PDILT, a Divergent Testis-specific Protein Disulfide Isomerase with a Non-classical SXXC Motif That Engages in Disulfide-dependent Interactions in the Endoplasmic Reticulum*

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Protein disulfide isomerase (PDI) is the archetypal enzyme involved in the formation and reshuffling of disulfide bonds in the endoplasmic reticulum (ER). PDI achieves its redox function through two highly conserved thioredoxin domains, and PDI can also operate as an ER chaperone. The substrate specificities and the exact functions of most other PDI family proteins remain important unsolved questions in biology. Here, we characterize a new and striking member of the PDI family, which we have named protein disulfide isomerase-like protein of the testis (PDILT). PDILT is the first eukaryotic SXXC protein to be characterized in the ER. Our experiments have unveiled a novel, glycosylated PDI-like protein whose tissue-specific expression and unusual motifs have implications for the evolution, catalytic function, and substrate selection of thioredoxin family proteins. We show that PDILT is an ER resident glycoprotein that liaises with partner proteins in disulfide-dependent complexes within the testis. PDILT interacts with the oxidoreductase Ero1alpha, demonstrating that the N-terminal cysteine of the CXXC sequence is not required for binding of PDI family proteins to ER oxidoreductases. The expression of PDILT, in addition to PDI in the testis, suggests that PDILT performs a specialized chaperone function in testicular cells. PDILT is an unusual PDI relative that highlights the adaptability of chaperone and redox function in enzymes of the endoplasmic reticulum.

Disulfide bond formation is an essential step in the folding of many secretory and cell surface proteins. Spontaneous formation of disulfide bonds is inefficient, and therefore, this process needs to be catalyzed by enzymes (1). One of these enzymes, protein disulfide isomerase (PDI),1 can transfer the disulfide bond between the two cysteines of its redox active site (CGHC) to substrate proteins and is required for co-translational folding of glycoproteins (2). Reduced PDI is recharged by Ero1alpha, an FAD-dependent oxidoreductase that utilizes oxygen as its terminal electron acceptor (3–5).

PDI is part of the thioredoxin superfamily (6). PDI has two thioredoxin domains containing the active site (denoted a and a’) and two inactive domains (b and b’). Other PDI family members, including PDIp, ERP57, ERP72, and ERdj5 contain one or more of these modules (7). PDI, ERP57, and ERP72 are ubiquitously expressed (8), but little is known about the in vivo activities or substrate specificity of other family members. In particular, the function of the recently identified PDI relatives ERP28 and ERP19 remains unclear.

Some thioredoxin superfamily proteins of the thioredoxin and glutaredoxin type are involved in redox reactions in other compartments of the eukaryotic cell, including the cytosol and mitochondria (9, 10). Whereas Trx1 is responsible for keeping the cytosol in a reduced state, PDI enables proteins to become oxidized. The redox potential of a thioredoxin family protein indicates whether oxidation or reduction is favored, based on the relative preference of the CGHC motif to form a dihithio or a disulfide. However, work on the bacterial periplasmic disulfide bond catalysts DsbA and DsbC has shown that redox potentials do not necessarily predict redox function in vivo (11). DsbA and DsbC have comparable redox potentials of −120 and −130 mV, respectively, but whereas DsbA is the primary oxidant of the periplasm, DsbC is responsible for disulfide isomerization. Dimerization of DsbC (and its interaction with DsbD) is a critical factor in its ability to act as an isomerase (12). The local redox microenvironment, governed by other proteins, oligomeric status, and small molecular weight thiols such as glutathione, determines in vivo oxidoreductase activity both in prokaryotes and eukaryotes. Relating in vitro experiments to their in vivo context is, therefore, an important challenge in this area of biochemistry.

PDI not only introduces disulfide bonds into substrates but can also rearrange existing ones. Mutational analysis has shown that both cysteines in the redox active site of PDI are required for disulfide bond formation but that the N-terminal cysteine of this motif is sufficient for isomerization (13). There has been considerable debate about the relative importance of the oxidase, isomerase, and reductase functions of PDI in vivo (14–16). This assessment is complicated by redundancy, as many of the PDI homologues in yeast and mammals can partially complement PDI function.

