Identification of the physiological substrates of PDIp, a pancreas-specific protein-disulfide isomerase family member

About 20 members of the protein-disulfide isomerase (PDI) family are present in the endoplasmic reticulum of mammalian cells. They are thought to catalyze thiol-disulfide exchange reactions within secretory or membrane proteins to assist in their folding or to regulate their functions. PDIp is a PDI family member highly expressed in the pancreas and known to bind estrogen in vivo and in vitro. However, the physiological functions of PDIp remained unclear. In this study, we set out to identify its physiological substrates. By combining acid quenching and thiol alkylation, we stabilized and purified the complexes formed between endogenous PDIp and its target proteins from the mouse pancreas. MS analysis of these complexes helped identify the disulfide-linked PDIp targets in vivo, revealing that PDIp interacts directly with a number of pancreatic digestive enzymes. Interestingly, when pancreatic elastase, one of the identified proteins, was expressed alone in cultured cells, its proenzyme formed disulfide-linked aggregates within cells. However, when pancreatic elastase was co-expressed with PDIp, the latter prevented the formation of these aggregates and enhanced the production and secretion of proelastase in a form that could be converted to an active enzyme upon trypsin treatment. These findings indicate that the main targets of PDIp are digestive enzymes and that PDIp plays an important role in the biosynthesis of a digestive enzyme by assisting with the proper folding of the proenzyme within cells.

Disulfide bonds, formed by oxidation of two cysteines, are important structural features of a great number of secretory and membrane proteins (1). In eukaryotes, formation of disulfide bonds in these proteins is mostly catalyzed by enzymes belonging to the protein-disulfide isomerase (PDI) family. The members of this protein family are characterized by the presence of one or more thioredoxin-like domains and their localization to the ER. The thioredoxin-like domain often, but not necessarily, has a CXXC active-site motif (cysteines separated by two amino acids) (2).

The two cysteines in the active-site motifs of PDI family members are mostly redox-active. They can exist in an oxidized (disulfide-bonded) or reduced (free) form. Most PDI family members use their redox-active cysteines to introduce, reduce, or isomerize disulfide bonds, leading to the formation of disulfide bonds between correct pairs of cysteine residues (2, 3). The latter two reactions, in particular, are thought to be required for correcting disulfide bonds formed between incorrect pairs of cysteines (4, 5). Importantly, the functions of PDI family members are not limited to the catalysis of oxidative folding. For example, ERdj5 can promote the ER-associated degradation of misfolded proteins that arise in the ER by cleaving disulfide bonds in the proteins, which is thought to be required for the efficient retrotranslocation of the proteins from the lumen of the ER and their subsequent degradation by proteasome in the cytosol (6, 7). Recent results suggest that some PDI family members participate in the regulation of ER stress response or Ca^{2+} homeostasis by modulating the redox status of ER stress sensors or Ca^{2+} pumps, leading to their activation or inactivation (8–10). More recently, PDI has been reported to contribute to the regulation of coagulation that takes place outside of cells (11).

In the ER of mammalian cells, there exist ~20 PDI family members (2, 12). They are supposed to play some roles within or outside of cells by modulating biological processes often through their redox activities. However, the physiological functions of some members still remain largely unknown (2, 12). One way to reveal their functions is to find their physiological substrates (4, 13–16).

PDIp was discovered as a PDI family member that is specifically expressed in the pancreas (17). Later studies revealed that

2 The abbreviations used are: PDI, protein-disulfide isomerase; ER, endoplasmic reticulum; NEM, N-ethylmaleimide; Suc-Ala-Pro-Ala-MCA, succinyl-l-Ala-l-Pro-l-Ala-7-methylcoumaryl-4-amide; DMEM, Dulbecco’s modified Eagle’s medium; IP, immunoprecipitation; FBS, fetal bovine serum.
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![Diagram](image)

**Figure 1. Strategy to identify the substrates of PDIp.** A, domain organization of PDIp. The CXXC active-site motifs are present in domains a and a', but not in domains b and b'. KEEL, ER retention signal; N, N-glycosylation site. B, thiol–disulfide exchange reactions catalyzed by PDI family members (PDIs). Note that each reaction is mediated by the attack on a disulfide by a thiolate anion (not shown). Thus, a thiol group of the attacking cysteine must be deprotonated for the reactions to take place. Therefore, strong acid quenches the reactions, resulting in the transient stabilization of the disulfide-linked complexes. C, stabilization of the disulfide-linked intermediates that arise in a mouse tissue.

PDIp possesses four thioredoxin domains, two of which have a CXXC active-site motif (Fig. 1A) (19, 20). Thiol–disulfide exchange reactions catalyzed by PDI family members proceed through a disulfide-linked intermediate formed between the enzyme and its substrate (Fig. 1B). Stabilization and purification of the intermediate complexes that arise in mammalian tissues and subsequent MS analysis of the proteins contained in the complexes may lead to the identification of the in vivo substrates of the enzyme.

To stabilize the disulfide-linked complexes between PDIp and its putative substrates that form in vivo, we disrupted mouse tissues in the presence of 10% TCA using a homogenizer immediately after excising the tissues from a mouse (Fig. 1C). We used this method because protonation of thiols of free cysteines by strong acid rapidly quenches thiol–disulfide exchange reactions in tissue samples (15). This acid-quenching step was followed by alkylation of free cysteines with N-ethylmaleimide (NEM), leading to the stabilization of the complexes.

**Disulfide-linked complexes containing PDIp are abundant in digestive tissues**

To detect PDIp and disulfide-linked complexes containing this enzyme, the NEM-treated tissue lysates were separated by SDS-PAGE under nonreducing conditions and subjected to immunoblotting with an antibody to PDIp (Fig. 2A). A band corresponding to the monomeric form of PDIp was observed at ~65 kDa. In addition to this band, a large number of bands of different apparent molecular masses were detected in the stomach and pancreas (Fig. 2A, lanes 11 and 12, vertical lines). We suggest that the latter bands represent disulfide-linked complexes containing PDIp for three reasons. First, they ran slower than the monomeric form of this protein. Second, when the samples were treated with the sulfhydryl-reducing reagent, DTT, these bands were reduced (Fig. 2B). Finally, when the NEM-treated pancreatic lysate was separated by two-dimensional electrophoresis in which the first dimension was nonreducing and the second was reducing, we detected several spots at ~65 kDa in the second dimension using the anti-PDIp antibody (Fig. 2C). Because PDIp in the spots indicated by a horizontal line migrated slower than monomeric PDIp in the first dimension, PDIp in these spots likely originated from disulfide-linked complexes containing PDIp (Fig. 2C).

Interestingly, the monomeric form of PDIp and/or complexes containing PDIp were observed in tissue samples prepared from digestive organs including the pancreas, stomach, and small intestine (Fig. 2A, lanes 11–13). Among the tissues, PDIp was most abundant in the pancreas (Fig. 2), in agreement with former publications (17, 18). Detection of disulfide-linked complexes of various sizes containing this protein indicates that PDIp interacts with a variety of proteins via a disulfide bond (Fig. 2A, lanes 11 and 12).

