J. Biochem. 126, 722-730 (1999)

Isolation and Characterization of Two 20S Proteasomes from the Endoplasmic Reticulum of Rat Liver Microsomes[1]

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Received May 17, 1999; accepted July 31, 1999

Two new forms of proteasomes, designated as the endoplasmic reticulum (ER) membrane-associated proteasome (ERa proteasome) and ER membrane-bound proteasome (ERb proteasome), were purified to homogeneity from 0.0125 and 2.5% sodium cholate extracts, respectively, of a rat liver microsomal fraction. SDS-PAGE analysis revealed that the purified ERa and ERb proteasomes were composed of multiple subunits similar to the cytosolic 20S proteasome. However, electrophoretic, structural and immunochemical differences between the ERa, ERb and cytosolic 20S proteasomes were observed on native PAGE, two-dimensional (2D) PAGE, and immunoblot analyses. Purification of ERb from a 2.5% sodium cholate extract of the trypsin-treated microsomal fraction yielded a trypsin-modified form of ERb (tERb), which lacked the C2 subunit at least. On the other hand, no ERa proteasome was obtained from the 0.0125% sodium cholate extract of the trypsin-treated microsomes, suggesting that ERa and ERb are ER membrane-associated and -bound proteasomes, respectively. The ERa, ERb, and cytosolic 20S proteasomes exhibited similar specificities as to peptide hydrolyzing activity, although differences in their activities were noted in the presence of SDS and phospholipid. With respect to the proteolysis of protein substrates, only the ERb proteasome cleaved β-casein, and it also degraded reduced and carboxymethylated lysozyme considerably faster than the cytosolic 20S and ERa proteasomes. Collectively our results suggest that the ERa and ERb proteasomes may play roles in intracellular proteolysis distinct from that of the cytosolic 20S proteasome.

Key words: endoplasmic reticulum, ER-associated degradation, proteasome, proteolysis, quality control.

Proteasome is the key enzyme in intracellular protein turnover, antigen processing on MHC class I antigen presentation, and many other cellular events (*1-3*). The 20S proteasome (20S), a threonine protease (*4*) and a member of the N-terminal nucleophile-amidohydrolase family (*5*), is the major cytosolic protease complex that forms the core of the 26S proteasome complex which selectively degrades ubiquitinated proteins in an ATP-dependent manner (*6*). This 750 kDa protease is composed of 28 subunits arranged in four heptameric stacking rings, $\alpha_7\beta_7\beta_7\alpha_7$, and forms a hollow cylindrical shape particle (*7*) that has at least five distinct peptide hydrolyzing activities, designated as chymotrypsin-like, trypsin-like, peptidyl glutamyl-peptide hydrolyzing (PGPH), branched chain amino acid preferring (BrAAP), and small neutral amino acid preferring (SNAAP) activities (*8*).

This multi-catalytic protease complex is now thought to play a major role in a quality control system in the endoplasmic reticulum (ER) (*9*). Thus, among newly synthesized proteins, misfolded or abnormal secretory products that are not suitable for export from the ER, in addition to unassembled subunits of oligomer proteins, are subjected to selective degradation within the ER (*10*). This ER-associated protein degradation, referred to as "quality control" mechanisms in the ER (*11*), has recently been studied extensively. The substrates for degradation are translocated from the ER to the cytosol through retrograde transport (*12*), where proteolysis is catalyzed by the proteasome (*13*). We also reported such degradations of various types of plasma proteins mutants, which was blocked by proteasome inhibitors (*14-16*).

[1] This study was supported in part by Grants-in-Aid for Scientific Research on Priority Areas, "Intracellular Proteolysis," and for Scientific Research (B) (both to T.K.) from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: Boc, *t*-buthyloxycarbonyl; CHES, *N*-cyclohexyl-2-aminoethanesulfonic acid; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; FR, Z-L-phenylalanyl-L-arginine-MCA; LLE, Z-L-leucyl-L-leucyl-L-glutamic acid-MCA; LLL, Z-L-leucyl-L-leucyl-L-leucine-MCA; LLVY, Suc-L-leucyl-L-leucyl-L-valyl-L-tyrosine-MCA; LRR, Boc-L-leucyl-L-arginyl-L-arginine-MCA; MCA, 4-methyl-coumaryl-7-amide; MG115, Z-L-leucyl-L-leucyl-L-norvalinal; MG132, Z-L-leucyl-L-leucyl-L-leucinal; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RCM, reduced and *S*-carboxymethylated; Suc, succinyl; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tricine, *N*-[Tris(hydroxymethyl)methyl]glycine; Z, carbobenzoxy.

