Protein Degradation by ERp72 from Rat and Mouse Liver Endoplasmic Reticulum*

(Received for publication, February 25, 1993, and in revised form, June 22, 1993)

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The endoplasmic reticulum (ER) resident protein, ER60, is a member of the protein disulfide-isomerase family and contains two copies of the internal thioredoxin motif, CGHC. Previously, ER60 was identified as a cysteine protease and named ER-60 protease (Urade, R., Nasu, M., Moriyama, T., Wada, K., and Kito, M. (1992) J. Biol. Chem. 267, 15152–15159; Urade, R., and Kito, M. (1992) FEBS Lett. 312, 83–86). Here, ERp72, the other member of the protein disulfide-isomerase family containing three CGHC motifs, was isolated from ER of rat and mouse livers through four sequential chromatographies on DEAE-Toyopearl 650, AF-heparin Toyopearl 650M, and TSK gel G3000SW twice. The purified rat protein was found to be homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, not being contaminated by ER-60 protease, as judged on immunoblot analysis using an anti-ER-60 protease antibody. The partial amino acid sequence of rat ERp72 was 93% homologous to that of mouse ERp72. The purified rat ERp72 degraded other ER resident proteins such as protein disulfide-isomerase and calreticulin. The purified mouse ERp72 also degraded those proteins. Though rat ERp72 did not basically require Ca2+ for the reaction, the degradation of protein disulfide-isomerase was enhanced, but the degradation of calreticulin was inhibited in the presence of Ca2+. The proteolytic activity of rat ERp72 was inhibited by cysteine protease inhibitors. Its sensitivity to protease inhibitors was the same as that of ER-60 protease. In addition, the proteolytic activity of rat ERp72 was inhibited by acidic phospholipids, also similar to ER-60 protease. Therefore, we propose that ERp72 be named ER-72 protease.

In a previous study, we purified a luminal protein of the endoplasmic reticulum (ER) exhibiting sequence similarity to phospholipid-specific phospholipase C (ALLM) from rat liver (2). This protein was characterized as a novel cysteine protease and named ER-60 protease (3). It is well known that cathepsins, intracellular cysteine proteases, contain a well conserved amino acid sequence around cysteine residues constituting the active site, as in the case of papain (4, 5). However, a similar amino acid sequence was not found in ER-60 protease, in contrast to calpain, a cytosolic cysteine protease (6). ER-60 protease contains seven cysteine residues, four of which constitute two copies of the internal thioredoxin motif, CGHC, and it is regarded as a member of the protein disulfide-isomerase family (7). However, it is unknown whether or not a cysteine of the CGHC motif in ER-60 protease comprises the active-site structure. Among ER proteases, ERp72 (8, 9) and P5 (10) are also known to be members of the protein disulfide-isomerase family, but their biological functions are unknown. Mouse ERp72 and human ERp72 contain no cysteine residue other than the six cysteine residues constituting their three CGHC motifs. Recently, a protein homologous to ERp72 was reported to exist in rat liver (11). It seems possible that a CGHC motif of ER-60 protease constitutes its active-site structure. This may be confirmed by examining whether or not ERp72 exhibits protease activity and properties similar to those of ER-60 protease.

In this study, we describe the purification and properties of ERp72 from rat and mouse liver ER.

EXPERIMENTAL PROCEDURES

Materials—Bacillus cereus phospholipase C (grade 1, 2000 units/mg), casein, N-acetyl-leucyl-leucyl-norleucinal (ALLM), and N-acetyl-leucyl-leucyl-methioninal (ALLM) were purchased from Boehringer Mannheim. Leupeptin and E-64 were from Peptide Institute, Inc. (Osaka, Japan). N-Tosyl-l-phenylalanyl chloromethyl ketone (TPCK) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). N-Tosyl-l-lysyl chloromethyl ketone (TLCK) was from Aldrich. Bovine liver protein disulfide-isomerase was obtained from Takara Shuzo Co., Ltd., Kyoto, Japan. Bovine serum albumin was purchased from Miles Laboratories Inc. Lysoylendopeptidase (2 units/mg) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A DEAE-Toyopearl 650 prepacked column, AF-heparin Toyopearl 650M resin, and a TSK gel G3000SW high performance liquid chromatography (HPLC) column were obtained from Tosoh (Tokyo). A µBondapack C8, HPLC column was purchased from Waters. Polyvinylidene difluoride (PVDF) protein sequencing membranes were obtained from Bio-Rad. A Protoblot™ immunoblotting system was purchased from Promega Biotech. All other chemicals were of reagent grade.