**Purification and identification of disulfide-linked partners of PDIp from pancreas**

To identify the physiological substrates of PDIp, we purified PDIp-containing complexes from the pancreas, because the complexes were most abundant in this tissue. For this purpose, the NEM-treated lysate of pancreas was subjected to immunoprecipitation with the antibody to PDIp (Fig. 3A). After washing the immune complexes, the monomeric form of PDIp and the complexes containing PDIp were released from the immune complexes by incubating them in Laemmli sample buffer. As a control, immunoprecipitation was also performed using IgG from normal mouse serum. The released proteins were then separated by nonreducing SDS-PAGE and detected by silver staining (Fig. 3B). The monomeric form of PDIp was purified by the antibody to PDIp but not by the control IgG, confirming that the control IgG does not pull down PDIp (Fig. 3B). Some of
the bands in the area indicated by a vertical line in lane 2 (marked by arrowheads) were specific to the immunoprecipitate obtained with the antibody to PDIp and ran slower than the monomeric form of PDIp. Thus, they may represent the disulfide-linked complexes containing PDIp. In contrast, a few bands were specific to the sample purified with the antibody to PDIp but ran faster than the monomeric form of PDIp (Fig. 3B, lane 2), likely representing proteins that interacted with PDIp noncovalently. To identify the disulfide-linked partners of PDIp, we cut off the area of the gel lane (Fig. 3B, lane 2, vertical line), performed in-gel digestion with trypsin, and identified the digested polypeptides by MS. To specify the potential binding partners of PDIp, the same analysis was performed using normal IgG as a negative control, and any proteins thus identified were excluded from the list of potential partners of PDIp (Table 1).

Characteristics of the proteins identified by mass spectrometry analysis

The identified proteins are those that reside in the ER or those on the secretory pathway. In addition, each of them contains at least one cysteine. Thus, they can be the substrates of PDIp or the regulator of the function of PDIp. The identified proteins can be categorized into four groups: PDIp family members, PDIp oxidases, digestive enzymes and their regulators, and coagulation factors. Among them, PDIp family members and PDIp oxidases were excluded from further analysis, as they can be functional regulators or collaborators of PDIp rather than the substrates of this enzyme (see “Discussion”).

Coagulation factors including fibrinogens (Table 1), produced mainly in the liver, were also excluded from further analysis because PDIp may have interacted with them artificially during the tissue sample preparation because of their high abundance in the blood (22) and the high reactivity of their cysteine to PDIp (16). In this connection, we previously detected...
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Table 1

| Group and name     | Description                                      | Accession | Coverage (%) | Disulfides (cysteines) | Location |
|--------------------|--------------------------------------------------|-----------|--------------|------------------------|----------|
| **PDIp family members** |                                                 |           |              |                        |          |
| PDHB               | Protein-disulfide isomerase (PDI)                | NP_035162 | 78           | 2                      | ER       |
| PDI6               | Protein-disulfide isomerase A6 (P5)              | NP_082235 | 32.4         | 2                      | ER       |
| PDI5               | Protein-disulfide isomerase A5 (PDIR)            | NP_082571 | 19.4         | 4                      | ER       |
| PDI3               | Protein-disulfide isomerase A3 (ERp57)           | NP_031978 | 17.1         | 4                      | ER       |
| TXNDC5             | Thioredoxin domain–containing protein5 (ERp46)    | NP_063342 | 11.8         | 3                      | ER       |
| **PDIp oxidases**  |                                                 |           |              |                        |          |
| PRDX4              | Peroxiredoxin-4                                 | NP_058044 | 44.9         | 2                      | ER       |
| ERO1B              | ERO1-like protein β                              | NP_080460 | 35.5         | 6                      | ER       |
| **Digestive enzymes and their regulators** |                                                 |           |              |                        |          |
| AMY2A              | Pancreatic α-amylase                             | NP_001036176 | 72.4       | 5                      | Secreted |
| CEL2A2             | Chymotrypsin-like elastase family member 2A (elastase-2A) | NP_031945 | 59.8         | 4                      | Secreted |
| CTRL               | Chymopasin                                       | NP_075671 | 47           | 5                      | Secreted |
| CLPS               | Colipase                                         | NP_079745 | 41.6         | 5                      | Secreted |
| CTRL1              | Chymotrypsinogen B                               | NP_079859 | 38           | 5                      | Secreted |
| TRY7               | Trypsinogen 7                                    | NP_075822 | 29.6         | (12)                   | Secreted |
| CELA1              | Chymotrypsin-like elastase family member 1 (elastase-1) | NP_291090 | 28.6         | 4                      | Secreted |
| SYCN               | Syncollin (regulation of exocytosis)             | NP_080992 | 26.9         | (8)                    | Secretory vesicle |
| PNLIP               | Pancreatic triacylglycerol lipase                | NP_081201 | 24.3         | 6                      | Secreted |
| CELA3B             | Chymotrypsin-like elastase family member 3B (elastase-3B) | NP_080695 | 21.2         | 4                      | Secreted |
| PL2A1G1B           | Phospholipase A2                                 | NP_035237 | 19.9         | 7                      | Secreted |
| TRY4               | Trypsin-4                                        | NP_035776 | 19.1         | 6                      | Secreted |
| PRSS2              | Anionic trypsin-2                                | NP_033456 | 18.6         | 6                      | Secreted |
| CPB1               | Carboxypeptidase B                               | NP_083982 | 18.6         | 3                      | Secreted |
| PNLIPPR1           | Inactive pancreatic lipase-related protein 1     | NP_061362 | 14.4         | 6                      | Secreted |
| PRSS1              | Anionic trypsin-1                                | NP_444473 | 12.2         | 6                      | Secreted |
| **Coagulation factors** |                                                 |           |              |                        |          |
| FG5G               | Fibrinogen γ chain                               | NP_598623 | 26.8         | 8                      | Secreted |
| FGA                | Fibrinogen-α chain                               | NP_001104518 | 21.7     | 8                      | Secreted |
| FGB                | Fibrinogen-β chain                               | NP_862897 | 21.6         | 5                      | Secreted |
| F13A1              | Coagulation factor XIII A chain                  | NP_001159863 | 10.8       | (9)                    | Secreted |

a The value of percent coverage.

b The number of disulfides that are known to or deduced to form. Where not known, the number of total cysteine residues in the protein is given in parentheses.

the product of artificial interaction between DsbA, a bacterial periplasmic disulfide bond–introducing enzyme, and EF-Tu, an abundant cytoplasmic protein with a highly reactive cysteine (23). Whether or not the observed interactions between PDIp and coagulation factors represent artificial ones remains unclear and requires detailed study.

**PDIp is expressed in pancreatic exocrine acinar cells but not in endocrine islet cells**

There are two types of secretory cells in the pancreas: exocrine acinar cells that produce digestive enzymes, and endocrine islet cells that produce hormones such as insulin and glucagon. Our MS analysis detected a variety of digestive enzymes but not hormones (Table 1), which implies that pancreatic hormones may not be the substrates of PDIp. Notably, former immunofluorescence analysis suggested that PDIp is expressed in the acinar cells of the pancreas (18, 24). To further study the localization of PDIp in the pancreas by immunoblotting, we separated pancreatic acinar and islet cells from the mouse pancreas and analyzed the distribution of PDIp using the antibody to PDIp. PDIp was clearly detected in the acinar cells (Fig. 3C, lane 1), but not in the islet cells (lane 2). Taken together, these results unambiguously show that PDIp is specifically expressed in the exocrine cells in the pancreas. This finding explains well our failure to detect pancreatic hormones in our MS analysis.

**PDIp interacts with α-amylase or proenzymes of pancreatic proteases via an intermolecular disulfide bond(s)**

To further test the interaction between PDIp and the identified proteins, we immunoprecipitated PDIp with the antibody to PDIp from the NEM-treated pancreatic lysate. The immunoprecipitates were then subjected to immunoblotting with an antibody to pancreatic α-amylase. Separation of the immunoprecipitates under nonreducing conditions detected several bands (Fig. 4A, lane 3, closed arrowheads). For the following reasons, these bands likely represent disulfide-linked complexes formed between PDIp and α-amylase. First, these bands were specifically immunoprecipitated by the anti-PDIp antibody and detected by the anti-α-amylase antibody (Fig. 4A, lanes 2 and 3). Second, when the samples were reduced with DTT before electrophoresis, these bands disappeared, giving rise to a band corresponding to the reduced form of α-amylase (Fig. 4A, lanes 3 and 6). Thus, PDIp interacted with α-amylase via an intermolecular disulfide bond(s). Among the disulfide-linked complexes, the smallest one (a faint band that ran at around 130 kDa) likely represents a heterodimer between PDIp and α-amylase because the apparent molecular masses of the complex correlate well with the sum of the molecular masses of these two proteins: 55 kDa for α-amylase and 65 kDa for PDIp. The other bands likely represent even higher-order complexes containing more than two proteins because they run slower than the presumed heterodimer. In a similar manner, we obtained evidence for disulfide-mediated interaction between
PDIp and chymotrypsinogen B (Fig. 4B) or trypsinogens (Fig. 4C). Thus, PDIp interacts with α-amylase or proenzymes of pancreatic proteases via an intermolecular disulfide bond(s).

