Physical and Functional Interaction of Transmembrane Thioredoxin-related Protein with Major Histocompatibility Complex Class I Heavy Chain: Redox-based Protein Quality Control and Its Potential Relevance to Immune Responses

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In the endoplasmic reticulum (ER), a variety of oxidoreductases classified in the thioredoxin superfamily have been found to catalyze the formation and rearrangement of disulfide bonds. However, the precise function and specificity of the individual thioredoxin family proteins remain to be elucidated. Here, we characterize a transmembrane thioredoxin-related protein (TMX), a membrane-bound oxidoreductase in the ER. TMX exists in a predominantly reduced form and associates with the molecular chaperon calnexin, which can mediate substrate binding. To determine the target molecules for TMX, we apply a substrate-trapping approach based on the reaction mechanism of thiol-disulfide exchange, identifying major histocompatibility complex (MHC) class I heavy chain (HC) as a candidate substrate. Unlike the classical ER oxidoreductases such as protein disulfide isomerase and ERp57, TMX seems not to be essential for normal assembly of MHC class I molecules. However, we show that TMX–class I HC interaction is enhanced during tunicamycin-induced ER stress, and TMX prevents the ER-to-cytosol retrotranslocation of misfolded class I HC targeted for proteasomal degradation. These results suggest a specific role for TMX and its mechanism of action in redox-based ER quality control.

INTRODUCTION

Many secretory and membrane proteins are cotranslationally transported into the endoplasmic reticulum (ER), in which they acquire their correct conformation (Ellgaard and Helenius, 2003). The formation of disulfide bonds between cysteine residues is critical for the proper folding and assembly of proteins entering the secretory pathway (Sevier and Kaiser, 2006). The ER contains several oxidoreductases classified in the thioredoxin superfamily that catalyze the formation and rearrangement of disulfide bonds. Thioredoxin has been demonstrated to catalyze the reduction of disulfide bonds and thereby participate in many thiol-dependent cellular processes (Nakamura, 2005). Most of the ER oxidoreductases contain thioredoxin-like domains with their characteristic CXXC active site motifs that are responsible

for catalyzing thiol-disulfide exchange reactions (Ellgaard and Ruddock, 2005).

Although many ER oxidoreductases, such as protein disulfide isomerase (PDI) and ERp57, are soluble proteins in the ER lumen, there also exist several membrane-bound oxidoreductases. In a search for genes induced by transforming growth factor- β , we identified a member of the thioredoxin family, transmembrane thioredoxin-related protein (TMX) (Akiyama *et al.*, 2000; Matsuo *et al.*, 2001). TMX contains one catalytic thioredoxin-like domain with a unique active site motif, CPAC, and a single transmembrane region. TMX orthologues have been found in other animal species, including mammals, *Drosophila melanogaster*, and *Caenorhabditis elegans* (Ko and Chow, 2002) but not in plants, fungi, or prokaryotes. The thioredoxin-like domain of TMX is present in the ER lumen and shows reductase and isomerase activity *in vitro* (Matsuo *et al.*, 2004). TMX2 and TMX3 have also been reported in the literature as human transmembrane oxidoreductases (Meng *et al.*, 2003; Haugstetter *et al.*, 2005). The physiological roles of these TMX proteins still remain unclear, but, considering their structural characteristics, these TMX proteins might preferentially act on membrane-anchored substrates.

The presence of a large variety of thioredoxin family members suggests a complex mechanism of redox regulation in the mammalian ER. Whether the family members are functionally redundant or each plays a separate and distinct role remains to be elucidated. For a better understanding of

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Abbreviations used: AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; CST, castanospermine; DPS, 4, 4'-dipyridyl disulfide; DTT, dithiothreitol; ER, endoplasmic reticulum; HC, heavy chain; HLA, human leukocyte antigen; MHC, major histocompatibility complex; NEM, N-ethylmaleimide; PDI, protein disulfide isomerase; UPR, unfolded protein response.

their mechanisms of action, it is necessary to determine the cellular substrates and binding partners for the individual proteins. In the present study, we applied a substrate-trapping approach based on the reaction mechanism of thiol-disulfide exchange. Using a trapping mutant of TMX that enables the stabilization of the mixed disulfide intermediates between the enzyme and substrate, we identify major histocompatibility complex (MHC) class I heavy chain (HC) as a target molecule for TMX. As shown in previous studies, ER oxidoreductases such as ERp57 and PDI associate with MHC class I molecules during biosynthesis (Hughes and Cresswell, 1998; Antoniou *et al.*, 2003), and they play critical roles in the assembly of the peptide-loading complex (Garbi *et al.*, 2006; Park *et al.*, 2006; Kienast *et al.*, 2007). Unlike these oxidoreductases in the ER lumen, TMX did not affect the surface expression of class I molecules, but we found that TMX prevents the retrotranslocation of class I heavy chain for proteasomal degradation, suggesting a specific role for TMX in the retention and refolding of misfolded proteins in the ER.

MATERIALS AND METHODS

Antibodies

Rabbit anti-TMX polyclonal antibody was used as described previously (Matsuo *et al.*, 2004). Mouse monoclonal antibodies to calnexin (AF8) (David *et al.*, 1993) and MHC class I heavy chain (HC10) (Stam *et al.*, 1986) were generously provided by Dr. Masahiko Sugita (Kyoto University, Kyoto, Japan). Rabbit polyclonal antibodies specific for calreticulin (SPA-600), calnexin (SPA-865), and PDI (SPA-890) were from Assay Designs (Ann Arbor, MI). Peroxidase-conjugated mouse monoclonal antibodies to myc or V5 were from Nacalai Tesque (Kyoto, Japan), and anti-myc agarose (9E10) was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-BiP was from BD Biosciences (San Jose, CA), mouse monoclonal anti- α -tubulin was from Sigma-Aldrich (St. Louis, MO), and fluorescein isothiocyanate (FITC)-labeled anti-human MHC class I (W6/32) was from eBioscience (San Diego, CA). Mouse anti-thioredoxin monoclonal antibody (mAb) (ADF11) was described previously (Kogaki *et al.*, 1996).

