We have cloned, sequenced, and expressed full length cDNA clones encoding two abundant, luminal endoplasmic reticulum proteins (ERp). ERp59/PDI and ERp72. ERp59/PDI has been identified as the microsomal enzyme protein disulfide isomerase (PDI). An analysis of the amino acid sequence of ERp72 showed that it shared sequence identity with ERp59/PDI at three discrete regions, having three copies of the sequences that are thought to be the CGHC-containing active sites of ERp59/PDI. Thus, ERp72 appears to be a newly described member of the family of CGHC-containing proteins. ERp59/PDI has the sequence KDEL at its COOH terminus while ERp72 has the related sequence KEEL. Removal of the KDEL of ERp59/PDI or the KEEL of ERp72 by in vitro mutagenesis of full length cDNA clones encoding these two proteins. Sequencing has revealed that ERp59 is the microsomal enzyme protein disulfide isomerase (PDI). Accordingly, ERp59 will be referred to as ERp59/PDI in this report. ERp72 has been identified as a novel relative of ERp59/PDI, having three copies of what are thought to be the CGHC-containing active sites of ERp59/PDI. Thus, ERp72 appears to be a newly described member of the family of CGHC-containing proteins. ERp59/PDI has the sequence KDEL at its COOH terminus while ERp72 has the related sequence KEEL. Removal of the KDEL of ERp59/PDI or the KEEL of ERp72 by in vitro mutagenesis of full length cDNA clones encoding these two proteins sequencing has revealed that ERp59 is the microsomal enzyme protein disulfide isomerase (PDI). Accordingly, ERp59 will be referred to as ERp59/PDI in this report. ERp72 has been identified as a novel relative of ERp59/PDI, having three copies of what are thought to be the active sites of ERp59/PDI. Thus, the discovery of ERp72 has expanded the catalogue of CGHC-containing, PDI-related proteins (for review, see Freedman, 1989). From the Department of Microbiology, St. Louis University School of Medicine, St. Louis, Missouri 63104

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ERp72, an Abundant Luminal Endoplasmic Reticulum Protein, Contains Three Copies of the Active Site Sequences of Protein Disulfide Isomerase*

We are interested in understanding the regulation of the synthesis and sorting of endoplasmic reticulum proteins (ERps). In earlier work, we described the chemical characteristics and biosynthetic sorting of two abundant, luminal ERPs, ERp59 and ERp72 (Lewis et al., 1985a, 1985b, and 1986). In this work, we report the cloning, expression, and in vitro mutagenesis of full length cDNA clones encoding these two proteins. Sequencing has revealed that ERp59 is the microsomal enzyme protein disulfide isomerase (PDI). Accordingly, ERp59 will be referred to as ERp59/PDI in this report. ERp72 has been identified as a novel relative of ERp59/PDI, having three copies of what are thought to be the active sites of ERp59/PDI. Thus, the discovery of ERp72

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† To whom correspondence should be addressed.

‡ The abbreviations used are: ERPs, endoplasmic reticulum proteins; ERp59, 59-kDa Erp; ERp72, 72-kDa Erp; BIP, immunoglobulin heavy chain-binding proteins; ERp99/GPR94, 94-kDa glucose-regulated protein; CGHC, one-letter amino acid code for Cys-Gly-His-Cys sequence; PDI, protein disulfide isomerase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Endo H, endo-β-N-acetylglucosaminidase; hp, base pair.

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is slowly secreted from the cells, while wild type ERp72 appears to be almost entirely retained within the cells. Removal of the KDEL of ERp59/PDI or the KEEL of ERp72 results in the secretion of both proteins, with the most dramatic effect seen in the case of ERp72. This finding formally extends ER retention signal function to at least one other KDEL-related sequence and raises the question of whether signal function within this family of COOH-terminal sequences is dependent on or independent of the carrier protein.