Recently, the number of PDI family members has grown, and it has become clear that the WCGHC motif is not invariant (17). ERP44 and the yeast Eug1p are examples of thioredoxin-domain proteins that contain non-classical CXXS motifs. Eug1p is a poor oxidase and isomerase when assayed in vitro but can support these functions when the CXXS motif is mutated to CXXC (18). SXXC thioredoxin proteins have been identified in bacteria, but none has been characterized biochemically (19). No expressed eukaryotic SXXC motif proteins have so far been identified and studied.
In this paper we describe for the first time a eukaryotic SXXC motif protein of the endoplasmic reticulum. This protein is a testis-specific, glycosylated, divergent eukaryotic PDI that contains an SXXS and an SXXC motif within its two thio-
doxin domains. We have named this protein PDILT (protein disulfide isomerase-like protein of the testis). PDILT forms specific complexes with the oxidoreductase Ero1α and engages in disulfide-dependent interactions in the endoplasmic reticu-
lim of testicular cells. The ability of PDILT to sustain these protein-protein complexes without a CXXC motif demonstrates that upstream active site cysteines are not necessary for a PDI-like protein to detect an oxidoreductase.

EXPERIMENTAL PROCEDURES

Cell Lines, Tissues, and Antibodies—Human fibrosarcoma HT1080 cells, human MeJJuSo cells, human embryonal kidney HEK293 cells, murine spermatogonia-like GC-1 cells (gift from P. Saunders), and rat Sertoli SK-11 cells (gift from R. Ivel) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen). SK-11 cells were propagated at 33 °C. Human cervical carcinoma HeLa cells were maintained in minimal Eagle’s medium (Invitrogen). All media were supplemented with 5% calf serum (Sigma), 2 mM glutamax, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). All cell lines were kept at 5% CO2.

Tissue samples were obtained from male Balb/c or CD1 mice. The polyclonal rabbit anti-sera against PDI (20) and polyclonal Ero1α (DS21) have been described. The antiserum against PDILT was raised against peptide RQKL1DNSTNKQELNC and was a kind gift of Dr. L. Ellgaard. The antiserum against Erp57 was a kind gift of Prof. N. Bulleid. The calnexin antiserum was a kind gift from Prof. I. Braakman. Polyclonal antiseras against BiP (Santa Cruz) and Erp72 (Calbiochem) and the anti-Myc antibody clone 9B11 (New England Biolabs) were commercially available.

Transformation—Transformations with LipofectAMINE 2000 (Invitrogen) were performed according to the manufacturer’s instructions. Sub-confluent 293T cells were washed with ice-cold (Invitrogen) and transfected with 1 μg of DNA for 6 h in the presence of Opti-MEM serum-free medium (Invitrogen). After 6 h the medium was replaced with complete medium, and the cells were analyzed 24 h post-transfection.

RNA Isolation and RT-PCR—Total RNA from cell lines and mouse tissues was extracted using TRI reagent (Sigma) according to the manufacturer’s protocol. Before performing RT-PCR, RNA concentrations were determined by spectrophotometry to ensure equal total RNA input (typically 50 ng per reaction). RT-PCR reactions were performed using the AccessQuick kit (Promega) according to the manufacturer’s protocol on a Peltier Thermal Cycler (MJ Research). Primers for PDILT were designed to detect human, mouse, and rat homologues: catcggctcgcttctcg (exon 4) and tattttggtactcttgaagct (exon 9). The control actin primers were: ctacctggcttgaagct and actacctggcttatccttctcg. The result-

ant PCR products were analyzed on 1% agarose gels.

Construction of PDILT and Myc-tagged PDILT Vectors—The original cDNA was obtained from the IMAGE consortium. The PDILT cDNA was cloned into the BamHI/Not1 restriction site of pcDNA3 and verified by DNA sequencing. The PDILT sequence corresponds to gi:28372543 and differs by three amino acids from gi:21757251.