We also studied interaction between PDIp and proelastase, a proenzyme of elastase (25). Because of the low reactivity and high background of our anti-elastase antibody (Fig. S1C), we failed to identify the products of interaction between proelastase and PDIp in the NEM-treated pancreatic tissue lysate.

Thus, to study the interaction between PDIp and proelastase, we inserted a c-Myc sequence in front of the ER retention signal of PDIp and fused a triple FLAG sequence (FLAG3) to the C terminus of proelastase (encoded by CELA2A), and expressed them in HeLa cells. Then the cellular proteins were treated with TCA, alkylated with NEM, immunoprecipitated with an antibody to c-Myc, and detected with an antibody to FLAG (Fig. 4D, lane 8). When the immunoprecipitates were separated under nonreducing conditions, note that the monomeric forms of the partner proteins run slightly faster under nonreducing conditions (lane 4) than they do under reducing conditions (lane 1). Note also that the antitrypsin antibody recognizes several pancreatic trypsin isoforms. Thus, we often detected two isoforms of trypsinogen in the samples from the pancreas (lanes 4 and 6). The closed arrowheads indicate disulfide-linked complexes formed between PDIp and its identified partners. The oxidized monomeric forms of the identified partner proteins are indicated by the open arrowheads, and their reduced forms are shown to the right of each panel. Note that the monomeric forms of the partner proteins run slightly faster under nonreducing conditions (lane 4) than they do under reducing conditions (lane 1). Note also that the antitrypsin antibody recognizes several pancreatic trypsin isoforms. Thus, we often detected two isoforms of trypsinogen in the samples from the pancreas (C, lanes 4 and 6). D–G, detection of interaction of PDIp with proelastase expressed in HeLa cells. D and E, HeLa cells were transfected with pTF006 and/or pON103 to express, as indicated, proelastase-FLAG3 and/or PDIp-c-Myc and grown in DMEM supplemented with 10% FBS for 24 h. The cells were collected, alkylated with NEM, and subjected to immunoprecipitation with an antibody to c-Myc to purify complexes containing PDIp-c-Myc. The immunoprecipitates were separated by SDS-PAGE, and proelastase-FLAG3 contained in the immunoprecipitates was detected with horseradish peroxidase–conjugated antibody to FLAG. Lanes 1–4 contained 0.03 μg of NEM-treated cell lysate (Input). Lanes 5–8 contained immunoprecipitates obtained from 60 μg of NEM-treated cell lysate. In E, proteins were reduced with 100 mM DTT before electrophoresis. The position of the monomer form of proelastase-FLAG3 is indicated on the right. Also indicated on the right in D are the positions of complexes containing both PDIp-c-Myc and proelastase-FLAG3 (closed arrowheads). A–E, the asterisk marks nonspecific bands. F and G, same as D and E except that the NEM-alkylated cell lysate was first subjected to immunoprecipitation with the antibody to FLAG and then to immunoblotting with the antibody to c-Myc.
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nonreducing conditions, the anti-FLAG antibody specifically detected two bands at the position of monomeric proelastase-FLAG3 (≈30 kDa) (Fig. 4D, lane 8), indicating that PDIp-c-Myc can interact with proelastase-FLAG3 noncovalently.

Interestingly, among the two monomeric proelastase-FLAG3 bands observed at around 30 kDa (Fig. 4, D and E, lanes 3 and 4), the relative intensity of the lower band increased upon reduction of samples with DTT before electrophoresis (Fig. 4, compare lanes 3 and 4 in D with lanes 3 and 4 in E). This finding implies that a portion of the monomeric proelastase with the greater electrophoretic mobility observed under reducing conditions existed as disulfide-linked complexes under nonreducing conditions. The identities of these two bands will be discussed later.

Importantly, the same anti-FLAG antibody detected bands with apparent molecular masses greater than the monomeric form of proelastase (Fig. 4D, lane 8, closed arrowheads). We suggest that they represent disulfide-linked complexes involving both proelastase-FLAG3 and PDIp-c-Myc for the following reasons. First, these high-molecular mass bands (Fig. 4D, lane 8, arrowheads) were specifically immunoprecipitated by the anti-c-Myc antibody and detected by the anti-FLAG antibody only when HeLa cells were expressing both PDIp-c-Myc and proelastase-FLAG3 (Fig. 4D, compare lane 8 with lane 7). Second, these high-molecular mass bands disappeared when the sample was treated with the reduc tant DTT before electrophoresis (Fig. 4, compare lane 8 in D with lane 8 in E), suggesting that they are disulfide-linked complexes. Finally, these high-molecular mass bands were also detected by the “anti-c-Myc” antibody when the same cell lysate was immunoprecipitated with the anti-FLAG antibody (Fig. 4F, lane 8). Among them, a very faint band at ≈100 kDa likely represents a disulfide-linked heterodimer formed between PDIp-c-Myc and proelastase as the apparent molecular mass of the protein band correlates well with the sum of the apparent molecular masses of PDIp-c-Myc (65 kDa) and proelastase-FLAG3 (30 kDa).

Note that we also detected the monomeric form of PDIp-c-Myc when the NEM-alkylated lysate of cells expressing both proelastase-FLAG3 and PDIp-c-Myc was immunoprecipitated by the anti-FLAG antibody (Fig. 4F, lane 8), confirming that PDIp-c-Myc can interact with proelastase-FLAG3 noncovalently. Thus, PDIp can interact with proelastase both noncovalently and covalently in HeLa cells. These results indicate that PDIp can interact with α-amylase, chymotrypsinogen B, trypsinogens, and proelastase via an intermolecular disulfide bond(s) within cells.

Role of PDIp in the production of proelastase

Among the interactors of PDIp, we decided to focus on proelastase in the following studies for two reasons. First, sensitive fluorogenic substrates are available for the measurement of elastase activity (30). Second, although cellular proteins were denatured with TCA and alkylated with NEM in the presence of SDS before immunoprecipitation experiments, we observed both covalent and noncovalent interactions between PDIp and proelastase in the immunoprecipitation experiments (see above), suggesting tight functional association between the two proteins.

We first explored the role of PDIp in the biosynthesis of proelastase in cells. For this purpose, we looked at the effect of co-expression of PDIp on the production of proelastase in HeLa cells. This is because all of the tested pancreatic cancer cell lines have lost the ability to produce PDIp for unknown reasons (18), making it impractical to study the role of PDIp in the biosynthesis of proelastase using these cell lines and knockdown experiments. Consistently, we failed to detect the expression of PDIp and pancreatic proteases including elastase in AR42J, a widely used pancreatic acinar cell line (Fig. S1, A, C, D, and E). In contrast, we detected α-amylase in AR42J (Fig. S1B) in agreement with the properties of this cell line (26, 27).