Purification of ER-72 Protease—Smooth ER of liver was prepared from 20 male Sprague-Dawley rats (8 weeks old) by the sucrose density gradient centrifugation method, as described previously (2). Unless otherwise specified, all procedures were carried out at 4°C. One liter of the smooth ER suspension (6577 mg of protein) was mixed with 4000 units of B. cereus phospholipase C, followed by incubation for 10 min at 37°C. The suspension was centrifuged for 1 h at 290,000 × g. The supernatant was supplemented with solid ammonium sulfate (516 g/liter) and then stirred for 30 min. The mixture was then centrifuged for 30 min at 13,000 × g. The resulting pellet was dissolved in 50 ml of 20 mM Tris-HCl, pH 7.4, containing 0.2 mM EDTA, 0.5 mM PMSE, and 10% glycerol (buffer A) and then dialyzed overnight against 3 liters of buffer A.
containing 50 mM KCl, 0.5 mM EGTA, 0.5 mM PMSF, and 10% buffer B. The dialysate (12 mg of protein) was applied to an AF-glycerol (buffer B) and then dialyzed overnight against 1 liter of and precipitated with ammonium sulfate as described above. The heparin Toyopearl 650M column (0.7 cm) was applied to the same column, as of which the amino acid sequences of internal peptide fragments were column was eluted with a linear gradient of 0-250 mM NaCl in buffer 400 mM KCl at the flow rate of 0.5 ml/min. Rat ERp72 was eluted from the column with 400 mM KCl. The eluted fraction was concentrated to 0.8 ml and then applied to the TSK gel G3000SW column again. The peak fractions of rat ERp72 (0.6 mg of protein) were collected and dialyzed overnight against 10 mM bis-Tris-HCl, pH 7.0.

Mouse ERp72 was purified from liver smooth ER prepared from 100 male ddY mice (8 weeks old) as described above.

**Purification of Calreticulin and Carboxylesterase—Calreticulin and carboxylesterase E1 were prepared from an extract of the smooth ER of rat liver by B. cereus phospholipase C treatment, through three sequential chromatographies on DEAE-Toyopearl 650M, and TSK gel G3000SW, as described previously (2).** The purified proteins were dialyzed overnight against 10 mM bis-Tris-HCl, pH 7.0.

**Amino Acid Sequencing of Internal Peptide Fragments—100 μg of rat ERp72 was precipitated with 6% trichloroacetic acid at 4 °C for 30 min and then centrifuged at 4 °C for 30 min at 1000 x g. The pellet was washed five times with 1 ml of water-saturated diethyl ether and then dried under N2 gas. The dried protein was digested with lysylendopeptidase, and then the peptide fragments produced were separated on a μBondasphere C4 HPLC column as described previously (2). The isolated peptides were analyzed with a Protein Sequencer (model 477A) equipped with an on-line HPLC (model 120A) (Applied Biosystems, Inc.).**

**Assaying of Proteolytic Degradation—The purified rat or mouse ERp72 and the indicated substrate protein were incubated for 3 h at 37 °C with 10 mM bis-Tris-HCl, pH 6.3, supplemented with 0.1 M β-mercaptoethanol and 1 mM EDTA or 1 mM CaCl2, in the absence or presence of an inhibitor, in a final volume of 9 μl. For determination of the effects of phospholipids, the reaction was carried out in 40 mM bis-Tris-HCl, pH 6.3, supplemented with 0.1 M β-mercaptoethanol. Phospholipids were sonicated for 5 min in 100 mM bis-Tris-HCl, pH 7, at 1 °C and then added to the reaction mixture. The reaction products were analyzed by SDS-PAGE.**