To create a Myc-tagged version of PDILT, fusion PCR was used to insert a Myc tag between amino acids Q574 and K575 of PDILT, upstream of the KEEl sequence. In a first PCR round, fragments of the 5’ part of PDILT with a 3’ Myc tag and a 3’ part of PDILT with a 5’ Myc tag were generated. These two fragments were used in a second PCR round to generate PDILT-Myc using the extreme 5’ and 3’ primers and the Myc overlap from both first round PCR fragments.

In Vitro Transcription—In vitro transcription of pcDNA3 PDILT con-
structs was performed using 5 μg of DNA linearized with MluI. T7 RNA polymerase-driven transcription reactions were performed using the Pro-

mega riboprobe system, according to manufacturer’s instructions.

Metabolic Labeling—All procedures were at 37 °C unless otherwise stated. Cells in 6-cm dishes were starved for 20 min in minimum Eagle’s medium lacking methionine and cysteine (I CN) but supplemented with 2 mM glutamax and 20 mM Heps. Cells were biosynthetically labeled with 50 μCi of [35S]methionine/cysteine (Amersham Biosciences) for 10 min. For pulse-labeling, the pulse was stopped by washing the cells twice with ice-cold Hanks’ balanced salt solution (Invitrogen) supplemented with 20 mM NEM to trap disulfide-linked complexes. For pulse-
chase, cells were washed once after the pulse with complete growth medium supplemented with 5 mM cysteine and methionine and 1 mM cycloheximide (chase medium) and then maintained in chase medium for the indicated times. Pulse-chased cells were then treated as for pulse-only cells. All cells were lysed at 4 °C in 300 μl of lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA) supplemented with freshly added 20 mM NEM and 10 μg/ml protease inhibitors (chymostatin, leupeptin, pepstatin, and antipain; Sigma). Post-nuclear supernatants were generated by centrifugation at 16,100 × g for 10 min at 4 °C and subjected to immunoprecipitation at 4 °C for 1 h using antibodies immobilized on protein A-Sepharose beads (Amersham Biosciences). Beads were washed three times in lysis buffer before boiling for 5 min in sample buffer in the presence or absence of 50 mM dithiothreitol. Samples were subsequently analyzed by 8% SDS-PAGE.

Western Blotting—Post-nuclear cell lysates or comparable amounts of mouse tissues were directly analyzed by SDS-PAGE or subjected to immunoprecipitation before analysis by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) at 150 mA for 2 h, and the membranes were blocked in 8% milk, Tris-buffered saline Tween for 30 min. The primary anti-Myc, anti-Ero1α, anti-
Erp57, anti-calnexin, anti-Erp72, and PDI antibodies were used at 1:1000, anti-PDILT was used at 1:333, and anti-BiP was used at 1:100 dilution. After washing three times with Tris-buffered saline Tween, membranes were incubated with corresponding secondary antibodies (HPR) washed extensively, and visualized by ECL (Amersham Biosciences) and exposure to film (Eastman Kodak Co.). Protein markers were from Bio-Rad.

Endoglycosidase H Treatment—Post-nuclear supernatants were subjected to endoglycosidase H treatment according to the manufacturers protocol (New England Biolabs). Briefly, β-mercaptoethanol-reduced SDS-denatured samples were incubated in 50 mM sodium citrate, pH 5.5, at 33 °C and taken up into sample buffer before analysis by 8% SDS-PAGE.

Conflcence—Transfected HeLa cells were grown on cover-
slips (VWR) and washed twice in PBS before fixing in ice-cold methanol for 10 min. The coverslips were blocked in 0.2% bovine serum albumin (Sigma) in PBS for 30 min and then incubated with primary antibodies in 0.2% bovine serum albumin, PBS, for 1 h. After washing twice in PBS, the coverslips were incubated with the relevant secondary antibody to bovine isothiocyanate-conjugated secondary antibodies (DAKO). Finally, the coverslips were washed twice in PBS before mounting with Vectashield (Vector laboratories) and analyzed with a Zeiss laser-scanning micro-
scope (LSM 510 META).