Expression Vectors

TMX cDNA was subcloned into pEF6/Myc-His A (Invitrogen, Carlsbad, CA) for the selection of stable cell lines with blasticidin. To construct TMX/C59A, TMX/C56A-C59A, and TMX/P101T, substitution mutations were introduced by polymerase chain reaction (PCR) by using the following primers: C59A, F1 and R2; C56A-C59A, F3 and R4; and P101T, F5 and R6. PCR products encoding TMX140 (primer set: F7 and R8), TMX180 (F7 and R9), or TMX215 (F7 and R10) were amplified from the TMX cDNA and inserted into pcDNA3.1/MycHis A vector (Invitrogen). For the expression of soluble TMX, an ER retention motif (KDEL) was introduced by PCR at the C terminus of TMX140-myc with primers F11 and R12, and the PCR products were cloned into pcDNA3.1(-) (Invitrogen). For the inducible expression of TMX/P101T-myc, the cDNA was subcloned into the tetracycline-regulated expression vector pTRE2pur (Clontech, Mountain View, CA). The coding sequences of human Ero1 α and Ero1 β were amplified by PCR using the first-strand cDNAs prepared from A549 cells (primers used were as follows: Ero1 α , F13 and R14; and Ero1 β , F15 and R16). V5-tagged Ero1s were generated by inserting the PCR products into pcDNA3.1/V5-His vector (Invitrogen). Human leukocyte antigen B27 (HLA-B27) cDNA in pSR α -neo was a kind gift from Dr. Masahiko Sugita (Kyoto University, Kyoto, Japan) (Sugita and Brenner, 1995). The full-length HLA-B27 coding sequence was amplified by PCR and inserted into pcDNA3.1/V5-His vector to add a V5 tag at the C terminus. Plasmids were transfected into cells with FuGENE6 (Roche Diagnostics, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen) for transient expression. The sequences of the primers used are given in Supplemental Table S1.

Cell Culture

Human lung adenocarcinoma cell line A549 (JCRB0076) and human embryonic kidney cell line 293 (JCRB0068) were obtained from Health Science Research Resources Bank (Osaka, Japan). A549, 293, and HeLa cells were maintained in DMEM (Sigma-Aldrich) supplemented with 10% fetal calf serum (Invitrogen), and human T cell leukemia line Jurkat was cultured in RPMI-1640 medium (Sigma-Aldrich) with 10% fetal calf serum. To induce the unfolded protein response, cells were treated with brefeldin A, thapsigargin, and tunicamycin from Nacalai Tesque for the indicated periods. To generate cells stably expressing TMX-myc, pEF6-TMX-myc was transfected into A549 cells by using the Nucleofector system (Amara Biosystems, Gaithersburg,

MD). Cells were cultured in selective medium containing 2 μ g/ml blasticidin (InvivoGen, San Diego, CA), and resistant colonies were picked and cultured. For the inducible expression of the trapping mutant, pTRE2pur-TMX/P101T-myc was transfected into T-REx 293 cells (Invitrogen) stably expressing the tetracycline repressor using FuGENE6 transfection reagent (Roche Diagnostics). A clonal cell line was established by selection with 0.5 μ g/ml puromycin (InvivoGen) and 100 μ g/ml G418 (Nacalai Tesque).

Determination of the Redox State

Cells were incubated for 10 min at 37°C with or without 0.5 mM 4,4'-dipyridyl disulfide (DPS; Nacalai Tesque) or 5 mM dithiothreitol (DTT; Nacalai Tesque), washed with ice-cold phosphate-buffered saline (PBS) containing 20 mM N-ethylmaleimide (NEM; Nacalai Tesque) immediately or after incubation in fresh medium to alkylate free thiols. The cells were lysed in 50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 20 mM NEM, and 0.5% NP-40, and the lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions. The redox state of TMX was determined by monitoring the mobility shift of oxidized versus reduced proteins in the immunoblot analysis. In an alternative method, after acid denaturation by trichloroacetic acid (TCA) proteins with free thiol groups were modified with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) (Kobayashi *et al.*, 1997). In brief, cells were treated with 10% TCA in PBS for 15 min and then lysed in 50 mM Tris-HCl, pH 6.8, 1% SDS, and 1 mM AMS. The samples were incubated for 30 min at room temperature and subjected to nonreducing SDS-PAGE followed by immunoblotting.

RNA Interference

For suppression of the endogenous TMX gene, the following synthetic oligonucleotides containing a 19-nt target sequence of human TMX (GGAGACTGGATGATAGAAT) were annealed and inserted into pSUPER RNAi vector (Oligoengine, Seattle, WA): 5'-GATCCCGGAGACTGGATGATAGAATTTCAAGAGAATTCTATCATCCAGTCTCCTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAGGAGACTGGATGATAGAATTCTCTTGAAATTCATATCATCCAGTCTCCGGG-3'. Either the resulting plasmid pSUPER-TMX or control pSUPER vector was transfected into HeLa cells together with pUCDV-BSD (Kaken Pharmaceutical, Tokyo, Japan) containing the blasticidin resistance marker. Transfection was performed using the Nucleofector system (Amara). The cells were cultured in selective medium containing 2 μ g/ml blasticidin, and resistant colonies were isolated. The level of TMX gene knockdown was evaluated by immunoblot analysis with anti-TMX antibody.

Measurement of Cell Viability

Cell viability was measured using cell count reagent SF containing the tetrazolium salt WST-8 (Nacalai Tesque) following the manufacturer's instructions. Each sample was assayed in triplicate, and the experiments were repeated twice.

Immunoprecipitation and Immunoblotting

Cells expressing TMX-myc were incubated in PBS containing 20 mM NEM for 10 min on ice to preserve mixed disulfides. Cells were then lysed in 50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 20 mM NEM, and 0.5% NP-40, and insoluble materials were precipitated by centrifugation at 20,000 $\times$ g for 10 min. The resulting supernatants were subjected to immunoprecipitation with anti-myc agarose. Immunoprecipitates were washed with lysis buffer and eluted in buffer containing 150 μ g/ml c-myc peptide (Sigma-Aldrich). For myc/calnexin sequential immunoprecipitation, the myc peptide eluates were diluted in lysis buffer and further immunoprecipitated using anti-calnexin antibody. In immunoprecipitation experiments using anti-calnexin or anti-MHC class I heavy chain, immune complexes were recovered with protein A-Sepharose and eluted with SDS-PAGE sample buffer. Samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane Immobilon-P (Millipore, Billerica, MA) followed by blocking in Tris-buffered saline/Tween 20 containing 0.5% skim milk (Nacalai Tesque). Immunoblots were probed with the indicated antibodies, and immunodetection was carried out using enhanced chemiluminescence reagents (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom).