**Nondenaturing Gel Electrophoresis of ERp72—The purified rat ERp72 (15 μl) was mixed with 6 μl of sample buffer (40% glycerol, 250 mM Tris-HCl, pH 6.8, 20% β-mercaptoethanol, 0.02% bromphenol blue). A 7.5% non-denaturing slab gel (8.5 × 5.5 cm) was prepared using the discontinuous buffer system (12). Samples were loaded onto the gel and electrophoresed at 4 °C. The ERp72 band was excised, crushed in a plastic tube with a spatula, and incubated.
The partial amino acid sequence of rat ERp72 was determined as described under "Experimental Procedures." Amino acid residues of mouse ERp72 different from those of rat ERp72 are shown below the sequences of peptide fragments of rat ERp72. Regions of identity between mouse and rat ERp72s are as noted by periods.

<table>
<thead>
<tr>
<th>Peptide fragment</th>
<th>Amino acid sequence</th>
<th>Corresponding mouse ERp72 sequence*</th>
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<tr>
<td>1</td>
<td>FDVSGYPTL</td>
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<tr>
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<td>Gin&lt;sup&gt;200&lt;/sup&gt;—Lys&lt;sup&gt;204&lt;/sup&gt;</td>
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<tr>
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</table>

* Data are from Mazzarella et al. (8).

RESULTS

Purification and Characterization of Rat ERp72—ERp72 is a soluble protein localized in the ER lumen and contains the COOH-terminal retention signal, KEEL (8). Thus, we started the purification of ERp72 in the first step with phospholipase C from B. cereus and then centrifuged to separate the released soluble proteins from the insoluble materials. The soluble proteins were applied to a DEAE column. 70-80-kDa proteins were eluted with a linear gradient of NaCl from the column as three peaks. Fractions containing a 74-kDa protein(s), which was eluted with 200-225 mM NaCl, were pooled and applied to a heparin column. Then, the effluent was applied to a TSK gel G3000SW column, and the 74-kDa protein(s) was eluted at two positions. The 74-kDa protein, which was eluted at a position corresponding to M<sub>r</sub> = 160,000, was determined not to be a protein corresponding to ERp72 by internal amino acid sequencing (data not shown). The 74-kDa protein, which was eluted at a position corresponding to M<sub>r</sub> = 70,000, was judged to be ERp72, because its internal amino acid sequences were determined to be similar to those of mouse and human ERp72. Then, the rat ERp72 fractions were pooled and applied again to the TSK gel G3000SW column. Rat ERp72 was eluted at a position corresponding to M<sub>r</sub> = 70,000 as a single peak with a small shoulder, suggesting that rat ERp72 is a monomeric protein (Fig. 1, A and B). The fractions obtained at each purification step were analyzed by SDS-PAGE. Rat ERp72 obtained on the second gel filtration chromatography was apparently homogeneous (Fig. 2, lane 6). Because ER-60 protease was separated from ERp72 by the first step DEAE column chromatography, contamination by ER-60 protease was not detected in the rat ERp72 preparation during immunoblot analysis (Fig. 2, lane 7).

NH<sub>2</sub>-terminal amino acid sequence analysis of rat ERp72 was unsuccessful. After rat ERp72 had been treated with lysylendopeptidase, the digest was separated into peptide fragments on a C<sub>18</sub> column. The amino acid sequences (131 amino acid residues) of the peptide fragments analyzed were 93% homologous to those of mouse ERp72 (Table I).