RESULTS

Identification of a Novel PDI Relative with an Unusual Active Site Motif—The function and specificity of many PDI homologues in the mammalian ER is not fully understood. We were particularly interested in investigating the importance of the PDI CGHC motif for partner/substrate selection and redox control. We set about identifying putative uncharacterized PDI proteins to help us understand this motif in more detail. When PDILT was used as an input sequence, the BLAST search engine (www.ncbi.nlm.nih.gov) readily identified known PDI homologues including PDIp, ERdj5, ERp57, Erp72, P5, PDIR, and ERp44. We also identified a number of interesting, uncharac-
terized sequences that were closely related to PDI. One of these was the hypothetical protein gi:28372543/gi:21757251 (abbre-
viated to GI28/21). GI28/21 shared 27% identity (48% similarity) with PDI and occupied a distinct branch of the PDI family tree (Fig. 1A). Orthologues of this hypothetical human protein can be found in several other species (Fig. 1B). Direct homologues of GI28/21 were not found in other non-vertebrate ge-

rases such as Saccharomyces cerevisiae (yeast), Drosophila melanogaster (fruit fly), and Arabidopsis thaliana (thale cress). GI28/21 has two predicted thioredoxin domains between amino acids 42–153 and 389–493, corresponding to the a and a’ domains of PDI, respectively. Notably, GI28/21 lacks the two classical redox-active CXXC motifs of PDI within the thioredoxin domains. Instead, GI28/21 has an SSKQS sequence that aligns with the WCGHC PDI a domain and a WSKKC sequence that aligns with the PDI a’ domain (Fig. 1C). The a’ domain is most strongly conserved between PDI and GI28/21, sharing 48% identity. In addition, GI28/21 has a short N-
terminal insertion and a C-terminal extension, giving rise to a putative 67-kDa protein, ~10 kDa larger than PDI itself. These additional sequences have no homology with other known proteins. Furthermore, GI28/21 contains a C-terminal KEEL sequence likely to function in ER retention/retrieval and nine putative glycosylation sites. A signal peptide was predicted by the Signal P prediction server (22).

A database analysis of the exon-intron structure of GI28/21 (www.ensembl.org) suggested that it was not a pseudogene and had 12 exons, initiating in exon 2, but otherwise with a similar overall exon structure to PDI (Fig. 1D). GI28/21, thus, represents a gene with a b' domain that is more closely related to PDI than Erp57 or Erp72 but with very different predicted “active site” residues. Given the representation of GI28/21 in
different EST databases, its predicted expression in other species, and its unusual thioredoxin motifs, we extensively characterized GI28/21.

GI28/21 mRNA Is Specifically Expressed in the Testis—Most mammalian PDI family members are widely, if not ubiquitously expressed. To establish whether and where GI28/21 was expressed, we designed GI28/21-specific primers spanning exons 4–9 and used RT-PCR to detect any GI28/21 mRNA. First, we verified the assay. RT-PCR was performed on DNase-treated, in vitro transcribed, DNase-treated mRNA. An mRNA specific product of the expected size (624 base pairs) was obtained. The behavior of the tagged and untagged permeabilized cells. The behavior of the tagged and untagged from various mouse tissues using gi:28372543-specific primers (upper panel) and actin primers as a positive control (lower panel). gi:28372543 mRNA was only expressed in the testis. C, RT-PCR on RNA extracted from HT1080, SK-11, and GC-1 cell lines using gi:28372543 and actin primers (upper and lower panels, respectively). The RT-PCR on testis RNA serves as a positive control. Expression of gi:28372543 could not be detected in the cell lines tested.

To further demonstrate that PDILT was an ER resident protein, we took advantage of the fact that PDILT has nine putative glycosylation sites (NX(S/T), which could potentially be modified upon translocation into the ER. To determine the glycosylation status of PDILT, lysates were treated with endoglycosidase H. Endoglycosidase H digests immature N-glycans but is unable to remove these sugars once they have been modified in the Golgi. Lysates of HeLa cells transfected with PDILT-Myc were subjected to endoglycosidase H digestion and analyzed by SDS-PAGE and Western blotting. Endoglycosidase H digestion results in an increased mobility of the entire pool of PDILT-Myc from ~76 kDa to around 65 kDa (Fig. 3B). The 65-kDa band corresponds to the predicted molecular mass of the mature PDILT-Myc protein without its signal sequence. Taken together, these results demonstrate that PDILT is an ER resident glycoprotein.