Two-dimensional Gel Electrophoresis

Immunoprecipitates were separated by SDS-PAGE under nonreducing conditions. The excised gel strip was incubated in SDS sample buffer containing 50 mM DTT for 30 min and placed horizontally on the top of a new SDS-PAGE gel. After separation on the second gel, proteins were transferred to the membrane and analyzed by immunoblotting.

Flow Cytometry

Cells were detached with enzyme-free cell dissociation buffer (Invitrogen) and stained with FITC-labeled mAb W6/32 for 20 min at 4°C. Cells were analyzed using a FACSCalibur (BD Biosciences) and Flowjo software (Tree-Star, Ashland, OR).

Subcellular Fractionation

Cells were fractionated into cytosolic and membrane fractions using Proteo-Extract subcellular proteome extraction kit (EMD Biosciences, San Diego, CA). For assessing the translocation of MHC class I heavy chain, cells were transfected with HLA-B27-V5. Forty-eight hours after transfection, cells were incubated with or without 10 μ M MG-132 (Peptide Institute, Osaka, Japan) for 4 h at 37°C and then fractionated into cytosolic and membrane fractions. The resulting samples were separated by SDS-PAGE followed by immunoblotting.

RESULTS

TMX Is Predominantly Reduced In Vivo

The activity of oxidoreductases classified in the thioredoxin family depends on a pair of cysteine residues in a CXXC active site motif, which shuttle between the oxidized (disulfide) and reduced (dithiol) states. To examine the redox state of TMX active site cysteines, three different cell lines (293, A549, and Jurkat) were treated with thiol-specific alkylating agent NEM before cell lysis to prevent thiol-disulfide exchange. In control samples, cells were incubated with DTT to reduce the proteins or DPS to oxidize them before NEM treatment. Cell lysates were separated by SDS-PAGE under nonreducing conditions, and the redox state of TMX was determined by immunoblot analysis using an anti-TMX antibody. The oxidized proteins with intramolecular disulfide bonds were more compact and migrated faster than the reduced forms (Figure 1A, lanes 2 and 3, lanes 5 and 6; and E, lanes 2 and 3). At steady state, TMX was predominantly reduced in all cell lines tested (Figure 1A, lanes 1 and 4; and E, lane 1) although a small amount of oxidized proteins were present in 293 and Jurkat cells.

To ensure the reliability of the results, we used an alternative method for examining the redox state of oxidoreductases (Kobayashi *et al.*, 1997). 293 cells were treated with trichloroacetic acid to denature and precipitate whole cell proteins, and the precipitates were dissolved in buffer containing alkylating agent AMS, which modifies free thiol groups. Covalent modification with AMS increases the molecular mass by ~0.54 kDa per thiol group, thereby causing a shift in mobility upon separation by SDS-PAGE, and reduced (AMS-modified) proteins migrate slower than oxidized forms. As shown in Figure 1B, the majority of TMX migrated with the mobility of the reduced protein. TMX contains seven cysteines, two of which constitute the active site. To test whether the mobility shift after modification with AMS reflects alkylation of the active site cysteines, we examined the redox state of exogenously expressed wild-type TMX and its mutant, in which two cysteines within the active site were replaced with serines. In cells transfected with wild-type TMX, two bands corresponding to reduced and oxidized proteins were detected (Figure 1C, lane 1), which was consistent with the redox properties of endogenous TMX. In the case of the cysteine mutant, however, we could only see a single band of the protein irrespective of DTT pretreatment (Figure 1C, lanes 3 and 4). Thus, the decreased mobility after AMS modification truly indicated that the active site was in the reduced state.

In the ER, there exist cellular mechanisms controlling the redox state of the oxidoreductases. In mammalian cells, glutathione, a low-molecular-weight thiol compound, has been shown to directly reduce an oxidoreductase and maintain the protein in a reduced form (Jessop and Bulleid, 2004). In comparison, Ero1 has been shown to reoxidize PDI for further catalytic cycles in an oxidative folding pathway (Frandsen and Kaiser, 1999; Mezghrani *et al.*, 2001). Considering that TMX is partially oxidized in cultured human cells, we investigated the redox state of TMX in cells overexpressing

Figure 1. TMX is predominantly reduced in cultured cells. (A) A549 or 293 cells were either left untreated (lanes 1 and 4) or treated with DTT (5 mM, lanes 2 and 5) or DPS (0.5 mM, lanes 3 and 6) for 10 min. After cell lysis in NEM-containing buffer, the redox state of TMX was analyzed by immunoblotting under nonreducing conditions. The reduced (Red) and oxidized (Ox) forms of TMX are indicated. (B) The redox state of TMX was determined by rapid acidification and AMS-alkylation technique as described in *Materials and Methods*. Samples from DTT-pretreated cells (+) were used as a marker for the reduced form of the protein. (C) 293 cells were transfected with wild-type TMX-myc (WT) or C56S-C59S mutant (CS), and the redox state of the exogenous TMX protein was analyzed as described in B. (D) 293 cells were transfected with control vector (V), Ero1 α -V5 (α), or Ero1 β -V5 (β), and 48 h after transfection the redox state of TMX was determined as described in A. Bottom, samples separated under reducing conditions were analyzed by immunoblotting with anti-V5 antibody for detecting Ero1-V5. (E) Jurkat cells were treated as described in A, and the redox state of TMX was determined. In lane 4, cells were treated with DPS and further incubated in fresh medium without DPS for 3 h before cell lysis.

either human Ero1 α (Cabibbo *et al.*, 2000) or Ero1 β (Pagani *et al.*, 2000). Unlike the Ero1-PDI pathway, both Ero1 α and Ero1 β could not alter the redox state of TMX (Figure 1D), suggesting that TMX seemed not to be a substrate for Ero1. Recovery from protein oxidation by DPS also was examined. In Figure 1E, DPS-treated Jurkat cells were further incubated in the absence of DPS, and the shift of the redox state was monitored. Three hours after removal of the oxidizing agent, we observed that some proteins were restored to the reduced forms, which were not detectable immediately after DPS treatment (Figure 1E, lane 4). Thus, TMX seemed to be maintained in a reduced state with exposed cysteine residues, which may be involved in the reduction/isomerization of the target molecules.