Notably, when we expressed proelastase-FLAG3 in HeLa cells, we observed two bands at the position of monomeric proelastase-FLAG3 (≈30 kDa) (Fig. 5, A and B), as we already mentioned in Fig. 4. We suggest that the lower band represents proelastase whose signal sequence was processed, and the upper band represents proelastase whose signal sequence remained unprocessed for the following two reasons. First, the upper band migrated slower than the lower band on a standard SDS-polyacrylamide gel (Fig. 5 (A and B), lane 2), implying that the molecular mass of the upper band is larger than that of the lower band. Second, proelastase is synthesized as a precursor containing a signal sequence of 16 amino acid residues at its N terminus (28). To examine whether the signal sequence is cleaved in the lower band, we constructed a version of proelastase containing a FLAG tag just after the signal sequence of proelastase. We will call it FLAG-proelastase. Because anti-FLAG M1 antibody from Sigma recognizes a FLAG tag only when the FLAG tag is present exactly at the N terminus of a protein (29), use of FLAG-proelastase and anti-FLAG M1 antibody may enable us to examine whether the signal sequence is processed in the lower band. For the analysis, we also used regular anti-FLAG M2 antibody, which recognizes a FLAG tag regardless of its position on a protein (unless otherwise stated, we used anti-FLAG M2 antibody as an antibody to FLAG). When we expressed FLAG-proelastase in HeLa cells, anti-FLAG M2 antibody recognized two bands at the position of monomeric FLAG-proelastase (≈28 kDa) (Fig. 5G, lane 2), consistent with the data obtained with cells expressing proelastase-FLAG3 (Fig. 5, A and B). By contrast, anti-FLAG M1 antibody recognized the lower band but not the upper band (Fig. 5G, lane 4), consistent with the lower band representing proelastase whose signal sequence was cleaved at the expected position. Hereafter, we will call the upper band “unprocessed proelastase” and the lower band “processed proelastase.”

Interestingly, in addition to these monomer bands, we also observed smear bands that ran more slowly than the monomeric proelastase when we expressed proelastase in the absence of PDIp (Figs. 4D (lane 3) and 5A (lane 2)). When the sample was reduced by DTT before electrophoresis, the majority of these bands were converted to its monomeric form (Figs. 4E (lane 3) and 5B (lane 2)), indicating that proelastase formed disulfide-linked high-molecular mass aggregates when expressed alone in HeLa cells. Remarkably, however, co-expression of PDIp with proelastase resulted in the decreased accumulation of the aggregates within cells and the enhanced
production of the processed monomeric form of proelastase within the cells (Figs. 4 (D and E, lanes 3 and 4) and 5 (A and B, lanes 2–5)) and in the medium fraction (Fig. 5 (C and D), lanes 2–5). Thus, PDIP prevented the formation of the aggregates of proelastase and promoted the production of the processed monomeric form of this protein.
We also examined the abilities of other typical PDI family members (PDI, P5, ERp57, and ERp46) (2, 3) to promote the production of the processed monomeric proelastase. For this purpose, we investigated the effects of overexpression of these PDI family members on the production of the processed monomeric proelastase in HeLa cells. To compare the expression levels of PDI family members expressed from plasmids, we fused a sequence encoding a c-Myc tag and the ER retention signal of PDIp (KEEL) at the C terminus of each PDI family member. Importantly, the levels of PDI, P5, ERp57, and ERp46 expressed from these plasmids were comparable with those of PDIp expressed from its plasmid (Fig. 5B, c-Myc).

Interestingly, when overexpressed from a plasmid, PDI, like PDIp, prevented the formation of the aggregates of proelastase within HeLa cells (Fig. 5A and B, lanes 2–6) and promoted the production of the processed monomeric proelastase in the cell fraction after treatment of the immunopurified proelastase with trypsin (Fig. 5C). PDIp is unique among the PDI family members tested in this experiment in that PDIp can both prevent the formation of aggregates of proelastase and enhance the production and secretion of the processed monomeric proelastase.

We next examined the effect of overexpressing P5, ERp57, or ERp46 on the biosynthesis of proelastase. Even when overexpressed from plasmids, these three PDI family members neither prevented the formation of the aggregates of proelastase within HeLa cells (Fig. 5A and B, lanes 2–6) nor promoted the production of the processed monomeric proelastase in the cell fraction (Fig. 5C). Thus, PDIp is unique among the PDI family members tested in this experiment in that PDIp can both prevent the formation of aggregates of proelastase and enhance the production and secretion of the processed monomeric proelastase.

**Role of PDIp in the production of active elastase**

Pancreatic elastase is synthesized and stored as proelastase, an inactive proenzyme, in the pancreatic acinar cells. Following meal ingestion, proelastase is secreted into the lumen of duodenum and processed to active enzyme through cleavage of the pro-sequence region by trypsin in the lumen (25). To further examine the role of PDIp in the production of active elastase, we partially purified proelastase from HeLa cells expressing proelastase-FLAG3 using an antibody to FLAG, treated the purified protein with a small amount of trypsin, and subjected it to an elastase activity assay using a fluorogenic substrate, Suc-Ala-Pro-Ala-MCA (30).

![Figure 6](https://example.com/figure6.png)

**Figure 6. PDIp promotes the generation of productive intermediate in the synthesis of elastase.** A, preparation of sample for elastase activity assay. HeLa cells were transfected with both pTF001 and pTF006 to express PDIp and proelastase-FLAG3. After 24 h, the cell and medium fractions were collected and treated with NEM to prevent disulfide rearrangements. To measure the elastase activity, proelastase-FLAG3 was immunopurified, treated with trypsin, and subjected to elastase activity assay using a fluorogenic substrate, Suc-Ala-Pro-Ala-MCA (30). The activity was expressed as nmol of 7-amino-4-methylcoumarin released upon hydrolysis of the substrate at 37 °C per min per mg of NEM-alkylated cell lysate used for the immunopurification. B, elastase activity in the cell fraction after treatment of the immunopurified proelastase with trypsin. Error bars, S.E. for three independent experiments. Fisher’s t test was performed to determine p value for each assay (**, p < 0.01).
tase in the cells resulted in a significant increase in the elastase activity (Fig. 6B). A similar increase in the elastase activity was also observed with the samples obtained from the medium fraction of the cells co-expressing PDlp with proelastase (Fig. 6C). Thus, PDlp promoted the production of proelastase that could be converted to the active enzyme upon treatment with trypsin.

Discussion

The ER of mammalian cells houses more than 20 PDI family members (2). PDlp is a poorly characterized PDI family member highly expressed in the pancreas. It has been shown that this protein can bind and store estrogen (21). However, it remained unclear whether this protein has physiological functions other than binding estrogen. To solve this question, we set out to identify the physiological substrates of this enzyme from a mouse tissue, utilizing the fact that PDI family members often form disulfide-linked complexes with their target proteins (15, 31). Our analysis on the role of PDlp in the biosynthesis of one of the identified proteins led to a clue as to the physiological function of this enzyme.

A number of findings obtained in this work highlight physiological roles of PDlp. First, a number of exocrine digestive enzymes, but not endocrine hormones, were identified as putative disulfide-linked partners of PDlp from the pancreas in our MS analysis, implying that PDlp acts on digestive enzymes. Second, our immunoblotting analyses clearly showed that, in the pancreas, PDlp is localized in the exocrine cell fractions, but not in the endocrine cell fraction, suggesting that this protein acts on proteins in the pancreatic exocrine cells but not on proteins in the pancreatic endocrine cells. Third, PDlp and/or disulfide-linked complexes that involve PDlp were also found in the stomach and small intestine. As the latter organs also produce digestive enzymes (32), this finding is consistent with our proposal that PDlp preferentially acts on digestive enzymes. Fourth, when pancreatic elastase was produced in the absence of PDlp in cultured cells, proelastase formed disulfide-linked aggregates within cells. Co-expression of PDlp, but not PDI, P5, ERp57, or ERp46, with the pancreatic elastase resulted both in a decrease in the amount of the aggregates and in an increase in the amount of the processed monomeric form of proelastase within cells and in medium fraction, indicating that PDlp is unique among the tested PDI family members in that this protein can prevent the formation of the aggregates of proelastase and enhance the production and secretion of the processed monomer. Finally, upon activation of proelastase with trypsin, both cell and medium fractions obtained from cells expressing proelastase in the presence of PDlp showed significantly higher elastase activities than those from cells expressing proelastase in the absence of PDlp, indicating that PDlp promoted the production of proelastase that could be readily converted to the active enzyme upon treatment with trypsin. Based on these findings, we propose that PDlp is a PDI family member dedicated to the production of exocrine digestive enzymes.