PDILT Forms Intermolecular, Disulfide-dependent Complexes during Its Biosynthesis and at Steady State—PDI forms transient intermolecular bonds with substrate proteins and with the disulfide donor Ero1α (20). To investigate whether PDILT also engages in disulfide-bonded complexes, we performed pulse-chase and Western-blotting experiments. HeLa cells mock-transfected or transfected with PDILT-Myc were metabolically labeled or labeled and chased for the indicated times and lysed in the presence of NEM to trap any disulfide-bonded intermediates. Post-nuclear supernatants were subjected to immunoprecipitation with α-Myc and analyzed by non-reducing (Fig. 4A, lanes 1–5) or reducing SDS-PAGE (Fig. 4A, lanes 6–10). PDILT-Myc clearly had a relatively long half-life in HeLa cells of ~24 h. The monomeric form of PDILT-Myc resolved as several discrete bands, probably because of variable usage of glycosylation sites in HeLa cells.

The presence of intramolecular disulfide bonds makes proteins more compact and causes them to migrate more rapidly in non-reducing gels compared with reducing gels. No changes in the migration of the monomeric PDILT-Myc were observed, suggesting that the solitary non-thioredoxin domain cysteine at position 135 does not form an intramolecular disulfide with the C-terminal cysteine of the α’ domain XXXC motif.

Anti-Myc immunoprecipitations analyzed under non-reducing conditions showed some higher molecular weight complexes in addition to the monomeric PDILT-Myc (Fig. 4A, lanes 2–5). These complexes disappeared when the samples were reduced with dithiothreitol, demonstrating that PDILT-Myc engaged in disulfide-dependent intermolecular linkages with other proteins. At least one of these species disappeared after a 60-min chase, suggesting that some complexes formed transiently during biosynthesis. No complexes were seen in this region of the gel in HeLa cells transfected with an unrelated Myc construct (not shown). Note the disappearance of the faster migrating species (presumably degradation products) after a 240-min chase.

FIG. 2. PDILT is specifically expressed in the testis. A, RT-PCR using primers specific for gi:28372543 was performed on in vitro transcribed, DNase-treated mRNA. An mRNA specific product of the expected size (624 base pairs) was obtained. B, RT-PCR on RNA extracted from various mouse tissues using gi:28372543-specific primers (upper panel) and actin primers as a positive control (lower panel). gi:28372543 mRNA was only expressed in the testis. C, RT-PCR on RNA extracted from HT1080, SK-11, and GC-1 cell lines using gi:28372543 and actin primers (upper and lower panels, respectively). The RT-PCR on testis RNA serves as a positive control. Expression of gi:28372543 could not be detected in the cell lines tested.
To determine the relationship between monomeric PDILT-Myc and its partner proteins at steady state, we performed a Western blotting experiment in which HeLa cells transfected with PDILT-Myc were lysed in the presence of NEM to trap disulfide-bonded intermediates. Samples were analyzed by SDS-PAGE under non-reducing and reducing conditions and transferred to polyvinylidene difluoride membranes for analysis (Fig. 4B). Monomeric PDILT-Myc resolved as a broad band that did not change mobility upon reduction, confirming the absence of an intramolecular disulfide bond. Examination of the non-reducing gel showed that a high percentage of PDILT molecules engaged in intermolecular, disulfide-linked complexes of 200 kDa and higher at steady state. Thus, PDILT could interact with partner proteins in the ER both during...
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FIG. 5. PDILT forms intermolecular disulfide-bonded complexes in testis. HeLa PDILT-Myc transfectants, mouse testis, and mouse liver were lysed in the presence of NEM and analyzed by 8% reducing (R) or non-reducing (NR) SDS-PAGE and Western blotting (WB). The PDILT protein is expressed in testis and forms intermolecular disulfide-bonded complexes in testis.

biosynthesis and at steady state. The molecular weights of the steady-state complexes meant that they were unlikely to be PDILT-Myc homodimers, but it is possible that PDILT dimers could engage in multimeric complexes with other accessory proteins. Thus PDILT interacts with proteins in the endoplasmic reticulum in a redox-dependent way despite the lack of a CXXC motif.