The retrograde transport of protein substrates has been proposed to be a pathway to the ER-associated degradation (*17-19*), and these substrates are digested rapidly by the cytosolic proteasome (*2, 19*). 20S is mainly localized in the cytoplasm and nucleus (*20*). However, approximately 14% of the total proteasome in rat hepatocytes has been reported to be closely associated with the ER (*20-22*). The ER-associated proteasome is distributed on the cytosolic surface of the ER (*23*), and is thought to contribute to the proteolysis and extraction of ER proteins (*24*). On the other hand, the ER membrane-bound cysteine proteases have also been reported to take part in the degradation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (*25*). Therefore, ER-associated protein degradation may be performed not only by the cytosolic proteasome but also driven by some other protein(s) that are involved in the retrograde transportation, extraction from the ER-membrane and digestion of substrates. To identify the proteases in the ER that might contribute to the ER-associated protein degradation in the quality control machinery, we have isolated and characterized proteases from a rat liver microsomal fraction.

Here, we report the isolation and characterization of two novel proteases, designated as the ERa and ERb proteasomes, from 0.0125 and 2.5% sodium cholate extracts, respectively, of rat liver microsomes, and also report on differences in their structural and enzymological properties in comparison to those of the cytosolic 20S.

EXPERIMENTAL PROCEDURES

Materials—Sprague Dawley (SD) strain male rats were purchased from CLEA Japan (Tokyo), and Japanese White (JW) female rabbits were purchased from KAC (Kyoto). The chromatography media were all purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), except for POROS HS (PE Biosystems Japan, Chiba). The anti-human C2 subunit (HC2) monoclonal antibody, mAb 2-17 (*26*), was a generous gift from Dr. Keiji Tanaka (The Tokyo Metropolitan Institute of Medical Science, Tokyo). Various peptidyl 4-methyl-coumaryl-7-amide (MCA) substrates, carbobenzoxy (Z)-Phe-Arg-MCA (FR), Z-Leu-Leu-Glu-MCA (LLE), Z-Leu-Leu-Leu-MCA (LLL), succinyl (Suc)-Leu-Leu-Val-Tyr-MCA (LLVY), and *t*-buthyloxycarbonyl (Boc)-Leu-Arg-Arg-MCA (LRR), were purchased from Peptide Institute (Osaka). Pepstatin, chymostatin, Z-leucyl-leucyl-norvalinal (MG115), and Z-leucyl-leucyl-leucinal (MG132) were purchased from Peptide Institute (Osaka). Dithiothreitol (DTT), benzamidine, *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), and lysozyme were purchased from Nacalai Tesque (Kyoto). *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), trypsin from porcine pancreas (type IX), trypsin inhibitor from soybean (type II-S), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), β-casein, and bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO). Lactacystin prepared from *Streptomyces* sp. OM-6519 (*27*) was a generous gift from Dr. Satoshi Omura (The Kitasato Institute, Tokyo). Other materials were of the highest grade commercially available.

Isolation of a Microsome Fraction—Starved SD rat liver was perfused with a 0.25 M sucrose solution and then washed with 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). The washed liver was homogenized in 9 volumes of 20 mM Tris-HCl buffer, pH 7.5, containing 50 mM KCl, 2 mM $MgCl_2$, and 0.25 M sucrose (homogenization buffer). In the following steps, all procedures were carried out at 4°C unless otherwise specified. After centrifugation for 20 min at 12,000 × *g*, the supernatant was recovered and subsequently centrifuged for 1 h at 105,000 × *g*. The resulting microsome fraction (precipitate) was collected, resuspended in a 0.15 M KCl solution, pH 7.5, containing 1 mM EDTA, and again centrifuged for 1 h at 105,000 × *g*. The pellet (washed microsome fraction) was collected, resuspended in homogenization buffer at 40 mg/ml (protein concentration) and, if not processed immediately, stored at −80°C until cholate extraction.