EXPERIMENTAL PROCEDURES

**Sequenceing of Full Length cDNA Clones for ERp59/PDI and ERp72**—Partial cDNA clones were first identified by hybridization selection techniques using monospecific antisera against murine ERp59/PDI and murine ERp72 by using procedures described by Mazzarella and Green (1987). The ERp59/PDI and ERp72 cDNA cloning procedures were identical. The anti-ERp59/PDI antiserum had been extensively characterized in previous work (Lewis et al., 1985a, 1985b, and 1986). These antisera were used in all the experiments described in this report. Recombinant pcD vectors carrying full length ERp59/PDI and ERp72 cDNA inserts were identified in a mouse fibroblast cDNA library (a generous gift of Dr. Hiroto Okayama, NIH) using partial ERp59/PDI and ERp72 cDNA clones by cloning procedures described by Mazzarella and Green (1987). The entire ERp59/PDI and ERp72 cDNA inserts were excised from the pcD vector by digestion at flanking BamHI sites. The BamHI fragments were cloned into M13mp19 in both orientations and the ERp59/PDI and ERp72 cDNA inserts were found to be unstable in M13mp19, but the pcUC119 vector (a gift from Dr. Paul Gold of Gold Biotechnology, Inc.) was determined to be compatible with the insert and was used for the sequence analysis for this clone.

Nestled sets of both strands of each clone were prepared by the method of Dale et al. (1985), as described by Mazzarella and Green (1987). Specific subclones from the cDNA inserts were constructed to sequence regions that were not represented in the nested sets. Dideoxy sequencing (Sanger et al., 1987) reactions were done using either the Klenow fragment of Escherichia coli DNA polymerase, Sequenase (United States Biochemical Corp.), or Taq polymerase (Stratagene Cloning Systems). Chemical sequencing (Maxam and Gilbert, 1977) of one of ERp59/PDI subclones was also done.

**Construction of COOH-Terminal Deletion Mutants**—The Alter-Gene in vitro mutagenesis system (Gold Biotechnology, Inc.) was used to construct ERp59/PDI and ERp72 mutant lacking their respective COOH-terminal tetrapeptide. Full length ERp72 was excised from pcD72-1 using the flanking BamHI sites contained within the expression vector. This BamHI fragment was cloned into the Alter-Gene vector, pGBT518. For ERp59/PDI mutagenesis the 5'-EcoRI/BamHI fragment of each wild type clone were completely digested with EcoRI and then partially digested with BamHI. The 3'-EcoRI/BamHI fragment of each wild type clone was replaced with its respective mutant EcoRI/BamHI fragment to create full length ERp59/PDI and ERp72 clones lacking both the 5'-untranslated region and the KDEL or KEEL sequence, respectively.

**Expression of Full Length and Mutant ERp59/PDI and ERp72 Clones**—The full-length ERp59/PDI and ERp72 pcD clones as well as the COOH-terminal deletion retention signal mutants were screened for protein expression by transfection into COS cells as described by Mazzarella and Green (1987), except that in all experiments the labeling media contained dialyzed fetal calf serum at a final concentration of 5%. This was done to ensure optimal secretory activity. COS cells for screening were plated at 1.2 × 10⁵ per well in 12-well plates at 72 h after transfection and labeled for 4 h with [³⁵S]methionine at a concentration of 50 µCi/ml. Pulse-chase experiments were performed to determine the secretion kinetics of the full-length and mutant clones. At 72 h after transfection, COS cells were labeled for 15 min in labeling media containing 100 µCi/ml of [³⁵S]methionine. The media was removed and replaced with cold media supplemented with 0.3 mM unlabeled methionine. At appropriate time intervals, the medium was removed, and the cells were washed and then lysed to release intracellular protein. Both the cell lysate and the media were incubated with anti-ERp59/PDI or anti-ERp72 in order to immunoprecipitate the relevant protein. The immunoprecipitates were analyzed by SDS-PAGE, and the autoradiograms were quantitated by densitometry using an LKB laser densitometer Ultrascan (model Z-102) coupled to an automatic integrator (LICB model 220 Bromma).