Endogenous PDILT Engages in Disulfide-bonded Complexes in the Mouse Testis—PDILT-Myc disulfide-bonded complexes can easily be detected in HeLa transfectants (Fig. 4). To study endogenous PDILT in testis, we used an antiserum raised against a PDILT peptide. Non-tagged PDILT and PDILT-Myc from HeLa transfectants were both specifically detected by the anti-PDILT serum (Fig. 5, compare lanes 2 and 7 with lanes 3 and 8). This confirmed that both tagged and non-tagged PDILT behaved identically and engaged in disulfide-dependent complexes. Anti-PDILT decorated two bands in reduced mouse testis lysate, of which the upper band corresponded to mouse PDILT (Fig. 5, lane 9). The lower band was a cross-reactive protein also present in liver (Fig. 5, lane 10) and kidney (data not shown). The non-reducing gel showed that endogenous, disulfide-dependent mouse PDILT protein-protein interactions occurred in the testis (Fig. 5, lane 4). These complexes were disrupted by dithiothreitol (Fig. 5, lane 9) and were not present in liver or non-transfected HeLa cells (Fig. 5, lanes 1 and 5), demonstrating their specificity. The shift in molecular weight of the endogenous high molecular weight complexes indicates that a larger testis-specific partner is selected by PDILT (Fig. 5, compare lanes 2–4). Our results prove that PDILT is highly expressed in testis and interacts with partner proteins under endogenous conditions.

PDILT, PDI, ERp57, and ERp72 Are All Expressed at High Levels in the Testis—PDILT expression might compensate for the absence of other ER chaperones in the testis, or PDILT might have a unique tissue-specific function. To address this question, we compared the protein levels of ER chaperones and PDI homologues in mouse testis and mouse liver using HeLa transfectants as a positive control. PDILT was detected in the testis, and only the nonspecific background band could be detected in liver (Fig. 6, top left panel). The same samples were used to examine the levels of other chaperones. PDI, ERp57, ERp72, BiP, and calnexin had similar expression when comparing testis and liver (Fig. 6). The oxidoreductase Ero1α, which is expressed at low levels in most cell lines and tissues, was weakly detected in testis and hardly detectable in liver. We conclude that the testes express PDILT in addition to PDI, ERp57, and ERp72 and that PDILT may, therefore, perform a unique function in the testis.

PDILT Can Interact with Ero1α—Ero1α and PDI form complexes in mammalian cells to facilitate disulfide bond formation (20, 21) and ER retro-translocation. Given the strong sequence similarity between PDI and PDILT and having seen that Ero1α and PDILT are both expressed in testis, we investigated whether PDILT could bind to Ero1α. Because HeLa cells, other cell lines, and testis express low levels of endogenous Ero1α and the PDILT antisera does not immunoprecipitate, HeLa cells were co-transfected with PDILT-Myc and Ero1α. The cells were lysed in the presence of NEM, and the post-nuclear supernatants were subjected to immunoprecipitation with αMyc or anti-Ero1α. The immunoprecipitations (split equally) and total cell lysates were run on reducing SDS-PAGE and analyzed by Western blotting. Both Ero1α (~60 kDa) and PDILT (76 kDa) could be detected in the lysates (Fig. 7A, lanes 1 and 5). The anti-Ero1α immunoprecipitate brought down both Ero1α and PDILT-Myc (Fig. 7A, lanes 6 and 4, respectively), and the αMyc immunoprecipitate brought down both PDILT-Myc and Ero1α (Fig. 7A, lanes 2 and 8), indicating that PDILT and Ero1α interact. The anti-Ero1α D5 antibody is inefficient in immunoprecipitations, and immunoprecipitated a relatively small pool of Ero1α from the transfected cells (Fig. 7A, lane 6). Thus, the amount of Ero1α associated with PDILT-Myc corresponded to a sizeable fraction of the immunoprecipitated pool of molecules (compare Fig. 7A, lane 6 with lane 8). A longer exposure of this region of the gel is shown in the lower panel of Fig. 7A. Note that the 50-kDa antibody heavy chains from the antibodies used in the immunoprecipitations cross-reacted with the secondary antibody, as expected (Fig. 7, lanes 2, 4, 6, and 8). The additional 60–65-kDa bands in Fig. 7A and B, lanes 1 and 2, may represent degradation products of PDILT.

