ca^{2+} efflux [164]. Under conditions of high $[Ca^{2+}]_{ER}$, the pump activity of SERCA2b is down-regulated by the binding of ERp57 to a luminal loop in SERCA2b. The down-regulation requires the active sites in ERp57 (not depicted) as well as the presence of CRT that recruits ERp57 to SERCA2b [165]. The two conserved cysteine pairs in the SERCA2b and IP_3R1 luminal loops (depicted in brown) potentially constitute redox switches, but their in vivo redox state is currently not known (question marks, see text for further details).

their in vivo redox state is currently unknown. Cysteine-replacement mutants in the L4 loop of SERCA2b are more active, presumably due to their inability to form a disulfide or interact with ERp57 [165]. Although the binding of ERp57 to SERCA2b depends on its active sites [165], it remains to be established whether ERp57 is directly involved in oxidizing the L4 cysteines or depends on a preformed disulfide bridge for interaction. On the other hand, it is clear that both the binding of ERp44 to IP_3R1 and the inhibition of the channel by co-expressed ERp44 depend on the two L3V cysteines being in the reduced state [164]. However, a fraction of L3V likely exists in the disulfide state, because treatment of cells with a reducing agent results in increased cross-linking of IP_3R1 to ERp44 [164]. It is therefore conceivable that regulated disulfide-bond reduction in SERCA2b and IP_3R1 leads to an increase in $[Ca^{2+}]_{ER}$. Since such redox regulation requires catalysis, it is attractive to speculate that other PDI family members are involved in the process.

9. Conclusions and perspectives

For decades, PDI itself was the only recognized family member and in the first description of the PDI family in 1994 four members were known [166]. In this perspective, the recent expansion of the family is remarkable. It would be surprising though, if the thousands of proteins made in the ER did not have specific needs in terms of folding and maturation, and if these needs could all be fulfilled by a single or a few PDIs. It is therefore to be expected that individual PDIs have different (types of) substrates, and that they perform distinct tasks when cooperating during the folding of proteins like thyroglobulin. The identification of new endogenous substrates will be paralleled by the challenge of investigating the specificity of PDIs for these substrates in vitro. At the same time, a certain redundancy with respect to substrate recognition has been observed in vivo. For instance, when preventing substrate interaction by ERp57, other PDIs have been found to associate with the particular substrates investigated [99,167]. In addition, inactivation of ERp57 usually only retards but does not seem to block the maturation of its substrates.

To gain a better understanding of the multi-domain structures found in most PDIs and the function of the different domains in their natural context, structure determination of more PDIs is needed. The similarity in domain orientation observed for Pdi1p and ERp57**bb′** with calsequestrin – a calcium storage protein that contains three thioredoxin-like domains only very distantly related to the PDIs at the sequence level [168] – is intriguing, and it will be interesting to see if this is a general feature of the PDIs. Crystallographic studies should be combined with NMR spectroscopic investigation of interdomain dynamics in solution. In general, given the similarity between many redox-active thioredoxin-like domains, the characterization of non-catalytic domains could well hold the key to understanding the differences between the human PDIs.

The cellular redox regulation of PDIs – and of the ER environment in general – is an exciting but complex topic with a number of unresolved questions. One deals with the ability of

different PDIs to serve as substrates for the Ero1 proteins, and another whether additional pathways for oxidation exist. Recently, it has been proposed that oxidases of the QSOX family [169], that quickly and efficiently introduce disulfides into reduced unfolded proteins in vitro [170,171], could also do so in the ER [80]. Here, the function of the PDIs would mainly be restricted to isomerize non-native disulfides introduced by the QSOX proteins. While the idea of such a pathway in the ER seems appealing, further cell biological studies are clearly needed to establish a potential function of the QSOX proteins in the ER, especially since these proteins reside further along the secretory pathway [172]. For instance, the trapping of mixed disulfides between QSOX and folding intermediates in vivo could be used to demonstrate QSOX-mediated thiol oxidation in the ER. Another open question relates to how the ER deals with the H_2O_2 generated by the Ero1 and potentially the QSOX pathway. One possibility would be to employ glutathione or thioredoxin peroxidases, but to date no such ER enzymes have been reported.

Does the ER harbor dedicated reductases? Among the PDIs, ERdj5 is the strongest candidate since it contains several active sites related to the CGPC motif in thioredoxin that are probably more reducing than the regular CGHC motif [173]. In analogy to the thioredoxin reductase/thioredoxin system in the cytosol, the potential function of ERdj5 in reducing disulfides could well depend on an as yet unidentified reductase to maintain a reduced fraction of ERdj5. Two other candidate reductases are the selenoproteins Sep15 and SelM. Both have thioredoxin folds and harbor an active-site selenocysteine in place of one cysteine, a feature that renders them more reducing than PDIs [174]. The determination of the in vivo redox characteristics and regulation of Sep15, SelM and ERdj5 are interesting topics for future work.

In conclusion, the PDIs perform an impressive array of cellular functions that we are just starting to take in. Even the well-understood thiol–disulfide exchange reactions are not always straightforward to grasp in the context of the cellular environment where they are regulated by various pathways. For instance, although PDI is the best-known substrate of Ero1 it is clear that its function is not restricted to oxidation. Underlying the versatility of the PDIs is virtually one single domain type, the thioredoxin fold, which has evolved to accommodate the variety of functions displayed by the PDIs.

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