Endoprotease Activity of Rat ERp72—The proteolytic activity of rat ERp72 was analyzed by SDS-PAGE after its incubation with a substrate protein. Rat ERp72 (Fig. 3A, lane 1) or each substrate protein alone (lanes 2, 5, and 8) was not autocatalytically degraded after a 3-h incubation at 37°C. When bovine protein disulfide-isomerase (Fig. 3A, lane 3), rat calreticulin (lane 6), or bovine serum albumin (lane 9) was incubated with rat ERp72, each substrate protein was degraded into small peptide fragments. Rat ERp72 remained unaltered after the incubation. Rat carboxylesterase E1 and casein were resistant to digestion by rat ERp72 (data not shown). The degradation of protein disulfide-isomerase by rat ERp72 was stimulated by the addition of CaCl<sub>2</sub> to the reaction mixture (Fig. 3A, lane 4). The degradation reached a plateau at 300 μM CaCl<sub>2</sub> (Fig. 3B). However, degradation was observed even in the presence of 1 mM EGTA (Fig. 3A, lane 3, and 3B).
The proteolytic degradation of protein disulfide-isomerase (PDI) by rat ERp72. A, bovine protein disulfide-isomerase (PDI) (1 μg of protein) (lanes 2–4), calreticulin (Cal) (1 μg of protein) (lanes 5–7), and bovine serum albumin (BSA) (2 μg of protein) (lanes 8–10) were incubated for 3 h at 37 °C without (lanes 2, 5, and 8) or with rat ERp72 (1 μg of protein) (lanes 3, 4, 6, 7, 9, and 10) in the presence of 1 mM EGTA (lanes 3, 5, 6, and 9) or 1 mM CaCl₂ (lanes 2, 4, 7, and 10) as described under "Experimental Procedures." Then the samples were subjected to SDS-PAGE (12.5% gel). Bovine serum albumin was degraded regardless of whether EGTA or CaCl₂ was present (lanes 3, 4, 6, 7, and 9). The proteins were stained with Coomassie Brilliant Blue R-250. 

Effects of Protease Inhibitors on the Proteolytic Activity of Rat ERp72—The effects of protease inhibitors on the degradation of protein disulfide-isomerase by rat ERp72 were determined. The degradation was prevented by pretreatment of the rat ERp72 with 1 mM p-chloromercuribenzoate or iodoacetic acid (Fig. 5, lanes 4 and 5), suggesting that the thiol group of rat ERp72 is essential for its proteolytic activity. Diisopropyl fluorophosphate or phenylmethylsulfonyl fluoride (PMSF) did not inhibit the proteolytic activity of rat ERp72.

FIG. 3. Proteolytic degradation of ER proteins by rat ERp72. A, bovine protein disulfide-isomerase (PDI) (1 μg of protein) (lanes 2–4), calreticulin (Cal) (1 μg of protein) (lanes 5–7), and bovine serum albumin (BSA) (2 μg of protein) (lanes 8–10) were incubated for 3 h at 37 °C without (lanes 2, 5, and 8) or with rat ERp72 (1 μg of protein) (lanes 3, 4, 6, 7, 9, and 10) in the presence of 1 mM EGTA (lanes 3, 5, 6, and 9) or 1 mM CaCl₂ (lanes 2, 4, 7, and 10) as described under "Experimental Procedures." Then the samples were subjected to SDS-PAGE (12.5% gel). Bovine serum albumin was degraded regardless of whether EGTA or CaCl₂ was present (lanes 3, 4, 6, 7, and 9). The proteins were stained with Coomassie Brilliant Blue R-250.

FIG. 4. Proteolytic activity of rat ERp72 fraction on a nondenaturing gel. A, the purified rat ERp72 (2 μg of protein) was electrophoresed on a nondenaturing polyacrylamide gel at 100 V for 2 h and stained with silver. B, after rat ERp72 (2 μg of protein/lane) was electrophoresed in parallel on the nondenaturing polyacrylamide gel, ERp72 bands were excised. One of the ERp72 bands was subjected to SDS-PAGE (10% gel) and stained with silver (lane 1). The other ERp72 band was incubated for 3 h at 37 °C in a reaction mixture, containing protein disulfide-isomerase (PDI) and then subjected to SDS-PAGE as described under "Experimental Procedures" (lane 3). The reaction mixture containing PDI incubated for 3 h at 37 °C without gel slices was analyzed by SDS-PAGE (lane 2). The proteins were stained with Coomassie Brilliant Blue R-250.