Importantly, overexpression of PDlp or PDI, but not that of P5, ERp57, or ERp46, prevented the formation of aggregates of proelastase, resulting in the accumulation of the processed monomeric form of proelastase within cells. However, unlike PDlp, PDI failed to promote the secretion of the processed monomeric form of proelastase into the medium. It has been reported that expression of PDI reduces insulin secretion (31) and induces the accumulation of proinsulin within pancreatic β-cells (33, 34). Thus, PDI may sometimes act to retain secretory proteins within cells.

Interestingly, our MS analysis identified five of the PDI family members, including P4HB (PDI), PDA6 (P5), and PDA3 (ERP57) (2, 14), as putative disulfide-linked partners of PDlp. They may be interacting directly with PDlp for exchange of electrons, as has been proposed for the interaction between several PDI family members (4, 15, 35, 36). Alternatively, these enzymes may be acting on a substrate protein at the same time as PDlp, generating oligomers linked together via disulfide bonds. In addition, our MS analysis identified two of the PDI-oxidizing enzymes, PRDX4 (Prx4) (37, 38) and ERO1LB (Ero1β) (39, 40), as possible disulfide-linked partners of PDlp. Thus, it may be possible that both Prx4 and Ero1β act as oxidizing enzymes of PDlp in vivo. Further studies are required to reveal the physiological meanings of these interactions.

Unexpectedly, fibrinogens were also identified by our MS analysis as possible interactors of PDlp. Recent results have established that secreted PDI is required for the coagulation that takes place in vitro and in vivo (11, 41, 42). Thus, it may happen that PDlp plays some role in coagulation in the pancreas. Alternatively, PDlp may have interacted with fibrinogens artificially during sample preparation, as we have suggested under “Results.”

Interestingly, in vitro studies have identified peptides that bind to PDlp (19, 43). Mutating tyrosine and tryptophan residues or introducing an acidic residue next to these residues on the peptides resulted in loss of their ability to bind to PDlp (43). These findings led to a model that tyrosine residues or tryptophan residues within the folding polypeptide trigger its binding to PDlp, except when adjacent to a negative charge (43). Consistently, the proteins identified in our MS analysis commonly have the above motif on their amino acid sequences (Table S1). However, the residues with the same feature are also present in proteins, including LDL receptor (44), that are expressed in a wide variety of mammalian tissues (Table S1). Thus, the presence or absence of these residues will not be the only feature that makes a substrate necessitate PDlp for its efficient folding.

Researchers have used redox-active site mutants of PDI family members to stabilize the disulfide-linked complexes between the enzymes and their substrates, allowing them to purify and identify the targets of these enzymes (4, 13, 16). Previously, we reported that, by combining acid-quenching and free cysteine modification, it was possible to detect, purify, and identify the disulfide-linked partners of ERdj5 from the epididymis of mice (15). The uniqueness of this approach is that the method does not require genetic engineering of mice for the identification of putative substrates of a PDI family member in a specific tissue. Here, we have shown that it is also possible to do so with PDlp from the pancreas. Thus, this approach seems applicable to a wider range of PDI family members and thereby will help define the roles of the enzymes at the animal or tissue level.

In conclusion, our findings suggest that PDlp is a PDI family member dedicated to the biosynthesis of digestive enzymes.
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However, it is still unknown why PDIp is suited as the catalyst of the biosynthesis of digestive enzymes. It may be possible that the folding pathways of the digestive enzymes share some common feature that requires the assistance of PDIp. Obviously, detailed biochemical and structural studies will be required to solve the question.

Experimental procedures

Preparation of mouse tissue lysates treated with NEM

Eight-week-old male C57BL/6j mice, purchased from CLEA Japan, were used to prepare tissue lysates. A tissue, dissected from the body of a mouse, was immediately disrupted in 1 ml of ice-cold 10% TCA using a homogenizer. After 20 min of incubation on ice, the proteins were collected by centrifugation and washed twice with ice-cold acetone to remove acid. The free cysteines in the proteins were then alkylated with NEM using NEM alkylation buffer (100 mM Tris-HCl (pH 6.8), 2% SDS, 50 mM NEM) supplemented with 10 μg/ml pepstatin A, 10 μg/ml benzamidine, and 1 mM phenylmethylsulfonyl fluoride essentially as described before (15). All experimental protocols involving animals were approved by the Committee on Animal Research at Nara Institute of Science and Technology (NAIST) and were performed in accordance with the institutional guidelines of NAIST.

Isolation of the islets and acinar cells from mouse pancreas

To isolate the islets and acinar cells from the mouse pancreas, intraductal perfusion of the pancreas was performed using 3 ml of Hanks’ balanced salt solution containing 1.5 mg/ml collagenase P (Roche Applied Science). The pancreas is then excised whole and subjected to further digestion with collagenase P at 37 °C for 20 min. The perfused pancreas was subjected to centrifugation using Histopaque-1077 (Sigma-Aldrich) to obtain the acinar cells and a fraction containing the pancreatic islets. The islets were then isolated from the fraction by hand-picking under a dissecting microscope. The isolated cells were dissolved in Laemmli sample buffer and separated by SDS-PAGE under reducing conditions to detect PDIp by immunoblotting.

Plasmids

All of the primers used for plasmid construction are listed in Table S2. Human cDNA clones for pancreatic elastase (ELA2A), and ERP46 (TXNDC5) were obtained from the Riken BioResource Research Center through the National BioResource Project of MEXT/AMED, Japan (45). Plasmids for PDIp-FLAG, PDI-FLAG, P5-FLAG, and ERP57-FLAG were kindly provided by Adrian Salic (Harvard Medical School). The expression vector pcDNA3.1+ was purchased from Invitrogen. Plasmid pTF001 (encoding human PDIp on pcDNA3.1+) was constructed by amplifying a region encoding PDIp from a plasmid encoding PDIp-FLAG using primers (PDIp-f and PDIp-r) and cloning it between the KpnI and XbaI sites of pcDNA3.1+. Plasmid pTF006 (encoding proelastase-FLAG3, human proelastase fused with a triple FLAG sequence on pcDNA3.1+) was constructed by amplifying a region encoding human pancreatic elastase using primers (CEL2-f and CEL2-FLAG-r) and cloning it between the HindIII and BamHI sites of pcDNA3.1+.

To construct pTF007 (encoding proelastase on pcDNA3.1+), the elastase gene was amplified using primers (CEL2-f and CEL2-r) and cloned into the HindIII and BamHI sites of pcDNA3.1+. Plasmid pTF016 (encoding FLAG-proelastase on pcDNA3.1+) was constructed by assembling two fragments using the Gibson Assembly Cloning Kit (New England Biolabs). The fragments used were a 7.0-kb fragment amplified from pTF007 with primers pTF016-F and pTF016-R and a 80-bp fragment produced by annealing two primers (pTF016-INS-F and pTF016-INS-R). Plasmid pON101 (encoding PDIp-FLAG3 on pcDNA3.1+) was constructed by assembling two fragments using the Gibson Assembly Cloning Kit (New England Biolabs). The fragments used were a 7.0-kb fragment amplified from pTF001 with primers pON101-f1 and pON101-r1 and a 111-bp fragment amplified from pTF006 with primers pON101-f2 and pON101-r2. To construct pON103 encoding PDIp-c-Myc on pcDNA3.1+, two primers (BspEI-Myc-Agel-F and BspEI-Myc-Agel-R) were annealed, digested with BspEI and Agel, and cloned into pON101. Plasmid pON104 (encoding PDI-c-Myc on pcDNA3.1+) was constructed by amplifying a region encoding human PDI from a plasmid encoding PDIp-FLAG using primers pON104-f and pON104-r and cloning it between the KpnI and BspEI sites of pON103. Plasmid pON105 (encoding ERP46-c-Myc on pcDNA3.1+) was constructed by amplifying a region encoding human ERP46 from an ERP46 cDNA clone using primers pON105-f and pON105-r and cloning it between the KpnI and Agel sites of pON103. Plasmid pON106 (encoding P5-c-Myc on pcDNA3.1+) was constructed by amplifying a region encoding human P5 from a plasmid encoding P5-FLAG using primers pON106-f and pON106-r and cloning it between the HindIII and BspEI sites of pON103. Plasmid pON107 (encoding ERP57-c-Myc on pcDNA3.1+) was constructed by amplifying a region encoding human ERP57 from a plasmid encoding ERP57-FLAG using primers pON107-f and pON107-r and cloning it between the KpnI and BspEI sites of pON103. All clones were verified by sequencing of the entire ORF.