To demonstrate the specificity of the PDILT-Ero1α interaction, PDILT-Myc HeLa transfectants were analyzed as above and subjected to immunoprecipitation with αMyc or αPDI (instead of αEro1α) followed by Western blotting to detect either PDILT-Myc or PDI (Fig. 7B). No interaction could be detected between PDILT-Myc and PDI in either the αMyc or αPDI immunoprecipitates (Fig. 7B, lanes 8 and 4, respectively) even though the detection levels of PDI were similar to those of Ero1α, and the αPDI antibody was more efficient in immunoprecipitation than

FIG. 6. PDILT and chaperone expression in the testis. Cell and tissue lysates were prepared as in Fig. 5 and analyzed by reducing SDS-PAGE and Western blotting with the indicated antisera.

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FIG. 7. PDILT can interact with Ero1α in the testis. A. The 50-kDa antibody heavy chains from the antibodies used in the immunoprecipitations cross-reacted with the secondary antibody, as expected (Fig. 7, lanes 2, 4, 6, and 8). The additional 60–65-kDa bands in Fig. 7A and B, lanes 1 and 2, may represent degradation products of PDILT.
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D5 (compare Fig. 7, A, lanes 5 and 6, and B, lanes 5 and 6). Thus, PDILT specifically interacts with Ero1α in the endoplasmic reticulum despite the lack of a conventional CGHC motif. Conserved, non-redox active sequences shared by PDI and PDILT are, therefore, important determinants of Ero1α binding.

**DISCUSSION**

Disulfide bonds are an essential structural and regulatory feature of many secretory and plasma membrane proteins (23). The formation and isomerization of disulfide bonds is catalyzed by PDI and PDI-related proteins, and although the function of PDI has been well studied in vitro and in living cells, much less is known about the function of its relatives. It is clear that many different folding enzymes are expressed in the ER, and the challenge now is to understand why they are so numerous and what contribution they all make to protein secretion and degradation. Here, we have characterized a new, tissue-specific member of the PDI family, which we have called PDILT because of its presence in the testis. The conserved exon structure of PDILT and PDI emphasizes their relatedness, and the appearance of PDILT in vertebrates but not lower organisms suggests that it has arisen recently to perform a gonad-specific function. PDILT shares 27% identity (48% similarity) with the presence of physiological concentrations of zinc ions (29). The range of testis-specific redox proteins is remarkable, described (33), and calmegin knock-out mice are infertile (34).

A more probable alternative is that an SXXC protein has evolved to fulfill a different specialist function in the ER. In support of this idea, Erp28 (Erp29 in rat), the homologue of wind, is a PDI family member with no CXXC motif that has been postulated to have a chaperone-only function (30). In vivo experiments together with detailed studies of purified PDILT in vitro will be required to establish whether PDILT has the potential to perform chaperone, isomerase, or other redox reactions.

PDILT is not the only unusual ER protein to reside in the testis. The testes are home to a number of ER resident selenoproteins such as Sep15 (31) and sulfhydryl oxidase (32). A testis-specific homologue of calnexin called calmegin has been described (33), and calmegin knock-out mice are infertile (34). An entire testis-specific thioredoxin system also exists in developing spermatids, comprising Sptrrx-1, Sptrrx-2, and Sptrrx-3 (35). The range of testis-specific redox proteins is remarkable, yet the precise function of ER proteins in the gonads is poorly understood. The special pH, redox, and temperature environments in testis or the need to produce a certain type of substrate protein may necessitate the expression of these additional, testis-specific, chaperones, and folding factors. We have shown that PDILT can form intermolecular complexes with proteins in the ER of transfected cells (Fig. 4) and in the testis (Fig. 5). The identification of PDILT-interacting proteins or the aberrantly folded glycoprotein is degraded (by the proteasome) (25). In this respect it will be interesting to determine what effect glycosylation has on PDILT function and partner selection.