RESULTS

**Identification, Expression and Sequencing of Full Length cDNA Clones for ERp59/PDI and ERp72**—In earlier experiments, ERp59/PDI and ERp72 cDNA clones were identified by using hybrid selection techniques to screen a cDNA library prepared in pBR322 from 15–18 S fraction of murine placental poly(A)+ mRNA (data not shown). These experiments followed the procedures that had been used to isolate and characterize a full length cDNA clone encoding ERp99/GRP94, an abundant glycoprotein of the ER (Mazzarella and Green, 1987). The rabbit anti-murine ERp59/PDI and anti-murine ERp72 used in these experiments and in the subsequent experiments discussed in this report had been extensively characterized in previous work (Lewis et al., 1985a, 1985b, and 1986). In a screen of 800 independent transformants, one ERp59/PDI cDNA clone of ~500 bp and two ERp72 cDNA clones of ~300 bp were found. A Northern blot analysis of the length of ERp59/PDI and ERp72 mRNA showed that ERp59/PDI mRNA was approximately 3000 nucleotides long while ERp72 mRNA was approximately 2800 nucleotides long. The original cDNA clones were used to rescreen the pBR322 library, but no cDNA clones corresponding to full length ERp59/PDI or ERp72 cDNA were found. The original cDNA inserts were then used to screen a cDNA library prepared in the pcD shuttle vector (Okayama and Berg, 1983) using mRNA isolated from mouse fibroblasts. Several clones containing either ERp59/PDI or ERp72 sequences were isolated. Since neither type of clone contained an internal BamHI site, digestion with BamHI was used to determine the size of the cDNA inserts. Those clones of each type with inserts greater than ~2000 bp were used as a source of plasmid DNA for transient expression experiments in COS cells. In the case of ERp59/PDI (Fig. 1), only the ERp59/PDI-related band seen in mock-transfected (lane 6) or untransfected cells (lane 7) was the cross-reacting monkey ERp59/PDI. This was an expected finding based upon our earlier observations concerning the antigenic relatedness of ERp59/PDI from various species (Lewis et al., 1986). Of all the pcD plasmids tested, only pcD9-11 (lane 4) yielded any murine ERp59/PDI in the

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2 R. A. Mazzarella, unpublished results.
transfection assay. In the case of ERp72, preliminary transfection experiments indicated that two of the three pcD plasmids containing ERp72 sequences were capable of yielding full-length ERp72 upon transfection. Unexpectedly, the murine ERp72 expressed in the COS cells by pcD72-1 and pcD72-4 consisted of two closely migrating bands of equal intensity rather than a single protein species as is the case with cellular ERp72. Restriction enzyme analysis of these two clones showed that pcD72-1 was only ~50 bp longer at 5' end than pcD72-4. In order to determine whether the change in migration was the result of N-linked glycosylation, the expression experiment was repeated with the three ERp72 clones, and the immunoprecipitated expressed protein was treated with Endo H before SDS-PAGE (Fig. 2A). Endo H treatment had no effect on the migration on either band of the doublet. The expressed products were then immunoprecipitated with anti-BiP antibody, in order to determine if the doublet was due to complex formation between some of the overexpressed ERp72 and BiP (Fig. 2B). The level of BiP expression was the same in all the cell cultures in the expression experiment and was not influenced by overexpression of ERp72. In addition, BiP did not migrate at the position of the lower, newly observed band but only slightly below the position of authentic murine ERp72.

The cDNA inserts in pcD59-11 and pcD72-1 were selected for sequencing. The entire sequence of both inserts was determined by isolating a nested set of subclones for each strand of each of the inserts and by filling in any gaps in the sequence by subcloning specific restriction fragments of the cDNAs. The ERp59/PDI amino acid sequence derived from the nucleotide sequence is presented in Fig. 3. The ERp59/PDI precursor encoded by the cDNA consists of 509 amino acids with a calculated molecular mass of 57,058 Da. The AUG codon used for initiation is preceded by a G at position -3 and followed by a C in the +4 position. This is typical of only a minority of functional initiation sites in eukaryotic mRNA (Kozak, 1983). The consensus poly(A) addition signal is present 15 nucleotides from the poly(A) tail. ERp59/PDI is synthesized as a precursor protein with a cleavable signal peptide. The NH2-terminal sequence of mature murine ERp59/PDI has not been determined. Comparison with the rat protein, however, leads to the prediction that the cleavage site would be between Ala14-Leu15 yielding a mature protein with a molecular mass of 54,903 Da. If this is the case, the ERp59/PDI signal peptide would have the characteristics of a typical leader peptide. It would possess a hydrophobic central section preceded by a positively charged NH2 terminus and followed by a cleavage site consisting of two small hydrophobic residues separated by one amino acid. There are no potential N-glycosylation sites in the ERp59/PDI sequence. This is consistent with our earlier results showing that ERp59/PDI is not sensitive to Endo H (Lewis et al., 1986). ERp59/PDI has a COOH-terminal KDEL sequence which has been shown by Munro and Pelham (1987) to be an ER retention signal for the luminal protein BiP.