Cell culture, transfection, and sample preparation

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Nacalai Tesque) supplemented with 10% FBS at 37 °C in 5% CO2 air. Cells were transfected with plasmids using ViaFectTM (Promega). For the transfection, HeLa cells were plated onto a 6-well plate at 1.5 × 105 cells/well, cultured for 24 h, and transfected with 1.2 μg of pTF006 (encoding proelastase-FLAG3) and 0.8 μg of pTF001 (encoding PDIp), following the manufacturer’s instructions. At 24 h after the transfection, cells were washed twice with PBS, treated with 10% TCA, and subjected to alkylation with NEM essentially as described above. To analyze the proteins in the medium fraction, we exchanged the medium to 1 ml of prewarmed, serum-free Opti-MEM I (Gibco) 20 h after transfection and continued the growth for another 4 h. The culture supernatant was subjected to brief centrifugation to remove any cell debris, treated directly with 10% TCA, and alkylated with NEM essentially as described above to obtain an NEM-treated medium fraction.
Antibodies

Rabbit antibody that recognizes both mouse and rat elastase (ab21593) and rabbit antibody that recognizes both mouse and human pancreatic α-amylase (ab21156) were purchased from Abcam. Goat antibody to chymotrypsin B of mouse, rat, and human origin (sc-16500), rabbit antibody to trypsins (trypsin-1, trypsin-2, trypsin-3, and trypsin-10) of mouse and rat origin (sc-67388), and mouse mAb to c-Myc (9E10) were purchased from Santa Cruz Biotechnology, Inc. Guinea pig polyclonal anti-insulin antibody (A0564) was purchased from Dako and has been described (31). Mouse anti-FLAG M1 and M2 antibodies were purchased from Sigma-Aldrich. Mouse mAb to PDIp was obtained by the following procedure. Mice were immunized with the membrane fraction from unfertilized eggs of Xenopus laevis (46). Hybridomas producing various antibodies were collected. We obtained one hybridoma, which secretes an mAb that recognized mammalian PDIp. The ascites fluid from a mouse inoculated with the hybridoma was used as the source of the antibody. Unless otherwise stated, the specificity of the antibodies used (except for the anti-FLAG M1 and M2 antibodies and anti-c-Myc (9E10) antibody) was characterized using mouse tissue lysates. Each of the antibodies recognized a protein(s) with the expected size and tissue distribution (see Fig. 2 and Fig. S2).

Immunoblotting

Proteins from NEM-treated tissue lysates or cell lysates were separated by 10 or 12% SDS-PAGE, blotted onto an Immobilon-P membrane (Millipore), incubated with a primary antibody and then with an appropriate secondary antibody, and detected with Clarity Western ECL Substrate (Bio-Rad). For the visualization of a protein fused with a FLAG3 or c-Myc sequence by immunoblotting, we used horse-radish peroxidase–conjugated antibody to FLAG or c-Myc tag, respectively.

Immunoprecipitation of PDIp and mixed-disulfide complexes containing PDIp from mouse pancreas

Three hundred μg of NEM-alkylated mouse pancreatic tissue lysate was diluted 10-fold with ice-cold IP buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40) and centrifuged at 15,000 × g for 10 min at 4 °C to obtain NEM-treated cleared pancreatic lysate. To purify PDIp and PDIp-containing disulfide-linked complexes from the cleared lysate, mouse anti-PDIp antibody was bound to Surebeads™ protein G magnetic beads (Bio-Rad), following the supplier’s instructions. The resulting beads were incubated with the NEM-treated cleared pancreatic lysate (see above) for 3 h at 4 °C. The immune complexes were collected by magnetization and washed at least four times with the same IP buffer. The immunosolanes were then released by incubating the sample at 37 °C for 30 min with 2× Laemmli sample buffer.

Identification of disulfide-linked partners of PDIp by mass spectrometry

One-third of the total purified proteins obtained using the above procedure were separated by 10% SDS-polyacrylamide gels, and the proteins that ran slower than the position of the monomeric form of PDIp were excised from the gel, digested with trypsin, and analyzed by LC/tandem MS (LTQ-Orbitrap Velos, Thermo Fisher Scientific). The MS/MS data file obtained was analyzed using the Mascot version 2.6 search engine (Matrix Science) against the NCBI nr database (May 2015; 66,387,522 sequences) with Mus musculus as species restriction. The Mascot search results were accepted if a protein hit included at least three peptides with ion scores above the identity threshold (p < 0.05) and the sequence coverage of the protein was more than 10%. Affinity purification using conjugated antibodies often causes a portion of the conjugated antibodies to be released from the beads, resulting in their co-purification with the target proteins. Furthermore, biological samples are often contaminated with keratins from environment. We thus excluded Igs and keratins from the list of identified proteins. To identify the potential binding partners of PDIp, the same analysis was performed using normal mouse serum IgG as a negative control, and any proteins identified in the sample were excluded from the list of potential partners of PDIp.

Co-immunoprecipitation experiment

The NEM-treated pancreatic lysate was subjected to immunoprecipitation with the appropriate antibody or control serum as described above. The immunoprecipitates were incubated in the presence (reducing) or absence (nonreducing) of 100 mM DTT for 10 min prior to immunoblotting with appropriate antibody.

Preparation of NEM-alkylated sample for elastase activity assay

HeLa cells were plated in a 10-cm culture dish at 7.5 × 10^5 cells, cultured for 24 h, and transfected with 6 μg of pTF006 (encoding proelastase-FLAG3) and 4 μg of pTF001 (encoding PDIp) as described above. At 24 h after the transfection, the cell and medium fractions were prepared from the culture as follows. To prepare the medium fraction, the culture supernatant (10 ml) was centrifuged at 100 × g for 5 min to remove any floating cells and/or debris, concentrated to 500 μl by ultrafiltration using an Amicon Ultra-15 3k device, and incubated with 500 μl of NEM alkylation buffer (100 mM Tris-HCl (pH 8.0), 0.5% Triton X-100, 2 mM EDTA, 50 mM NEM) for 30 min at 37 °C to obtain NEM-alkylated medium fraction. To prepare the cell fraction, cells after removal of the culture supernatant were washed twice with PBS, dissolved in 300 μl of ice-cold NEM alkylation buffer (see above), and incubated for 30 min at 37 °C to obtain NEM-alkylated cell fraction. Alkylation of the proteins in the cell and medium fractions with NEM was performed to prevent any disulfide rearrangements that could occur during sample preparation. The resulting NEM-alkylated samples were subjected to immunoisolation with anti-FLAG M2 magnetic beads (Sigma) to purify proelastase (expressed from pTF006 encoding proelastase-FLAG3) (800 μl). This purification step was required to remove any contaminants that could interfere with the activity of elastase. The purified proelastase was then activated with 50 μl of immobilized tosylphenylalanlanyl chloromethyl ketone–treated trypsin (Thermo
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Scientific) for 1 h at 37 °C in a total of 500 μl of activation buffer (100 mM Tris-HCl (pH 8.0), 2 mM CaCl₂, 5 mM NEM) and stored at −80 °C.