PDI needs both cysteines of its redox active site for oxidase activity, but the N-terminal cysteine is sufficient for isomerase function. In our current understanding, PDILT, with only the C-terminal cysteine, should not be redox-active. In terms of mechanism, however, little is known about how electron transfer is coupled between the protein to be oxidized and the (C/S)XXC(C/S) motif of the PDI. Apart from the Drosophila wind protein (26), no PDI family crystal structures are available. Individual PDI domains have been solved by NMR (see e.g. Refs. 27 and 28), but substrate docking to PDI itself has never been visualized. Because isomerization requires only one free Cys residue, an SXXC motif might dock with a substrate and form a mixed disulfide with its single free cysteine. Although unlikely, it should also be noted that many thioredoxin family proteins can dimerize, and PDILT dimers might co-operate with other proteins to form redox active, high molecular weight complexes. In fact, PDI has recently been shown to dimerize in the presence of physiological concentrations of zinc ions (29).
substrates in the testis itself will, therefore, help address this issue. Surprisingly, little is known about ER chaperone expression and coordinated function in the testis. Our experiments reveal that PDI, Erp57, Erp72, BiP, and calnexin proteins are all expressed here (Fig. 6) despite the existence of other specialized ER factors. Thus, PDILT does not replace PDI, Erp57, or Erp72 but, rather, has a distinct function in the male reproductive system.

We have used PDILT to explore the molecular requirements for PDI family proteins to bind to ER oxidoreductases (Ero1α) (Fig. 7). It has been previously demonstrated that Ero1α is required to oxidize PDI and maintain the oxidative protein folding cycle in the ER (21). The only other protein known to form complexes with Ero1α is Erp44 (36), which plays a part in thiol-mediated ER retention of Ero1α and immunoglobulins (37). Ero1α does not interact with Erp57, Erp72, P5, and PDIR (20), and the biochemical basis for this Ero1α selectivity remains unknown. The interaction of PDILT with Ero1α demonstrates that an intact CGHC motif with an N-terminal cysteine is not required for a PDI to interact with an ER oxidoreductase per se. Thus, we envisage a model in which non-covalent “docking” followed by disulfide exchange interactions is required for PDI binding and electron transfer to Ero1α. The residues important for docking but not electron transfer are likely conserved between PDI and PDILT. In this respect it is noteworthy that ERp57, which interacts with calnexin rather than Ero1α, has a divergent b’ domain that determines its substrate selectivity for lectins (38). PDI and PDILT share a number of charged and hydrophobic b’ domain amino acid residues that are not conserved in ERp57. Of particular interest are the PDILT sequences L279-LF and K306-ILFILVDAD (Fig. 1). These residues are almost identical in PDI and are important in PDI substrate binding (39). Given that Ero1p has a disordered, solvent-exposed peptide loop (40), our results with PDILT will enable us to test the role of the PDI b’ domain in Ero1α binding by using informed site-directed mutagenesis.

Ero1α is expressed at low levels in testis, and the inefficiency of PDILT and Ero1α antibodies in immunoprecipitation prevents the interaction from being analyzed in the tissue itself. Could a PDILT-Ero1α interaction be biologically important in the testis? It is possible that PDILT is redox active as an isomerase and passes electrons to Ero1α. However, the position of the PDILT SXCC cysteine makes this unlikely. Ero1α and PDI also contribute to ER protein degradation (reduction and unfolding), and perhaps the interaction between PDILT and Ero1α is related to ER degradation. Another possibility is that PDILT retains chaperone-only function and sees Ero1α as a substrate to be folded. If this latter explanation were true, it would imply that Ero1α requires specialist chaperone help in the testis. Regardless of their physiological importance, further study of PDILT-Ero1α complexes will provide useful information about both the covalent and non-covalent interactions required for a PDI protein to associate with an Ero protein.

The details of how thioredoxin-domain proteins contribute to ER function and protein folding are beginning to emerge. We have identified and characterized the first eukaryotic SXCC motif protein in the endoplasmic reticulum, and we have shown that such a protein participates in disulfide-dependent inter-