Extraction with Sodium Cholate—The washed microsome fraction resuspended in homogenization buffer at 40 mg/ml (protein concentration) was mixed with 2 volumes of homogenization buffer containing 2 mM benzamidine, 2 mM DTT, 2 mM EDTA, and 2 μg/ml pepstatin (extraction buffer). A one-third volume of a 0.05% sodium cholate solution was added and then the mixture was incubated for 1 h with gentle agitation. The slurry was then centrifuged for 1 h at 105,000 × *g*. The supernatant (0.0125% cholate extract) was collected, fractionated by 50-75% saturated ammonium sulfate precipitation and then dialyzed against 20 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 1 mM benzamidine, 1 mM DTT, 1 mM EDTA, 1 μg/ml pepstatin, and 0.1 M NaCl (A-buffer). The pellet obtained on centrifugation was resuspended in homogenization buffer at 40 mg/ml, mixed with 2 volumes of extraction buffer and 1 volume of 10% sodium cholate, and then incubated for 1 h with gentle agitation. After centrifugation for 1 h at 105,000 × *g*, the supernatant (2.5% cholate extract) was recovered and dialyzed against 20 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 1 mM benzamidine, 1 mM DTT, 1 mM EDTA, and 1 μg/ml pepstatin (B-buffer).

Trypsin Treatment of the Microsome Fraction—Washed microsomes were treated with a 1/10 protein weight of trypsin for 1 h at 4°C in homogenization buffer containing 1 mM benzamidine, 1 mM DTT, 1 mM EDTA, and 1 μg/ml pepstatin. The reaction was stopped by the addition of a twofold molar excess of soybean trypsin inhibitor. After centrifugation for 1 h at 105,000 × *g*, the resulting pellet was extracted with sodium cholate as described above. To determine the localization of the purified proteases, the trypsin digestion was also performed in the presence of 1% (v/v) Triton X-100.

Column Chromatographies—The 0.0125% cholate extracts fractionated by 50-75% saturated ammonium sulfate precipitation were applied to a DEAE-Sephacel column (2.6 × 6 cm) equilibrated with A-buffer. After washing the column with A-buffer, bound materials were eluted with 300 ml of a linear gradient of 0.1-0.4 M NaCl in the same buffer, at the flow rate of 20 ml/h. The active fractions were pooled and applied to a POROS HS column (2.7 ml), equilibrated with 50 mM acetate buffer, pH 5.5, containing 0.25 M sucrose, 1 mM DTT, 1 mM EDTA, and 1 μg/ml pepstatin (C-buffer). Bound materials were eluted with 30 ml of a linear gradient of 0-0.3 M NaCl in C-buffer, at the flow rate of 1 ml/min. The active fractions were pooled, dialyzed against 50 mM Tris-HCl buffer, pH 7.5, contain-

ing 1 mM DTT, 1 mM EDTA, and 20% glycerol (D-buffer), and then concentrated by ultrafiltration with a YM-10 membrane (Amicon, Beverly, MA).

The protease from the 2.5% cholate extracts was purified by sequential chromatographies on a DEAE-Sephacel column (2.6×20 cm), an SP-Sepharose column (2.2×14 cm), a POROS HS column (2.7 ml), and Superdex 200 column (1.0×30 cm). The buffers used were B-buffer for DEAE-Sephacel, and C-buffer for SP-Sepharose and POROS HS. The materials bound to the ion-exchange columns were eluted with either 1,000 ml of a linear gradient of 0-0.4 M NaCl in B-buffer, at the flow rate of 40 ml/h, for DEAE-Sephacel, 500 ml of a linear gradient of 0-0.3 M NaCl in C-buffer, at the flow rate of 40 ml/h, for SP-Sepharose, or 30 ml of a linear gradient of 0-0.3 M NaCl in C-buffer, at the flow rate of 1 ml/min, for POROS HS. After concentration, the fractions eluted from the POROS HS column were applied to a Superdex 200 column equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM DTT, 1 mM EDTA, and 0.15 M NaCl, and eluted at the flow rate of 0.25 ml/min. POROS HS column chromatography and Superdex 200 gel filtration were performed using an FPLC (Fast Protein Liquid Chromatography) system (Pharmacia, Uppsala, Sweden). The active fractions were pooled, dialyzed against D-buffer and then concentrated by ultrafiltration.