**Fluorometric assay for elastase activity**

We measured elastase activity using a fluorogenic substrate, succinyl-1-alanine-1-proline-1-alanine-4-methylcoumaryl-7-amide (Suc-Ala-Pro-Ala-MCA) (Peptide Institute, Osaka, Japan) (30). Increase of fluorescence at 460 nm with excitation at 380 nm was monitored at 37 °C in a total of 400 μl of reaction buffer (10 mM Tris-HCl (pH 8.8), 2 mM EDTA, 0.1% (w/v) Brij35, 5 mM NEM) containing 20 μM Suc-Ala-Pro-Ala-MCA and 200 μl of the trypsin-activated NEM-treated proelastase sample.

**Author contributions**—T. F., O. N., M. S., A. T., and M. M. data curation; T. F., O. N., M. M., K. K., and K. I. formal analysis; T. F. and H. K. writing-original draft; A. T. resources; A. T., M. M., K. K., and K. I. formal analysis; T. F. and H. K. conceptualization; H. K. supervision; H. K. project administration; T. F. performed most of the experiments; O. N. performed experiments related to the elastase activity assays; M. S. performed all animal experiments.

**Acknowledgments**—We thank Mizuho Oda (Kyushu University) for the gift of plasmids. The MS analysis was performed in the Collaborative Research Project Program of the Medical Institute of Bioregulation, Kyushu University.

**References**

1. Kodokura, H., and Beckwith, J. (2010) Mechanisms of oxidative protein folding in the bacterial cell envelope. *Antioxid. Redox Signal.* 13, 1231–1246 CrossRef Medline

2. Okumura, M., Kodokura, H., and Inaba, K. (2015) Structures and functions of protein disulfide isomerase family members involved in proteostasis in the endoplasmic reticulum. *Free Radic. Biol. Med.* 83, 314–322 CrossRef Medline

3. Sato, Y., and Inaba, K. (2012) Disulfide bond formation network in the three biological kingdoms, bacteria, fungi and mammals. *FEBS J.* 279, 2262–2271 CrossRef Medline

4. Oka, O. B. V., Pringle, M. A., Schopp, I. M., Braakman, I., and Bulleid, N. J. (2013) ERDj5 is the ER reductase that catalyzes the removal of non-native disulfides and correct folding of the LDL receptor. *Mol. Cell* 50, 793–804 CrossRef Medline

5. Poet, G. J., Oka, O. B., van Lith, M., Cao, Z., Robinson, P. J., Pringle, M. A., Arnér, E. S., and Bulleid, N. J. (2017) Cytosolic thioredoxin reductase 1 is required for correct disulfide formation in the ER. *EMBO J.* 36, 693–702 CrossRef Medline

6. Hagiwara, M., Maegawa, K., Suzuki, M., Ushioda, R., Araki, K., Matsumoto, Y., Hoseki, J., Nagata, K., and Inaba, K. (2011) Structural basis of an ERAD pathway mediated by the ER-resident protein disulfide reductase ERDj5. *Mol. Cell* 41, 432–444 CrossRef Medline

7. Maegawa, K. L., Watanabe, S., Noi, K., Okumura, M., Amagai, Y., Inoue, M., Ushioda, R., Nagata, K., Ogura, T., and Inaba, K. (2017) The highly dynamic nature of ERDj5 is key to efficient elimination of aberrant protein oligomers through ER-associated degradation. *Structure* 25, 846–857.e4 CrossRef Medline

8. Eletto, D., Eletto, D., Dersh, D., Gidalevitz, T., and Argon, Y. (2014) Protein disulfide isomerase A6 controls the decay of Ire1α signaling via disulfide-dependent association. *Mol. Cell* 53, 562–576 CrossRef Medline

9. Higa, A., Takui, S., Lhomond, S., Jensen, D., Fernandez-Zapico, M. E., Simpson, J. C., Pasquet, J.-M., Schekman, R., and Chevet, E. (2014) Endoplasmic reticulum stress-activated transcription factor ATF6 requires the disulfide isomerase PDI5 to modulate chemo resistance. *Mol. Cell Biol.* 34, 1839–1849 CrossRef Medline

10. Ushioda, R., Miyamoto, A., Inoue, M., Watanabe, S., Okumura, M., Maegawa, K., I. Uegaki, K., Fujii, S., Fukuda, Y., Umitsu, M., Takagi, J., Inaba, K., Mikoshika, K., and Nagata, K. (2016) Redox-assisted regulation of Ca²⁺ homeostasis in the endoplasmic reticulum by disulfide reductase ERdj5. *Proc. Natl. Acad. Sci.* 113, E6055–E6063 CrossRef Medline

11. Bowley, S. R., Fang, C., Merrill-Skoloff, G., Furie, B. C., and Furie, B. (2017) Protein disulfide isomerase secretion following vascular injury initiates a regulatory pathway for thrombus formation. *Nat. Commun.* 8, 14151 CrossRef Medline

12. Braakman, I., and Bulleid, N. J. (2011) Protein folding and modification in the mammalian endoplasmic reticulum. *Annu. Rev. Biochem.* 80, 71–99 CrossRef Medline

13. Jessop, C. E., Chakravarti, S., Garbi, N., Hämmerling, G. J., Lovell, S., and Bulleid, N. J. (2007) ERp57 is essential for efficient folding of glycoproteins sharing common structural domains. *EMBO J.* 26, 28–40 CrossRef Medline

14. Jessop, C. E., Watkins, R. H., Simmons, J. J., Tasab, M., and Bulleid, N. J. (2009) Protein disulfide isomerase family members show distinct substrate specificity: P5 is targeted to BiP client proteins. *J. Cell Sci.* 122, 4287–4295 CrossRef Medline

15. Kadokura, H., Saito, M., Tsuru, A., Hosoda, A., Iwawaki, T., Inaba, K., and Kohn, K. (2013) Identification of the redox partners of ERdj5/PDI, a PDI family member, from an animal tissue. *Biochem. Biophys. Res. Commun.* 440, 245–250 CrossRef Medline

16. Stopa, J. D., Baker, K. M., Grover, S. P., Flammenga, R., and Furie, B. (2017) Kinetic-based trapping by intervening sequence variants of the active sites of protein-disulfide isomerase identifies platelet protein substrates. *J. Biol. Chem.* 292, 9063–9074 CrossRef Medline

17. Desilva, M. G., Lu, J., Donadel, G., Modi, W. S., Xie, H., Notkins, A. L., and Lan, M. S. (1996) Characterization and chromosomal localization of a new protein disulfide isomerase, PDIP, highly expressed in human pancreas. *DNA Cell Biol.* 15, 9–16 CrossRef Medline

18. Fu, X. M., Dai, X., Ding, J., and Zhu, B. T. (2009) Pancreas-specific protein disulfide isomerase has a cell type-specific expression in various mouse tissues and is absent in human pancreatic adenocarcinoma cells: implications for its functions. *J. Mol. Histol.* 40, 189–199 CrossRef Medline

19. Klappa, P., Stromer, T., Zimmermann, R., Ruddock, L. W., and Freedman, R. B. (1998) A pancreatic-specific glycosylated protein disulfide-isomerase binds to misfolded proteins and peptides with an interaction inhibited by oestrogens. *Eur. J. Biochem.* 254, 63–69 CrossRef Medline

20. Fu, X.-M., and Zhu, B. T. (2010) Human pancreas-specific protein disulfide-isomerase (PDIP) can function as a chaperone independently of its enzymatic activity by forming stable complexes with denatured substrate proteins. *Biochem. J.* 429, 157–169 CrossRef Medline