TMX Expression Is Not Induced by the Unfolded Protein Response

Cells respond to the accumulation of unfolded proteins in the ER by activating an intracellular signaling pathway called the unfolded protein response (UPR) (Ron and Walter, 2007). Activation of the UPR promotes the expression of molecular chaperones and folding catalysts such as BiP and PDI. We tested whether the expression of TMX was up-regulated by cellular stresses that induce the UPR. A549 cells were treated

Figure 2. TMX is not up-regulated by the unfolded protein response. A549 cells were treated with brefeldin A (BFA; 1 μ g/ml), thapsigargin (TG; 0.5 μ M), or tunicamycin (TM; 5 μ g/ml) for the indicated times to induce the UPR. Cell lysates were analyzed by immunoblotting with anti-BiP and anti-TMX antibodies (left). α -Tubulin (TUB) was used as loading control. Fold changes of BiP and TMX expression were determined after normalization to α -tubulin (right). Experiments were repeated at least three times, and representative data are shown.

with brefeldin A, thapsigargin, and tunicamycin. These agents induced the UPR, as indicated by the up-regulation of the UPR marker BiP (Figure 2). Under these stress conditions, we observed no significant increase in TMX protein expression, suggesting that TMX gene may not be directly regulated by the UPR. Similar results were obtained in 293 cells (data not shown).

Effects of TMX Gene Knockdown against ER Stress

To investigate the effect of TMX down-regulation, we used RNA interference to generate stable knockdown of TMX expression. HeLa cells stably expressing short interfering RNA (siRNA) against TMX were established. Immunoblot analysis showed that TMX expression was efficiently reduced in these TMX knockdown cells (Figure 3A, bottom), and specific gene knockdown was confirmed by unaltered expression of α -tubulin (Figure 3A, third panel). TMX down-regulation did not affect the expression of BiP and another oxidoreductase PDI (Figure 3A, top and second panels, respectively). The unaltered expression of these UPR markers indicated that, under normal culture conditions, TMX knockdown did not cause severe ER dysfunction, leading to the activation of the UPR. When cells were treated with ER stress-inducing reagent tunicamycin, the early

Figure 3. Effects of TMX down-regulation against ER stress. (A) HeLa cells stably expressing siRNA against TMX were established. Lysates from control (Ctrl) and TMX knockdown cells (KD1 and KD2) were analyzed by immunoblotting for BiP, PDI, α -tubulin (TUB), and TMX. (B) Parental HeLa cells and the cell clones (Ctrl, KD1, and KD2) were treated with tunicamycin (TM; 2 μ g/ml) to induce the UPR for the indicated times. Cell lysates were prepared and analyzed by immunoblotting with anti-BiP and anti-TUB. (C) Control and TMX knockdown cells were incubated in the absence or presence of TM (2 or 5 μ g/ml) for 48 h, and cell viability was examined by colorimetric WST-8 assay. Results are expressed as mean $\pm$ SD of triplicate samples. * p < 0.05 (Student's t test). Data are representative of two experiments.

stress response of the TMX knockdown cells seemed to be normal, because the induction of BiP after tunicamycin treatment was unaffected (Figure 3B). After longer incubation, however, down-regulation of TMX rendered cells more sensitive to tunicamycin, resulting in decreased cell viability (Figure 3C), suggesting the protective role of TMX under stress conditions in these cells.

TMX Associates with Calnexin but Not with Calreticulin

Apart from PDI and Erp57 with broader substrate specificities, less is known about interacting proteins for other oxidoreductases in the ER. To clarify the specific function of TMX, we performed coimmunoprecipitation experiments for the identification of proteins interacting with TMX. We first tested whether TMX can interact with ER molecular chaperones. Cells stably expressing myc-tagged TMX were lysed and immunoprecipitated with anti-myc antibodies. We found that calnexin, a membrane-bound chaperone in the ER, was coprecipitated with TMX-myc (Figure 4A, top). Furthermore, endogenous TMX was coprecipitated by anti-calnexin antibodies, confirming their specific interaction (Figure 4B). This association might not be due to lectin-like

Figure 4. Association of TMX with calnexin. (A) Lysates (left) and anti-myc immunoprecipitates (right, IP: myc) from A549 cells stably transfected with control vector (V) or TMX-myc (TMX) were analyzed by immunoblotting (IB) with antibodies to calnexin (CNX), calreticulin (CRT), and myc. (B) 293 cells were subjected to immunoprecipitation with control (Ctrl) or anti-calnexin (CNX) antibody, and immunoblotted with antibodies to calnexin and TMX. (C) Top, schematic structure of TMX and its C-terminally truncated variants. SP, signal peptide; TRX domain, thioredoxin-like domain; TM, transmembrane domain. Bottom, 293 cells were transfected with different TMX-myc variants as indicated. Cell lysates (lanes 1–3) and anti-myc immunoprecipitates from the culture supernatants (lanes 4–6) were analyzed by immunoblotting with anti-myc antibody. (D) Left, TMX140-myc with (–) or without (+) ER-retention signal KDEL was transfected into 293 cells. The cells were fractionated into cytosolic (C) and membrane fractions (M), and the culture supernatants (S) were immunoprecipitated with anti-myc. The resulting samples were immunoblotted with anti-myc. Right, separation of cytosolic and membrane fractions was confirmed by immunoblotting with anti-thioredoxin (TRX, cytosolic marker) and anti-TMX (ER). (E) 293 cells transfected with control vector (V), TMX-myc (WT), TMX215-myc (215), TMX140-mycKDEL (140+), TMX/C56S-C59S-myc (CS), or TMX/C56A-C59A-myc (CA) were subjected to immunoprecipitation with anti-myc and immunoblotted with anti-CN and anti-myc.

chaperone activity of calnexin because TMX does not contain any consensus sequence for *N*-glycosylation and carries no *N*-linked glycans recognized by calnexin (Williams, 2006). The interaction was rather specific for calnexin, as calreticulin, a soluble homologue of calnexin in the ER lumen, was not coprecipitated with TMX-myc (Figure 4A, middle).

Transmembrane Domain Is Responsible for the Binding of TMX to Calnexin

To identify the domain of TMX required for binding to calnexin, we constructed several C-terminally truncated variants (Figure 4C). Each of the TMX variants contains a myc epitope tag at the C terminus for immunoprecipitation experiments. When we deleted only the cytoplasmic tail of TMX (TMX215-myc), the proteins were retained within the cells (Figure 4C, lane 3), whereas the proteins lacking the predicted transmembrane domain (TMX140-myc and TMX180-myc) were secreted into the culture medium (Figure 4C, lanes 4 and 5, respectively). Thus, TMX was anchored in the membrane by a hydrophobic stretch of amino acid residues (aa 181–203) located near the C terminus of the protein. Soluble TMX without the transmembrane domain was produced by adding a 'KDEL' ER retention motif at the C terminus of the luminal part of TMX (TMX140-mycKDEL), thereby targeting the protein to the ER (Munro and Pelham, 1987). The KDEL sequence prevented the secretion of the protein, and after subcellular fractionation TMX140-mycKDEL was found in the membrane fraction containing the ER (Figure 4D, left) exactly like endogenous TMX. The purity of the membrane fraction was confirmed by the absence of a cytosolic marker thioredoxin (Figure 4D, right).