The fractions eluted from each column were monitored as to the absorbance at 280 nm, and the MCA substrate hydrolyzing activity towards FR and LLVY in the presence of 0.025% SDS. The fractions possessing LLVY hydrolyzing activity were pooled for further purification.

Assaying of Peptidase Activity—The procedures were performed essentially as described by Tanaka *et al.* (*28*). An aliquot of a sample (10 μl) was added to 90 μl of the fluorogenic substrate solution. The final reaction mixture was composed of 50 μM MCA substrate in 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM DTT. After 10 min incubation at 37°C, the reaction was stopped by the addition of 150 μl of a 0.9 N acetic acid solution, the mixture was made up to 1 ml with distilled water, and then the fluorescence of released 7-amino-4-methylcoumarin (AMC) was measured with excitation at 380 nm and emission at 440 nm using an F-3010 Fluorescence Spectrophotometer (Hitachi). The same assay was performed in both the presence and absence of 0.025% SDS. For the assay involving FR in the absence of SDS, 20 mM acetate buffer, pH 5.5, was used instead of 50 mM Tris-HCl buffer, pH 8.0.

Degradation of Protein Substrates—Five microliters of a substrate protein (1 mg/ml) in 100 mM Tris-acetate buffer, pH 8.0, was mixed with an equal volume of each protease (40 μg/ml). After incubation at 37°C for various times, the reaction was stopped by the addition of 2 μl of 10% SDS. Samples were then subjected to Tris-tricine SDS-PAGE. After staining substrate proteins with Coomassie Brilliant Blue, the visualized bands were quantitated with a BioImage analyzer (BioImage, Ann Arbor, MI).

Polyacrylamide Gel Electrophoresis—Native PAGE was performed in 5% slab gels for 8 h at 5 mA according to the method described by Davis (*29*). SDS-PAGE was performed by the method of Laemmli (*30*) in 12% gels containing 0.1% SDS or by the method of Schägger and von Jagow (*31*) in 15% gels containing 0.1% SDS using a Tris-tricine buffer system. The gels were stained with Coomassie Brilliant Blue or silver stain reagents (Daiichi Pure Chemicals, Tokyo).

Two-dimensional (2D) PAGE was carried out essentially according to the method of O'Farrell (*32*). Samples were dissolved in a solution containing 9 M urea, 2% NP-40, 5% mercaptoethanol, and 2% carrier ampholytes, *i.e.* Ampholine pH 3.5-10 (Amersham Pharmacia Biotech, Uppsala, Sweden), Bio-lyte pH 6-8 (Bio-Rad, Hercules, CA), and Pharmalyte pH 8-10.5 (Amersham Pharmacia Biotech, Uppsala, Sweden) in a ratio of 8:3:1, respectively. The first dimension was non-equilibrium pH gradient electrophoresis towards the cathode at 200 V (2 h), 400 V (14 h), and then 800 V (1 h). The second dimensional electrophoresis was performed on a 12% SDS polyacrylamide gel.

Preparation of Antisera against the Purified Proteases—Polyclonal antibodies against the purified proteases were raised in JW rabbits. Each antigen (25 μg) was injected with Freund's complete adjuvant subcutaneously at 2-week intervals after the first injection. The antibody titer was analyzed by means of an enzyme-linked immunosorbent assay (ELISA).

Western Blotting—Proteins resolved by SDS-PAGE were electrophoretically transferred to PVDF (polyvinylidene difluoride) membranes (Clear Blot Membrane P, ATTO, Tokyo) with a semi-dry blotter using 25 mM Tris-192 mM glycine-20% (v/v) methanol. Non-specific binding was blocked by soaking the membranes in 2% fat-free dried milk in 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl (TBS-milk) for at least 1 h. Each blot was incubated overnight with primary antibodies in TBS-milk at 4°C. Then the membranes were washed 3 times with 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 0.05% (v/v) Tween 20 (TBS-Tween) for 45 min. Antibodies bound to blots were detected by incubation for 1 h at room temperature with alkaline phosphatase-conjugated goat antibodies to rabbit IgG (EY Lab., San Mateo, CA), diluted 1:3,000 in TBS-milk, followed by washing as described above, and visualized with 173 mM nitrobluetetrazorium/115 mM 5- bromo-4-chloro-3-indolyl phosphate.