As we were completing the analysis of the ERp59/PDI clone, we learned that the anti-ERp59/PDI antibody that we had raised originally against gel-purified murine ERp59/PDI (Lewis et al., 1986) cross-reacted with purified bovine PDI (Paver et al., 1989). A comparison of the derived amino acid sequence of murine ERp59/PDI against the PDI sequences from rat, human, and chicken demonstrated unequivocally that ERp59 was murine PDI (Fig. 4). The murine enzyme is 96% identical to rat PDI, 92% identical to human PDI, and 86% identical to chicken PDI.
The ERp72 amino acid sequence derived from the nucleotide sequence of the pcD72-1 insert is shown in Fig. 5. The ERp72 precursor encoded by the cDNA consists of 638 amino acids with a calculated molecular mass of 71,973 Da. The AUG initiation codon is preceded by an A in the -3 position and followed by an A in the +4 position. This sequence is one of the favored sequences of functional initiation of the precursor, however, allows the prediction that the translation will be initiated. The putative polyadenylation signal is indicated by the presence of the T at position 2537.
FIG. 4. Amino acid sequence conservation of ERp59/PDI. A comparison of the amino acid sequences of ERp59/PDI from mouse, rat, human, and chicken (Geetha-Habib et al., 1988). Identity to murine ERp59/PDI is indicated by a dot. In cases where the sequences differ, the particular amino acid found at that position is indicated. A dash is used to indicate where gaps were introduced to produce optimal alignment.

An examination of the ERp72 sequence revealed a striking relationship between ERp59/PDI and ERp72 (Fig. 6). ERp72 possesses three repeats of the amino acid sequences that are thought to be the two active sites of PDI. There is complete conservation of the 11 amino acid sequence EFYAPWCGHC of the COOH-terminal region of the pcD72-2 and pcD72-4 cDNA clones.

Evaluation of the Role of COOH-terminal Sequences as ER Retention Signals for ERp59/PDI and ERp72—In order to determine whether the KDEL terminus of ERp59/PDI or the KEEL sequence of ERp72 is involved in the retention of either of the proteins in the ER, mutants of each protein specifically lacking these sequences were prepared. The synthesis and secretion of the wild type and mutant forms of the proteins were evaluated in transient expression experiments in COS cells (Fig. 7). After 4 h of continuous labeling, a small amount of wild type ERp59/PDI was found in the medium. The removal of KDEL from ERp59/PDI resulted in an approximately 3-fold increase of the amount of ERp59/PDI found in the medium. Very little, if any, of the wild type ERp72 was found in the media after 4 h of labeling. After removal of the KEEL sequence, almost half of the ERp72 was found in the medium. The results of these screening experiments were confirmed by pulse-chase experiments designed to investigate the kinetics of secretion of wild type ERp59/PDI and ERp72 and their COOH-terminal deletion mutants (Fig. 7B). Wild type ERp59/PDI was secreted very slowly from the COS cells. About 20% of the pulse-labeled protein was found in the medium after 8 h of chase. ERp59/PDI lacking the KDEL sequence, however, was approximately completely secreted from the COS cells with a t<sub>½</sub> of secretion of 3.5 h. The results with ERp72 were even more striking. Little, if any, wild type ERp72 is secreted from the COS cells during the 8-h chase. ERp72 lacking the KEEL sequence, however, was almost completely and rapidly secreted from the cells with a t<sub>½</sub> of secretion of 1.0–1.5 h. In the course of these experiments it was noted that, in some cases, the overexpression of the ERp72 deletion mutant resulted in the appearance of a more slowly moving species that was not part of the original doublet seen for ERp72 expression (see Fig. 2). This species comigrates with the ERp72 precursor made during cell-free protein synthesis in the absence of exogenous membranes. It is likely therefore that this species represents ERp72 precursor with an uncleaved signal peptide. This species is not secreted.

DISCUSSION

The cloning, expression, and sequencing of ERp59/PDI and ERp72 has provided new insights into CGHC-containing proteins which may be involved in vivo in the correct folding of and disulfide bond formation in newly synthesized proteins and in other membrane-associated functions. In addition, the in vitro mutagenesis experiments described in this paper have confirmed earlier observations (Munro and Pelham, 1987) on the role of KDEL in ER retention and have extended these observations to the related sequence KEEL.