Using these TMX variants, we tested each for binding to calnexin. Although TMX without its cytoplasmic segments (TMX215-myc) was still capable of binding calnexin (Figure 4E, lane 3), no interaction could be detected between soluble TMX and calnexin (Figure 4E, lane 4), suggesting that the transmembrane domain is responsible for their association. We also examined whether the CXXC motif of TMX was involved in calnexin binding. Substitution of the two cysteines within the active site with alanine residues (C56A-C59A) did not alter the interaction with calnexin (Figure 4E, lane 6), demonstrating that an intact CXXC motif was not required for TMX to associate with calnexin. Although the C56A-C59A mutant retained the ability to interact with calnexin, cysteine-to-serine mutation (C56S-C59S) significantly reduced the binding affinity for calnexin (Figure 4E, lane 5). Potentially, the substitution to polar serine residues with hydroxyl groups induced conformational change in the protein and affected the stability of TMX-calnexin complex. Thus, TMX specifically interacts with calnexin despite the lack of *N*-linked glycans suggesting their cooperative behavior in the protein folding process in the ER.

Mutation of Cys59 or Pro101 of TMX Leads to the Accumulation of Disulfide-linked Complexes

During the thiol-disulfide exchange reactions, it is thought that a transient mixed disulfide bond is formed between an oxidoreductase and its substrate. To investigate whether TMX can participate in the formation of mixed disulfides with other proteins, cells transfected with myc-tagged TMX were treated with NEM to trap disulfide-linked intermediates and analyzed by immunoblotting under nonreducing conditions. Only the monomeric TMX-myc was observed, and we failed to detect any mixed disulfides (Figure 5A, lane 1). Because such transient mixed disulfides tend to be unstable, we attempted to stabilize the complex by mutating a

Figure 5. Trapping mutants of TMX. (A) Lysates from 293 cells transfected with the indicated TMX-myc variants were separated under nonreducing (lanes 1–3) or reducing conditions (lanes 4–6) and immunoblotted with anti-myc. (B) top, 293 cells harboring the tetracycline-inducible TMX transgene (293:tet-TMX/P101T-myc) were treated with doxycycline (dox; 0–0.8 μg/ml) for 24 h. The expression of endogenous TMX and TMX/P101T-myc was examined by immunoblotting with anti-TMX antibody. Bottom, 293:tet-TMX/P101T-myc was treated with castanospermine (CST; 0, 0.5, and 1.0 mM) for 8 h and further incubated in the presence of dox (0.4 μg/ml) for 15 h. The formation of mixed disulfides was analyzed as described in A. (C) 293 cells were transfected with control vector, TMX/P101T-myc (P101T), or TMX/C56A-C59A-myc (C56-59A), and the lysates were immunoprecipitated with anti-calnexin (CNX) antibody. The immunoprecipitates were assessed for the presence of TMX-substrate complexes by immunoblotting with anti-myc. A putative TMX dimer is indicated by an asterisk.

conserved amino acid residue within the thioredoxin-like domain. We found that mutating the C-terminal cysteine of the CXXC motif to alanine (C59A) successfully led to the accumulation of multiple bands reacting with the myc anti-

body under nonreducing conditions (Figure 5A, lane 2). These bands disappeared when the samples were treated with reducing agents (Figure 5A, lane 5), indicating that they represent disulfide-linked complexes containing TMX-myc. It has been reported that conversion of the *cis*-proline of DsbA, an *Escherichia coli* oxidase in the periplasm, resulted in the accumulation of disulfide-linked DsbA-substrate complexes (Kadokura *et al.*, 2004). The corresponding proline residue is also conserved among mammalian thioredoxin family members including TMX (Supplemental Figure S1), and mutation of Pro101 of TMX to threonine (P101T) had an effect in detecting mixed disulfides (Figure 5A, lane 3). Thus, these mutants could be useful for stabilizing mixed disulfides formed during the thiol-disulfide exchange, leading to identification of the substrates of TMX.

To determine whether calnexin is required for substrate recognition by TMX, we examined the effects of castanospermine (CST), an inhibitor of glucosidases I and II, on the formation of the disulfide-linked complex. The inhibition of glucose trimming by CST blocks the glycan-dependent interactions of glycoproteins with calnexin (Hammond *et al.*, 1994). We established 293 cells expressing the TMX/P101T trapping mutant under the control of a tetracycline-inducible promoter. After treatment with doxycycline at 0.4 μg/ml, TMX/P101T-myc was expressed at levels similar to those of endogenous TMX (Figure 5B, top). The cells were pretreated with increasing doses of CST and then cultured in the presence of doxycycline to induce the expression of TMX/P101T-myc. Cell lysates were separated by SDS-PAGE under reducing and nonreducing conditions and immunoblotted with anti-myc antibodies. Treatment with CST reduced the formation of mixed disulfides (Figure 5B, bottom), suggesting that some substrates of TMX contain monoglucosylated N-glycans and they might be recognized by TMX through the cooperative interaction with calnexin. The presence of TMX-substrate complexes in anti-calnexin immunoprecipitates supported this possibility. As shown in Figure 5C, several high molecular weight complexes containing TMX/P101T-myc were observed in anti-calnexin immunoprecipitates under nonreducing conditions (lane 5). These were disrupted upon treatment with DTT (lane 2), or by mutating both of the two active site cysteines of TMX (lane 6), indicating that they are TMX-substrate conjugates. Similar results were obtained in the experiments using C59A trapping mutant (Supplemental Figure S2). The only exception was a band of ~75 kDa. The band was susceptible to reduction by DTT, but under nonreducing conditions it was also present with TMX/C56A-C59A mutant containing no active site cysteines (lane 6, asterisk). Although its identity is still unclear, the 75 kDa band, judging from the size, most likely represents a disulfide-linked dimer of TMX-myc, and noncatalytic cysteines might contribute to dimerization. It should be noted that even in the presence of CST, mixed disulfide formation was not completely blocked, and we could still detect TMX-substrate complexes (Figure 5B), indicating that the association of TMX with several substrates was not dependent on glucose trimming.