Effects of Phospholipids—Fifty microliters of phosphatidylcholine (PC; 10 mg/ml chloroform), phosphatidylethanolamine (PE; 10 mg/ml chloroform), phosphatidylserine (PS; 10 mg/ml chloroform), or a phospholipid mixture (PC:PE:PS=60:20:5; 10 mg/ml) was evaporated under a N_2 stream. This PC:PE:PS=60:20:5 mixture mimicked the phospholipid composition of ER membranes, as described by van Meer (*33*). Then, 10 μl of 50 mM Tris-HCl buffer, pH 8.0, was added and the phospholipid was resuspended by sonication. An equal volume (10 μl) of a purified protease (20 μg/ml) was mixed with the phospholipid suspension. After incubation for 15 min at 37°C, 80 μl of the MCA substrate solution was added, followed by further incubation for 60 min at 37°C. The assay conditions were as described above except for the buffers; 20 mM Tris-HCl buffer (pH 7.5) was used for LLVY in the absence of SDS, and 20 mM Tricine-CHES buffer (pH 9.0) for LRR.

Effects of Various Inhibitors—Ten microliters of a purified protease (20 μg/ml) was mixed with the same volume of an inhibitor solution, containing either lactacystin (100 μM) (*27*), MG132 (200 μM), MG115 (200 μM), TPCK (400 μM), TLCK (200 μM), or chymostatin (200 μM). After incubation for 15 min at 37°C, 80 μl of the MCA substrate solution was added, followed by further incuba-

tion for 20 min at 37°C. Then, the residual peptidase activity was measured as described above.

Protein Determination—The protein contents of microsome and sodium cholate extracts were measured by the method of Bradford (*34*) with BSA as a standard. The protein concentrations of each fraction and the purified proteases were determined from the absorbance at 280 nm, assuming $E^{1\%}_{280}=9.61$ (*28*), according to the extinction coefficient of 20S.

RESULTS

Purification of Proteases from the Microsome Fraction—We prepared a crude microsome fraction from the livers of 8-week-old SD strain male rats as described under "EXPERIMENTAL PROCEDURES." The crude microsome fraction was washed with 0.15 M KCl-1 mM EDTA (pH 7.5), and then ultracentrifuged to quantitatively remove the cytosolic proteins. The microsome fraction was then treated with 0.0125% sodium cholate for 1 h to extract the proteins that had associated with the surface of the ER membrane. After recovering the 0.0125% cholate extract by ultracentrifugation, the precipitate was further treated with 2.5% sodium cholate for 1 h to extract the proteins that had bound to the ER membrane and/or were located within the ER lumen.

The 0.0125% sodium cholate extracts were purified as described under "EXPERIMENTAL PROCEDURES." On a DEAE-Sephacel column, the adsorbed proteins were eluted with a linear NaCl gradient, from 0.1 M to 0.4 M, and protease activities were monitored by assaying both LLVY and FR hydrolyzing activities (data not shown). The peak fractions exhibiting LLVY hydrolyzing activity were pooled, and subsequently applied on a POROS HS column. The fractions having LLVY hydrolyzing activity were eluted at NaCl concentrations between 0.15 and 0.25 M (data not shown). This fraction contained a pure protease which we designated as the ERa proteasome (ERa).

The 2.5% sodium cholate extracts were dialyzed against B-buffer and then applied to a DEAE-Sephacel column. The bound materials were eluted with a linear NaCl gradient (0-0.4 M), and protease fractions were monitored by assaying both LLVY and FR hydrolyzing activities (Fig. 1A). The peak fractions exhibiting LLVY hydrolyzing activity (shown as DE3) were pooled, and then applied to an SP-Sepharose column. The bound materials were eluted with an NaCl gradient, from 0 to 0.4 M, and fractions having LLVY hydrolyzing activity were pooled (Fig. 1B). Following the dialysis, this fraction was applied to a POROS HS column. The LLVY hydrolyzing activity peak