ERp59/PDI and ERp72 are resident luminal proteins of the ER. This observation taken together with the determination that ERp59/PDI has key sequence identities to those of ERp59/PDI requires us to ask questions about the structural and functional relationship between these proteins. The absolute conservation of the sequence of 11 amino acids at the five CGHC sites in the two proteins, the lower, but still considerable, degree of conservation of sequence in the region immediately surrounding these sites, and the fact that analogous CGHC regions are equally spaced in the two proteins are consistent with the idea that these proteins perform the same or closely related functions in the ER. The fact, however, that no 72-kDa PDI-like protein has been observed either in the studies that have been done to isolate and characterize PDI (Freedman, 1989) or in all of the studies that have resulted in the identification of a particular property of PDI (Boado et al., 1988; Cheng et al., 1987; Geetha-Habib et al., 1988; Tasanen et al., 1988) could be an argument against this idea. This lack of evidence for a PDI-related role for ERp72 could be due to several reasons. The activity of ERp72 could be intrinsically less stable than PDI. Alternatively, the two proteins could have an overlapping spectrum of functions and an overlapping spectrum of substrates. Thus, the substrates and reaction conditions used to study PDI might not be suitable for ERp72. In addition, the level of expression of the two proteins might vary among tissues or cell types. Our earlier, preliminary studies using antibodies specific for the...
Table showing nucleotide and amino acid sequences of ERp72 is Related to Protein Disulfide Isomerase. The nucleotides are numbered to the right of each row. The deduced amino acid sequence is shown below the nucleotide sequence. Amino acids are numbered from the first methionine of the open reading frame. The potential N-linked glycosylation site is identified by the asterisk. A poly(A) tail of more than 100 nucleotides was found following the C at position 2356.
two proteins have indicated that the relative levels of the two
proteins are significantly different in liver, muscle, and
spleen.\(^3\) The results of our earlier experiments examining the
synthesis of the ERps during LPS-induced lymphocyte dif-
ferentiation showed that the two proteins were differentially
regulated in the LPS-responsive cells (Lewis et al., 1985a,
1986). The specific synthesis of ERp72 had increased 3-4-
fold by 48 h of LPS treatment whereas no increase was seen
in ERp59/PDI synthesis by this time. In addition, recent
findings\(^4\) have shown that ERp72 expression is increased in
Chinese hamster ovary cells that have been cultured in the
presence of a calcium ionophore or tunicamycin, treatments
which increase the expression of the glucose-regulated pro-
teins, while the expression of ERp59/PDI is not significantly
altered in the same cells. Finally, the work of many labora-
tories has indicated that PDI and the PDI-related proteins
may be multifunctional components. Bennett et al. (1988)
have recently extended the range of functions that could be
attributed to this class of enzymes to that of phosphoinositide-
specific phospholipase C activity. From these findings, it is
clear that the determination of the cellular role of ERp72 will
involve the testing of many possibilities. We are currently
developing ways to express the protein in various vector-host
systems in order to obtain sufficient material for functional
and physical studies.

The capability of expressing ERp59/PDI and ERp72 in
transient expression assays allowed a direct test of the role of

\(^3\) M. J. Lewis, unpublished results.

\(^4\) A. Dorner, manuscript submitted.

Fig. 6. Comparison of ERp59/PDI and ERp72 amino acid
sequences. The sequences have been aligned for maximum homol-