TMX Forms a Mixed Disulfide with MHC Class I Heavy Chain

Given the association of TMX with calnexin, we next tested the possibility that a membrane protein recognized by calnexin could be a target of TMX. We found that endogenous MHC class I HC was coprecipitated by anti-myc antibodies from cells expressing myc-tagged TMX/C59A (Figure 6A, lane 2). Integrin β1, another substrate for calnexin (Lenter and Vestweber, 1994), was not coprecipitated with TMX

Figure 6. TMX forms a disulfide-linked complex with MHC class I heavy chain. (A) 293 cells transfected with control vector or the indicated TMX-myc variants were subjected to immunoprecipitation with anti-myc. Cell lysates and the immunoprecipitates were analyzed by immunoblotting with anti-class I HC and anti-myc. Monomeric class I HC (open arrowhead) and the TMX-class I HC conjugate (closed arrowhead) are indicated (top). Red, reducing; Nonred, nonreducing. (B) Anti-myc immunoprecipitates from 293 cells transfected with TMX/C59A-myc were separated on a nonreducing/reducing two-dimensional gel, and analyzed by immunoblotting with antibodies to calnexin (CNX), class I HC, and myc. Calnexin was visible on the gel diagonal between the two arrows (circled). The arrowhead indicates MHC class I HC originated from the disulfide-linked complex.

(data not shown), indicating the specific interaction between TMX and class I HC. It is noted that under nonreducing conditions bands of ~80 kDa were detected with anti-class I HC in immunoprecipitates from C59A-expressing cells (Figure 6A, lane 6). The molecular weight of the bands was consistent with a complex of TMX-myc (~37 kDa) and class I HC (~43 kDa), and they were susceptible to reduction with DTT, suggesting that the trapping mutant forms a disulfide-linked dimer with endogenous class I HC. TMX/P101T trapping mutant could also form a mixed disulfide with class I HC (Supplemental Figure S3A), but wild-type TMX-myc and C56A/C59A mutant did not capture any mixed disulfides (Figure 6A, lanes 5 and 7). Similar results were obtained when the immunoprecipitation was performed with anti-class I HC followed by immunoblotting with anti-myc (Supplemental Figure S3B).

To further confirm the formation of mixed disulfides between TMX and class I HC, protein complexes containing TMX/C59A-myc were separated by two-dimensional nonreducing/reducing SDS-PAGE and immunoblotted. Proteins with interchain disulfide bonds separated by this method would be detected as spots migrating below the

Figure 7. TMX is not essential for the oxidative folding of MHC class I heavy chain in HeLa cells. (A) Lysates from control (Ctrl) and TMX knockdown cells (KD1 and KD2) were analyzed by immunoblotting for class I HC, α -tubulin (TUB), and TMX. (B) Surface expression of MHC class I molecules. Control and TMX knockdown cells were stained with anti-MHC class I (W6/32) and analyzed by flow cytometry. NC, unstained sample. (C) Control (Ctrl) and TMX knockdown (KD1) cells transfected with HLA-B27-V5 were either left untreated (-) or treated (+) with 5 mM DTT for 5 min. Cells were lysed in NEM-containing buffer and analyzed by immunoblotting with anti-V5 under nonreducing or reducing conditions. DTT-sensitive (closed arrowhead) and DTT-resistant forms (open arrowhead) of class I HC are indicated.

diagonal. Associated calnexin was visible on the gel diagonal (Figure 6B, left, circled), showing that the interaction between TMX and calnexin was noncovalent. By contrast, coprecipitated class I HC was detected as a spot migrating below the line (Figure 6B, left, arrowhead), demonstrating the covalent interaction between the trapping mutant and class I HC. Anti-myc antibodies decorated a number of spots of free TMX/C59A-myc originating from multiple disulfide-linked complexes (Figure 6B, right). Similar results were obtained with the P101T trapping mutant (data not shown).

Oxidative Folding of Class I Heavy Chain Is Not Affected by TMX Gene Knockdown

To elucidate the functional significance of the interaction between TMX and MHC class I HC, we first examined the effects of TMX down-regulation on the expression of class I HC. Cell lysates prepared from control or TMX knockdown cells were analyzed by immunoblotting to quantify the total amount of class I HC. As shown in Figure 7A, there was no significant difference in the expression level of the class I HC between these cells. Furthermore, in the steady state, TMX gene knockdown did not substantially affect the surface expression of MHC class I molecules as quantified by flow cytometry (Figure 7B).

Many proteins on the secretory pathway, including MHC class I HC, contain disulfide bonds (Dick, 2004). During maturation, some disulfides are buried in the folded structure and become insensitive to reducing agents unless the proteins are denatured (Braakman *et al.*, 1992; Tatu *et al.*,

Figure 8. Role of TMX in the degradation of MHC class I heavy chain. (A) A549 cells stably expressing TMX-myc were cultured in the absence (–) or presence (+) of tunicamycin (TM; 2 μ g/ml) for 20 h. Cell lysates (lanes 1 and 2) and anti-myc immunoprecipitates (lanes 3 and 4) were analyzed by immunoblotting with anti-class I HC and anti-myc. Glycosylated (closed arrowhead) and nonglycosylated forms (open arrowhead) of class I HC are indicated. (B) A549 cells expressing TMX-myc were treated with or without tunicamycin as described in A. Cell lysates were first immunoprecipitated with anti-myc (1st IP), and the eluates were further immunoprecipitated with anti-calnexin (2nd IP). Coprecipitated class I HC was detected by immunoblotting (open arrowhead). *, coeluted antibody heavy chains (~50 kDa). (C) 293 cells transfected with HLA-B27-V5 were incubated with (–) or without (+) MG-132 (10 μ M) for 4 h and fractionated into cytosolic (C) and membrane (M) fractions. The translocation of MHC class I HC into the cytosol was examined by immunoblotting with anti-V5. The purity of each fraction was verified by immunoblots for ER marker calnexin. (D) 293 cells transfected with control vector (V) or HLA-B27-V5 (B27) were subjected to immunoprecipitation with anti-V5 and immunoblotted with anti-V5 and anti-TMX. Arrowhead denotes endogenous TMX coprecipitated with HLA-B27-V5. *, coeluted antibody light chains (~25 kDa). (E) HLA-B27-V5 was cotransfected into 293 cells either with control vector (V) or with TMX-myc (TMX), and the translocation of HLA-B27 was analyzed as described in C. The gel was stained with Coomassie Blue (CBB) to ensure equal loading.