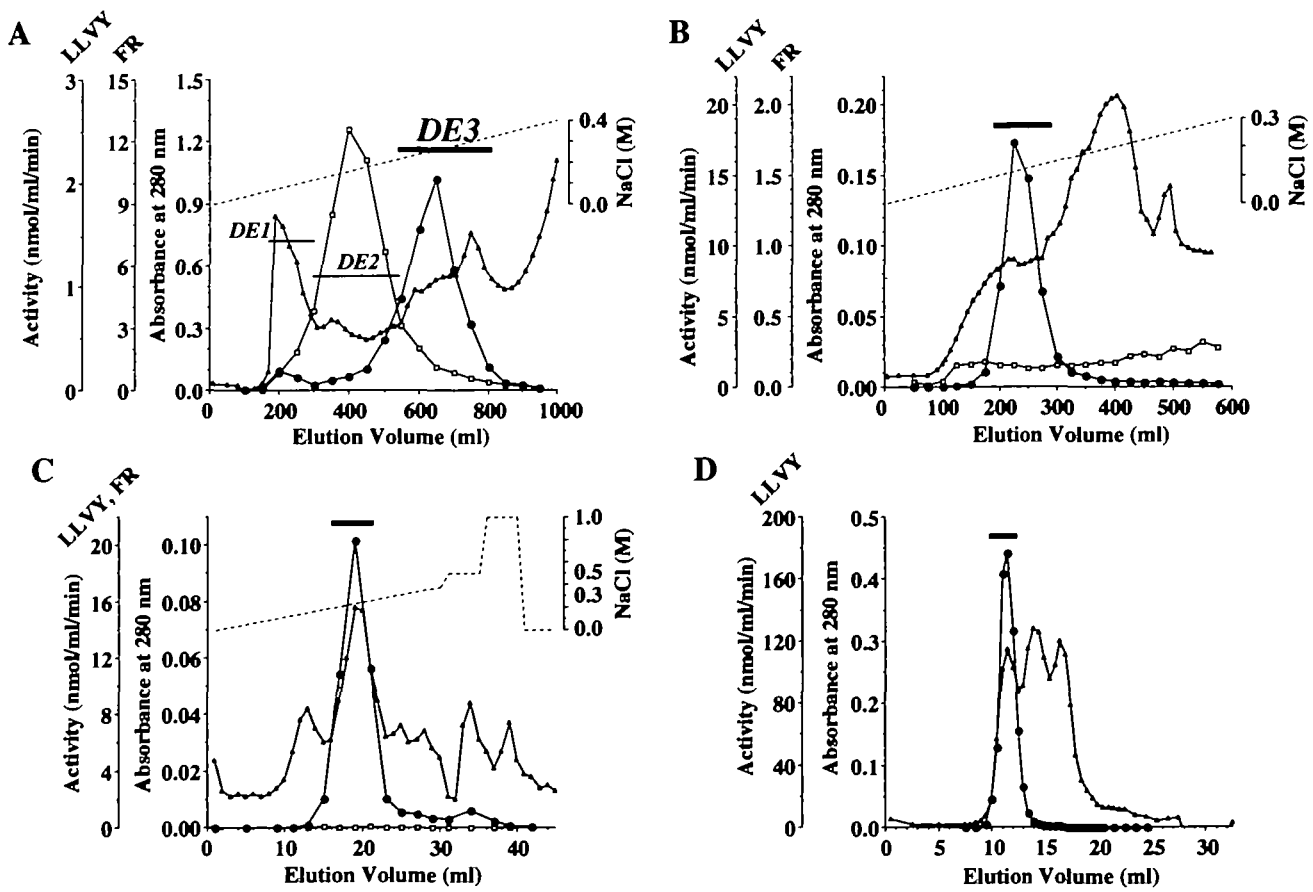

Fig. 1. **Elution profiles of 2.5% cholate extracts from DEAE-Sephacel, SP-Sepharose, POROS HS, and Superdex 200 columns.** The column sizes and elution conditions are given "EXPERIMENTAL PROCEDURES." A: Elution profile of 2.5% cholate extracts from a DEAE-Sephacel column. B: Elution profile of the DE3 fraction from a SP-Sepharose column. C: Elution profile from a POROS HS column. D: Elution profile from a Superdex 200 column. ●, LLVY hydrolyzing activity; ○, FR hydrolyzing activity; △, absorbance at 280 nm; ···, NaCl concentration. Horizontal bars indicate the pooled fractions.

was eluted at NaCl concentrations between 0.15 and 0.25 M (Fig. 1C). The pooled fractions were then applied to a Superdex 200 column (Fig. 1D), and the first peak fractions having LLVY hydrolyzing activity were pooled. This fraction was rechromatographed on the same Superdex 200 column (data not shown), and a pure protease preparation was obtained, which we designated as the ERb proteasome (ERb).