21. Fu, X., Wang, P., Fukui, M., Long, C., Yin, L., Choi, H. J., and Zhu, B. T. (2012) PDIP is a major intracellular oestrogen-storage protein that modulates tissue levels of oestrogen in the pancreas. *Biochem. J.* 447, 115–123 CrossRef Medline

22. Lang, T., Johanning, K., Metzler, H., Piepenbrock, S., Solomon, C., Rahe-Meyer, N., and Tanaka, K. A. (2009) The effects of fibrogenic levels on thromboelastometric variables in the presence of thrombocytopenia. *Anesth. Analg.* 108, 751–758 CrossRef Medline

23. Kodokura, H., Tian, H., Zander, T., Bardwell, J. C. A., and Beckwith, J. (2004) Snapshots of DsbA in action: detection of proteins in the process of oxidative folding. *Science* 303, 534–537 CrossRef Medline

24. Desilva, M. G., Notkins, A. L., and Lan, M. S. (1997) Molecular characterization of a pancreas-specific protein disulfide isomerase, PDIP, from an animal tissue. *FEBS J.* 245–250 CrossRef Medline

25. Pandol, S. J. (2010) *The Exocrine Pancreas*, pp. 29–36, Morgan & Claypool Life Sciences, San Rafael, CA

26. Logsdon, C. D., Moessner, J., Williams, J. A., and Goldfine, I. D. (1985) Glucocorticoids increase amylase mRNA levels, secretory organelles, and secretion in pancreatic acinar AR42J cells. *J. Cell Biol.* 100, 1200–1208 CrossRef Medline
27. Rajasekaran, A. K., Morimoto, T., Hanzel, D. K., Rodriguez-Boulan, E., and Kreibich, G. (1993) Structural reorganization of the rough endoplasmic reticulum without size expansion accounts for dexamethasone-induced secretory activity in AR42J cells. *J. Cell Sci.* 105, 333–345 Medline

28. Shirasu, Y., Yoshida, H., Matsu, S., Takemura, K., Ike, N., Shimada, Y., Ozawa, T., Mikayama, T., Iijima, H., Ishida, A., Sato, Y., Tamai, Y., Tanaka, J-I., and Ikenaga, H. (1987) Molecular cloning and expression in *Escherichia coli* of a cDNA encoding human pancreatic elastase 2. *J. Biochem.* 102, 1555–1563 CrossRef Medline

29. Knappik, A., and Plückthun, A. (1994) An improved affinity tag based on the FLAG peptide for the detection and purification of recombinant antibody fragments. *BioTechniques* 17, 754–761 Medline

30. Oshima, G., Akashi, K., and Yamada, M. (1984) pH dependence of salt activation of human leukocyte elastase. *Arch. Biochem. Biophys.* 233, 212–218 CrossRef Medline

31. Tsuchiya, Y., Saito, M., Kadokura, H., Miyazaki, J.-I., Tashiro, F., Imagawa, Y., Iwawaki, T., and Kohno, K. (2018) IRE1-XBP1 pathway regulates oxidative proinsulin folding in pancreatic β-cells. *J. Cell Biol.* 217, 1287–1301 CrossRef Medline

32. Rawlings, N. D., and Salvesen, G. (2013) *Handbook of Proteolytic Enzymes*, 3rd Ed., Elsevier, Amsterdam

33. Rajpal, G., Schuiki, I., Liu, M., Volchuk, A., and Arvan, P. (2012) Action of protein disulfide isomerase on proinsulin exit from endoplasmic reticulum of pancreatic β-cells. *J. Biol. Chem.* 287, 43–47 CrossRef Medline

34. Zhang, L., Lai, E., Teodoro, T., and Volchuk, A. (2009) GRP78, but not protein-disulfide isomerase, partially reverses hyperglycemia-induced oxidative stress in pancreatic β-cells. *J. Biol. Chem.* 284, 5289–5298 CrossRef Medline

35. Araki, K., Iemura, S., Kamiya, Y., Ron, D., Kato, K., Natsume, T., and Nagata, K. (2013) Ero1-α and PDIs constitute a hierarchical electron transfer network of endoplasmic reticulum oxidoreductases. *J. Cell Biol.* 202, 861–874 CrossRef Medline

36. Oka, O. B. V., Yeoh, H. Y., and Bulleid, N. J. (2015) Thiol-disulfide exchange between the PDI family of oxidoreductases negates the requirement for an oxidase or reductase for each enzyme. *Biochem. J.* 469, 279–288 CrossRef Medline

37. Sato, Y., Kojima, R., Okumura, M., Hagiwara, M., Masui, S., Maegawa, K., Saiki, M., Horibe, T., Suzuki, M., and Inaba, K. (2013) Synergistic cooperation of PDI family members in peroxiredoxin 4-driven oxidative protein folding. *Sci. Rep.* 3, 2456 CrossRef Medline

38. Tavender, T. J., Springate, J. J., and Bulleid, N. J. (2010) Recycling of peroxiredoxin IV provides a novel pathway for disulphide formation in the endoplasmic reticulum. *EMBO J.* 29, 4185–4197 CrossRef Medline

39. Pagani, M., Fabbri, M., Benedetti, C., Fassio, A., Pilati, S., Bulleid, N. J., Cabibbo, A., and Sitia, R. (2000) Endoplasmic reticulum oxidoreductin 1-Lβ (ERO1-Lβ), a human gene induced in the course of the unfolded protein response. *J. Biol. Chem.* 275, 23685–23692 CrossRef Medline

40. Zito, E., Chin, K. T., Blais, J., Harding, H. P., and Ron, D. (2010) ERO1-β, a pancreas-specific disulfide oxidase, promotes insulin biogenesis and glucose homeostasis. *J. Cell Biol.* 188, 821–832 CrossRef Medline

41. Kim, K., Hahn, E., Li, J., Holbrook, L. M., Sasikumar, P., Stanley, R. G., Ushio-Fukai, M., Gibbins, J. M., and Cho, J. (2013) Platelet protein disulfide isomerase is required for thrombus formation but not for hemostasis in mice. *Blood* 122, 1052–1061 CrossRef Medline

42. Schulman, S., Bendapudi, P., Sharda, A., Chen, V., Bellido-Martin, L., Ja-suja, R., Furie, B. C., Flaumenhaft, R., and Furie, B. (2016) Extracellular thiol isomerases and their role in thrombus formation. *Antioxid. Redox Signal.* 24, 1–15 CrossRef Medline

43. Ruddy, L. W., Freedman, R. B., and Klappa, P. (2000) Specificity in substrate binding by protein folding catalysts: tyrosine and tryptophan residues are the recognition motifs for the binding of peptides to the pancreas-specific protein disulfide isomerase PDIp. *Protein Sci.* 9, 758–764 Medline

44. Rudling, M. J., Reinhér, E., Einarsson, K., Ewerth, S., and Angelin, B. (1990) Low density lipoprotein receptor-binding activity in human tissues: quantitative importance of hepatic receptors and evidence for regulation of their expression in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 87, 3469–3473 CrossRef Medline

45. Ota, T., Suzuki, Y., Nishikawa, T., Otsuki, T., Sugiyama, T., Irie, R., Wakamatsu, A., Hayashi, K., Sato, H., Nagai, K., Kimura, K., Makita, H., Sekine, M., Obayashi, M., Nishi, T., et al. (2004) Complete sequencing and characterization of 21,243 full-length human cDNAs. *Nat. Genet.* 36, 40–45 CrossRef Medline

46. Sopha, P., Kadokura, H., Yamamoto, Y. H., Takeuchi, M., Saito, M., Tsuru, A., and Kohno, K. (2012) A novel mammalian ER-localized J-protein, DNAJB14, can accelerate ERAD of misfolded membrane proteins. *Cell Struct. Funct.* 37, 177–187 CrossRef Medline