1993). To analyze oxidative folding of class I HC, we assessed DTT sensitivity of HLA-B27 expressed in control and TMX knockdown cells (Figure 7C). Cells transfected with V5-tagged HLA-B27 were incubated in medium containing 5 mM DTT for 5 min. After washing out DTT, cell extracts were prepared, separated by nonreducing SDS-PAGE, and analyzed by immunoblotting with anti-V5 antibody. Although a small fraction of HLA-B27-V5 was reduced by DTT treatment (indicated by closed arrowhead), most of the proteins acquired a conformation resistant to DTT reduction both in the control and TMX knockdown cells (Figure 7C, lanes 2 and 4, open arrowhead). Thus, down-regulation of TMX did not affect the conversion of class I HC from DTT-sensitive to DTT-resistant form, and oxidative folding of class I HC seemed to proceed normally.

Tunicamycin Treatment Promotes the Formation of TMX–Class I Heavy Chain Complex

Next, we examined the requirement for N-linked glycans of MHC class I HC in its association with TMX. A549 cells stably expressing TMX-myc were cultured in the presence or absence of tunicamycin, a potent inhibitor of protein glycosylation. In tunicamycin-treated cells, nonglycosylated class I HC additionally appeared as a faster migrating species (Figure 8A, lane 2, open arrowhead). We noted that class I HC was not obviously detectable in the anti-myc immunoprecipitates from unstressed A549 cells (Figure 8A, lane 3). Interestingly, the amount of coprecipitated class I HC apparently increased in tunicamycin-treated cells (Figure 8A, lane 4). Furthermore, only the nonglycosylated forms of class I HC were coprecipitated, as judged by their mobility on SDS-PAGE, whereas no interaction was observed with the glycosylated proteins. Because TMX trapping mutants could recognize glycosylated class I HC (Figure 6), TMX seemed not to monitor the glycosylation status of class I HC. This led us to suspect that TMX might preferentially bind the incompletely folded proteins produced under conditions of ER stress.

To investigate whether calnexin is involved in this TMX–class I HC complex formation, the anti-myc immunoprecipitates from TMX-myc transfectants were further immunoprecipitated with anti-calnexin antibody. As shown in Figure 8B, nonglycosylated class I HC associated with TMX-myc also was bound to calnexin (lane 4, open arrowhead), suggesting that tunicamycin treatment induces the formation of a multiprotein complex containing TMX, class I HC and calnexin.

TMX Prevents the Degradation of HLA-B27

During conditions of stress, proteins are unable to adopt their native conformation, and terminally misfolded proteins are degraded by a process referred to as ER-associated degradation (Meusser *et al.*, 2005). Misfolded MHC class I molecules are retrotranslocated from the ER to the cytosol where they are deglycosylated and degraded by proteasomes. Previous studies have shown that when proteasomal proteolysis is inhibited characteristic deglycosylated forms

Representative data are shown, and the band intensities of the cytosolic HLA-B27 are quantified. The Graph shows the mean $\pm$ SD of four experiments. (F) Control (Ctrl) and TMX knockdown cells (KD1 and KD2) were transfected with HLA-B27-V5, and the translocation of HLA-B27 was analyzed in the absence (–) or presence (+) of MG-132. Amido black staining of the same blot confirmed equal loading. Data are representative of two experiments and the intensities of HLA-B27 bands in the cytosol (lanes 4–6, open arrowhead) are quantified as shown in the graph.

of class I HC accumulate in the cytosol (Wiertz *et al.*, 1996; Hughes *et al.*, 1997). To study the degradation process of class I HC, 293 cells transfected with V5-tagged HLA-B27, which is prone to misfold in the ER (Colbert, 2000), were fractionated into cytosolic and membrane fractions. We observed the accumulation of cytosolic HLA-B27 derivatives only in the presence of proteasome inhibitor MG-132, which seemed to be the deglycosylated degradation intermediates as judged by their smaller size (Figure 8C, top, lane 3). Calnexin was absent from the cytosolic fraction, which demonstrates the efficient separation of cytosolic and membrane components (Figure 8C, bottom). These results indicate that a portion of the exogenous HLA-B27 was targeted for proteasomal degradation and transported to the cytosol.

Using this assay system, we investigated whether TMX is involved in the mechanism that regulates the degradation of class I HC. We first tested whether TMX can interact with V5-tagged HLA-B27. Endogenous TMX was coprecipitated by anti-V5 antibody from cells expressing HLA-B27-V5 (Figure 8D, lane 4, arrowhead), but not from mock-transfected cells (lane 3), demonstrating the interaction between TMX and HLA-B27. In Figure 8E, HLA-B27-V5 was cotransfected either with TMX-myc or an empty vector into 293 cells, and after incubation with MG-132, cytosolic and membrane fractions were prepared. We found a decreased level of cytosolic deglycosylated HLA-B27 in cells expressing TMX-myc compared with vector control cells (Figure 8E, top, compare lane 1 to lane 2; the protein band intensities were quantified as shown in the graph), indicating that coexpression of TMX prevented the translocation of HLA-B27. TMX-myc was found only in the membrane fraction, and the ER-to-cytosol translocation of TMX itself was not detectable (Figure 8E, middle). Equal protein loading was confirmed by Coomassie blue staining of the gel (Figure 8E, bottom).

Next we assessed the effect of TMX down-regulation on the degradation process of class I HC. HLA-B27-V5 was transfected into control or TMX knockdown cells, and the translocation of HLA-B27 was examined. In the presence of MG-132, deglycosylated forms of HLA-B27 in the cytosol were increased in the two independent TMX knockdown clones compared with control cells expressing endogenous TMX (Figure 8F, compare lane 4 with lanes 5 and 6). Thus, the depletion of TMX enhanced the rate of degradation of HLA-B27, suggesting that TMX acts to retain MHC class I heavy chain in the ER and thereby protects the protein from degradation.