FIG. 5. Effects of protease inhibitors on the degradation of protein disulfide-isomerase by rat ERp72. The effects of the following protease inhibitors on the degradation of bovine protein disulfide-isomerase (PDI) (1 μg of protein) by rat ERp72 (1 μg of protein) were determined as described under "Experimental Procedures." Lanes 3, 8, and 10, no addition; lane 4, 1 mM p-chloromercuribenzoate (pCMB); lane 5, 1 mM iodoacetic acid (IAA); lane 6, 1 mM diisopropyl fluorophosphate (DFP); lane 7, 1 mM phenylmethylsulfonyl fluoride (PMSF); lane 9, 1 mM EDTA; lane 11, 10 μM ALLN; lane 12, 10 μM ALLM; lane 13, 10 μM E-64; lane 14, 10 μM leupeptin; lane 15, 10 μM TLCK; lane 16, 10 μM TPCK. In the case of lanes 4–7, the inhibitors and rat ERp72 were allowed to stand for 2 h at 4 °C before starting the incubation with PDI. Except in the case of lanes 8 and 9, the reactions were carried out in the presence of 1 mM CaCl₂. The samples were subjected to SDS-PAGE (12.5% gel). Lanes 1 and 2 contained untreated rat ERp72 and PDI, respectively.
Inhibition by Acidic Phospholipids of the Proteolytic Activity of Rat ERp72—We have reported that the proteolytic activity of ER-60 protease was inhibited by acidic phospholipids, such as phosphatidylinositol, phosphatidylinositol 4,5-bisphosphate, and phosphatidylserine (3). We examined the effects of acidic lipids on its proteolytic activity. An acidic phospholipid (phosphatidylinositol or phosphatidylserine) was added to the reaction mixture as mixed liposomes formed with phosphatidylcholine and phosphatidylethanolamine. The assay was performed in the absence of CaCl2. The liposomes composed of phosphatidylcholine and phosphatidylethanolamine did not have any effect on the degradation of protein disulfide-isomerase (Fig. 6, lanes 6-8). The liposomes containing phosphatidylserine inhibited the degradation (Fig. 6, lanes 9-11). The liposomes containing phosphatidylserine also had an inhibitory effect on the degradation (Fig. 6, lanes 12-14). Similar effects of these acidic phospholipids were observed on the degradation of calreticulin (data not shown).

Endoprotease Activity of Mouse ERp72—Mouse ERp72 was isolated from the ER of mouse liver by using the same purification procedures as those for rat ERp72. There was no visible silver-stained band of contaminating proteins on either SDS-PAGE or nondenaturing gel electrophoresis (Fig. 7A). The purified mouse ERp72 degraded bovine protein disulfide-isomerase and rat calreticulin (Fig. 7B, lanes 4 and 6). The degradation of protein disulfide-isomerase was stimulated in the presence of CaCl2 (Fig. 7B, lane 3).

**DISCUSSION**

In this paper, we described the purification of ERp72 from ER of rat and mouse livers. The internal amino acid sequences of rat ERp72 were almost identical to those of mouse ERp72 (8). Rat ERp72 is not N-glycosylated (data not shown). The purified rat ERp72 exhibits endoprotease activity, which is inhibited by cysteine protease inhibitors and acidic phospholipids, as was ER-60 protease (2, 3). It is unlikely that this proteolytic activity was due to contamination by other proteolytic enzymes, because there was no visible band of a contaminant on SDS-PAGE, and the elusion profile of the activity from the TSK gel G3000SW column coincided with that of the rat ERp72 protein. Furthermore, no contamination by ER-60 protease of the final rat ERp72 preparation was detected on Western blotting using the anti-ER-60 protease antibody. In addition, mouse ERp72 similarly exhibited endoprotease activity. Therefore, we call rat ERp72, ER-72 protease. ER-72 and ER-60 proteases may be regarded as a novel class of intracellular cysteine proteases, which differ from well-characterized intracellular cysteine proteases, lysosomal cathepsins, and cytosolic calpains, with regard to their primary structure, intracellular localization, and enzymatic properties.