Fig. 7. Analysis of COOH-terminal deletion mutants of
ERp59/PDI and ERp72. An ERp59/PDI mutant lacking only the
KDEL sequence and an ERp72 mutant lacking only the KEEL
sequence were prepared using the Alter-Gene in vitro mutagenesis
system. The synthesis and secretion of the wild type proteins (WT)
and the mutant proteins (−K) were determined in transient expres-
sion experiments using COS cells. In screening experiments (A), cells
were labeled for 4 h with \(^{[35]S}\)methionine (50 plCi/ml) at 72 h after
transfection. ERp59/PDI or ERp72 in the cells (C) or in the medium
(M) was determined by immunoprecipitation with the appropriate
antibody, followed by SDS-PAGE and autoradiography. The kinetics
of secretion of the wild type and mutant proteins was determined in
pulse-chase experiments (B). At 72 h after transfection, COS cells
were labeled for 15 min with \(^{[35]S}\)methionine (100 \(\mu\)Ci/ml). The
labeling media was removed, and the cells were incubated in complete
media containing 0.3 mM unlabeled methionine. The amount of each
protein in the cells or in the medium at the indicated times of chase
was determined by immunoprecipitation. The kinetics of secretion
is reported as the fraction of the total protein (cells + medium) remain-
ing in the cells at various times of chase. Protein was quantitated by
densitometric analyses of the autoradiograms.
the appearance of this doublet. Since both species of wild type ERp72 are retained within the cells and since each is secreted when the COOH-terminal KEEL is removed, our current hypothesis is that the difference between the two forms is a result of a modification or cleavage of the NH2-terminal portion of the protein. Another finding indicated that ~20% of the wild type ERp59/PDI was found in the media while only 10%, at most, of the wild type ERp72 was found outside the cells. The fact that any of the wild type protein is secreted at all could be due to the very high levels of expression of the protein obtained in transfection assays. Thus, the large amount of the KDEL- or KEEL-containing protein being produced could be saturating the mechanism for ER retention. The fact that there are differences between the basal level of export of these proteins could be due to differences in relative level of expression of the proteins. Alternatively, secretion of the wild type protein could be an idiosyncratic property of a particular protein when it is overexpressed. Consistent with this idea, is the fact that we failed to detect secretion of endogenous KDEL-containing proteins, such as BiP (Munro and Pelham, 1986) and ERp99/GRP94 (Mazzarella and Green, 1987) in the cells that were secreting wild type ERp59/PDI. Another difference observed between the proteins was in the kinetics of secretion of the mutants lacking their COOH-terminal retention signals. Although essentially all of each of the mutant proteins were secreted from the cells, ERp59/PDI was secreted with a t0 of 3.5 h, while ERp72 was secreted with a t0 of 1.0–1.5 h. This difference in rate of secretion could reflect the presence of other retention signals in ERp59/PDI. Alternatively, ERp59/PDI and ERp72 may interact with different proteins in the lumen of the ER and these different interactions may influence the rate at which each protein leaves the ER. Pertinent to these findings, are our earlier findings that, on the basis of subcellular fractionation procedures and biosynthetic sorting experiments, ERp72 appears to be more broadly distributed in the ER than does ERp59/PDI (Lewis et al., 1985a, 1985b, 1986). It is interesting to note that the results seen with these two proteins are quite different than those obtained with ERp99/GRP94 which was secreted only very slowly (12% after 8 h of chase) upon removal of its KDEL sequence. The slow rate of secretion of ERp99/GRP94 appears to be due, at least in part, to the presence of a 21-amino acid hydrophobic sequence in the NH2-terminal third of the protein.

The results reported in this paper have added to the KDEL signal family by showing that KEEL can also function as a retention signal for ERp72. Interestingly, the phosphoinositide-specific phospholipase C described by Bennett et al. (1988) has QEDL, another KDEL-related sequence, at its COOH terminus. In preliminary experiments, we have found that antibody against the phospholipase C recognizes ERp61, a luminal, resident ER protein and that the NH2-terminal 19 amino acids of ERp61 are identical to the sequences reported for the mature phospholipase C. It may prove interesting to see if other COOH-terminal tetrapeptide sequences which are closely related to KDEL could also function as ER retention signals and whether their signal function is dependent on or independent of the carrier protein.

Acknowledgments—We would like to thank Dr. Andrew Dorner of the Genetics Institute, Inc. for arranging the synthesis of the oligonucleotide used for mutagenesis of ERp72 and Dr. David G. Boile at Yale University for the gift of anti-BiP monoclonal antibody. Thanks are also due to Dr. James Lee for the use of the densitometer and to Nga Ho for her expertise and assistance with the COS cell transient expression studies. Finally, the authors would like to thank Dr. Arnold Kaplan for helpful discussions and recognize the excellent secretarial skills of Terrie Konisky.

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ERp72, an abundant luminal endoplasmic reticulum protein, contains three copies of the active site sequences of protein disulfide isomerase.
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