DISCUSSION

TMX differs from other ER oxidoreductases in that it is membrane bound and has a rather unusual active site motif (CPAC). The unique CPAC motif of TMX could affect the redox potential and contribute to its substrate specificity distinct from those of oxidoreductases with the common CGHC motifs. TMX was present mainly in the reduced form, which is consistent with previous observations that many ER oxidoreductases are predominantly reduced in mammalian cells (Mezghrani *et al.*, 2001; Jessop and Bulleid, 2004). We also found that a significant fraction of TMX was oxidized in some cell lines. Although it is uncertain whether the oxidized form is biologically active, TMX may have a physiological role as an oxidase like PDI. Alternatively, the oxidized TMX might be a reaction intermediate, which would subsequently be reduced. ERp57 is maintained in a reduced state, and after treatment with oxidizing agent it was restored to the reduced form very rapidly (Jessop and Bulleid, 2004). In contrast, the recovery of reduced TMX was

relatively slow, and oxidized TMX was still present three hours after removal of the oxidizing agent (Figure 1E). Therefore, the delay in the recovery from protein oxidation may provide an explanation for TMX seeming to be partially oxidized.

The diversity among thioredoxin family members in the ER may reflect their functional specialization. To determine whether these redox proteins share overlapping biological activities or they have separate and distinct functions, it is required to identify binding partners and substrate proteins for each oxidoreductase. PDI can interact directly with substrate proteins, and its b' domain is known to be the primary peptide binding site (Klappa *et al.*, 1998). ERp57, the closest homologue of PDI, has been shown to act as a glycoprotein-specific oxidoreductase through the interaction with the lectin-like chaperones calnexin and calreticulin (Molinari and Helenius, 1999; Oliver *et al.*, 1999; Jessop *et al.*, 2009). We found that TMX bound to calnexin. Given that the luminal part of TMX lacks a b'-like peptide binding domain and CST treatment partially inhibited the formation of mixed disulfides, TMX might require calnexin to recruit a portion of its substrates. Another issue to be considered is how TMX is retained in the ER. TMX lacks known ER retention signal like C-terminal dilysine motif (Jackson *et al.*, 1990), and no specific residues or sequence motifs required for its proper localization have been identified. The association with calnexin may be important for retaining TMX on the ER membrane.

Considering the instability of the intermediate mixed disulfides, experimental approaches have been used to trap substrates by mutating the C-terminal cysteine in the CXXC active site (Motohashi *et al.*, 2001; Jessop *et al.*, 2007; Schwertassek *et al.*, 2007). An alternative approach to mutate the conserved *cis* proline has been developed to identify substrates for *E. coli* oxidase DsbA (Kadokura *et al.*, 2004). We showed that the TMX mutant harboring a P101T substitution was capable of capturing the disulfide-linked complexes as seen with the C59A mutant, demonstrating that the mutation of the corresponding proline residue is also useful in mammalian systems for the detection of mixed disulfide intermediates.

The trapping mutants enabled us to identify MHC class I HC as a candidate substrate for TMX. Previous studies have shown that class I HC associates with ERp57 during the early events in its assembly (Lindquist *et al.*, 1998; Morrice and Powis, 1998). At a later stage, ERp57 has also been detected as a component of the peptide-loading complex (Hughes and Cresswell, 1998; Antoniou *et al.*, 2003), and B cell-specific deletion of ERp57 in mice resulted in reduced surface levels and stability of class I molecules (Garbi *et al.*, 2006). More recently, another ER oxidoreductase, PDI, has been identified as a component of the peptide-loading complex, and it stabilizes the peptide-receptive site of the class I molecule (Park *et al.*, 2006). Unlike these classical ER oxidoreductases, TMX was found not to be essential for the oxidative protein folding of class I HC under normal conditions. In exploring their functional relationship, however, we demonstrated that TMX prevented the degradation of class I HC expressed in excess in human cell lines. Furthermore, we see an increased interaction of TMX with nonglycosylated class I HC in tunicamycin-treated cells. Considering that protein overload or tunicamycin treatment should promote aberrant folding of proteins, TMX might act on proteins with malformed or incompletely folded structures that need to be refolded or degraded. Our data also suggest that calnexin is involved in the TMX-class I HC complex formation under stress conditions. Their interaction might

be mediated by the glycan-independent chaperone function of calnexin, which contributes to the quality control of misfolded proteins (Arunachalam and Cresswell, 1995; Ihara *et al.*, 1999; Swanton *et al.*, 2003).

The accumulation of misfolded species in the ER can interfere with cellular processes, and stressed cells cope with protein overload in the ER by increasing the folding capacity or activating the ER-associated protein degradation pathway. Several studies have shown that the retrotranslocation and degradation of misfolded proteins require a sequence of events, including unfolding, disulfide bond reduction, and deglycosylation (Kopito, 1997; Fagioli *et al.*, 2001). However, it is not well understood how cells discriminate between correctly-folded and misfolded proteins or regulate the decision of either repairing the protein or degrading it. It has been suggested that one possible mechanism involves regulation of the redox state of proteins. For example, ER-to-cytosol translocation is sensitive to agents that modify free thiols and alter the redox state of the cell (Tortorella *et al.*, 1998). PDI has been shown to act as a redox-dependent chaperone to unfold cholera toxin and facilitate retrograde transport of the A1 chain into the cytosol where it exerts toxic effects (Tsai *et al.*, 2001). On the contrary, ERp72 and ERp44, both of which belong to the thioredoxin family, act to retain their substrates in the ER (Anelli *et al.*, 2003; Forster *et al.*, 2006). It is possible that TMX mediates the ER retention of membrane proteins including class I HC through a mechanism analogous to that observed in the ER lumen. Reversible thiol-disulfide exchange reactions catalyzed by oxidoreductases must involve a large conformational change that may enable the cellular triage system to distinguish proteins to be refolded and proteins destined for degradation. Further investigation is required to clarify the underlying molecular mechanisms of this redox-based quality control system.

Alterations in the ER environment affect the protein folding, and the resulting accumulation of misfolded proteins is known to contribute to a variety of diseases (Kaufman, 2002). MHC class I is critically important as an antigen-presenting molecule in host defense against intracellular infections, but hyperexpression and misfolding of MHC class I could trigger the onset of autoimmune diseases (Singer *et al.*, 1997; Colbert, 2000). Increased interaction of TMX with class I HC under stress conditions raises the possibility that TMX plays a protective role in pathological conditions such as inflammation and infectious diseases under which large amounts of class I molecules are synthesized. Future development of knockout animal models will enable us to investigate the function of TMX *in vivo* and its relevance to pathophysiology associated with protein misfolding in the ER.

In this study, we have identified the physical and functional interaction between TMX and MHC class I HC. At present, it remains unclear whether TMX is involved in the redox regulation of other glycoproteins sharing common structures with MHC class I. The classification of substrate proteins for TMX will provide a better understanding of the precise function of this transmembrane oxidoreductase.

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