There are 6 cysteine residues in the amino acid sequences of mouse and human ERp72 (8, 9). All of the cysteine residues occur in the internal thioredoxin motif, CGHC. This implies that a cysteine residue in a CGHC motif functions as the active-site cysteine of ER-72 protease and ER-60 protease, because mouse ERp72 showed the proteolytic activity. Protein disulfide-isomerase contains two copies of the internal CGHC motif (7). However, no proteolytic activity was detected for the rat protein disulfide-isomerase purified from liver or the commercial bovine enzyme (data not shown). Protein disulfide-isomerase is known to be a noncovalently associated dimeric protein (14), whereas ER-72 and ER-60 proteases are monomeric proteins. The lack of proteolytic activity of protein

**FIG. 6. Inhibition of the proteolytic activity of rat ERp72 by acidic phospholipids in phosphatidylcholine/phosphatidylethanolamine liposomes.** Bovine protein disulfide-isomerase (PDI) was incubated with rat ERp72 (1 μg of protein) (lanes 3-14) in the absence (lanes 3-5) or presence of 300 μM phosphatidylcholine (PC) and 150 μM phosphatidylethanolamine (PE) (lanes 6-8), 300 μM PC, 150 μM PE, and 200 μM phosphatidylinositol (PI) (lanes 9-11) or 300 μM PC, 150 μM PE, and 200 μM phosphatidylserine (PS) (lanes 12-14) for 0.5 h (lanes 3, 6, 9, and 12), 1 h (lanes 4, 7, 10, and 13), or 3 h (lanes 5, 8, 11, and 14) at 37 °C as described under “Experimental Procedures.” Then the samples were subjected to SDS-PAGE (12.5% gel). Lanes 1 and 2 contained untreated rat ERp72 and PDI, respectively.

**FIG. 7. Proteolytic activity of mouse ERp72.** A, purified mouse ERp72 (1 μg of protein) was subjected to SDS-PAGE (10% gel) or nondenaturing gel electrophoresis (lane 2) and stained with silver. B, mouse ERp72 (1 μg of protein) was incubated for 3 h at 37 °C with or without (lane 1) protein disulfide-isomerase (PDI) (1 μg of protein) (lanes 3 and 4) or calreticulin (Cal) (1 μg of protein) (lane 6) in the absence (lanes 1, 4, and 6) or presence of 1 mM CaCl2 (lane 3), PDI (lane 2) and Cal (lane 5) were incubated for 3 h at 37 °C without ERp72. Subsequently, the samples were subjected to SDS-PAGE (10% gel). Proteins were stained with Coomassie Brilliant Blue R-250.
disulfide-isomerase may be due to its dimer structure. Alternatively, it seems likely that some structure, which is shared by ER-72 and ER-60 proteases other than the CGHC motif, may be essential for the proteolytic activity.

The degradation of protein disulfide-isomerase was stimulated in the presence of CaCl₂, suggesting that ER-72 protease is a calcium-dependent protease like calpain (15). However, unlike calpain, considerable degradation of protein disulfide-isomerase, calreticulin, and bovine serum albumin was observed even in the presence of EGTA. Calreticulin was not degraded in the presence of CaCl₂. Degradation of bovine serum albumin proceeded independently of the presence of EGTA or CaCl₂. These results suggest that Ca²⁺ enhanced the degradation of protein disulfide-isomerase through its interaction with protein disulfide-isomerase rather than the enzyme. Contrary to this, calreticulin was resistant to the degradation due to its interaction with Ca²⁺. Protein disulfide-isomerase (16) and calreticulin (17, 18) are known to be calcium-binding proteins.

Recent studies on the fate of proteins synthesized in ER in vivo have indicated the existence of a selective pre-Golgi degradation pathway (19). Not only unassembled proteins, such as T cell antigen receptor subunits (20, 21) and asialoglycoprotein receptor subunits (22), but also normal protein, such as 3-hydroxy-3-methylglutaryl-CoA reductase (23, 24), are degraded through this pathway. The degradation of these proteins in cells is inhibited by membrane-permeable cysteine protease inhibitors or sulfhydryl reagents (25–29). At present, no cysteine protease that acts on the pre-Golgi degradation system has been identified. It is possible that ER-72 and ER-60 proteases are components of the pre-Golgi degradation system, because the sensitivity of ER-60 and ER-72 proteases to cysteine protease inhibitors is similar to that of the in vivo degradation system. However, we have no direct evidence for this at present.

Acknowledgment—We thank Dr. Robert D. Simoni for critically reading the manuscript and valuable helpful discussions.

REFERENCES