For comparison with the ERa and ERb proteasomes preparations, the 20S proteasome (20S) was purified from the rat liver cytosol fraction by the same procedure as described for ERb proteasome purification (data not shown).

The yield of ERa:ERb:20S was 1-2:2-4:10-15, in ratio, and these proteasomes exhibited almost the same specific activity (340 ± 20 nmol/min/mg) for LLVY in the presence of 0.025% SDS.

Figure 2A shows the SDS-PAGE profiles of the purified proteases. Although ERa and ERb were each eluted as a single peak corresponding to an appropriate molecular weight of 750,000 from a Superdex 200 column, ERa (lane 1) and ERb (lane 3) migrated as multiple bands corresponding to molecular masses ranging from 20 to 32.5 kDa, which is quite similar to in the case of the cytosolic 20S proteasome (lane 2). On native PAGE (Fig. 2B), ERa (lane 1) migrated slightly faster than 20S (lane 2), and a small difference was detected between ERb (lane 3) and 20S (lane 2). These results indicate that ERa and ERb have a multisubunit structure similar to 20S. However, the electrophoretic properties of these proteases are distinct from each other.

Purification of Proteases from Trypsin-Treated Microsomes—To determine the membrane localization of ERa and ERb, we next attempted the purification of the proteases from trypsin-treated microsome preparations. Very small amounts of precipitates were recovered on ammonium sulfate fractionation of 0.0125% sodium cholate extracts of trypsin-treated microsomes, and no fraction exhibiting LLVY hydrolyzing activity was obtained from the DEAE-Sephacel column. We conclude that ERa or an ERa equivalent did not exist in the 0.0125% sodium cholate extracts of the trypsin-treated microsomes and that ERa was not bound, but rather associated with the surface of the ER membrane. In contrast, a protease having LLVY hydrolyzing activity was purified from 2.5% sodium cholate extracts of trypsin-treated microsomes (data not shown). This protease, designated as tERb, resembled 20S, ERa, and ERb in the SDS-PAGE pattern, but the highest molecular mass band(s) was not detected in the tERb pattern (Fig. 2A, lane 4).

The C2 (α6/PRE5) subunit is known to be the highest molecular mass subunit (29.5 kDa) among the cytosolic 20S proteasome components (*35*). Since tERb lost a high molecular mass subunit on trypsin digestion, we examined whether or not this subunit is the C2 subunit, using anti-human C2 (HC2) monoclonal antibodies (mAb 2-17) (*26*).

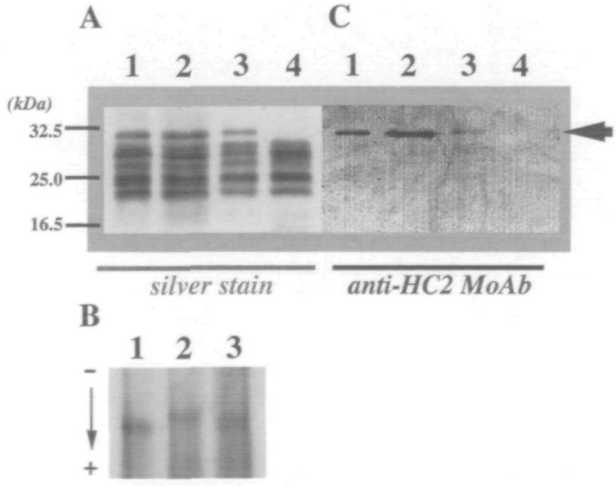

Fig. 2. **SDS-PAGE, native PAGE, and Western blot analysis of the purified proteases.** A: SDS-PAGE. B: Native PAGE. C: Western blot analysis with anti-HC2 monoclonal antibodies. SDS-PAGE was performed on 12% acrylamide gel and native PAGE on a 5% acrylamide gel. Protein bands were visualized by silver staining. Lanes 1, ERa proteasome; lanes 2, 20S proteasome; lanes 3, ERb proteasome; lanes 4, ERb proteasome equivalent (tERb) isolated from trypsin-treated microsomes. The molecular mass markers used for SDS-PAGE are triosephosphate isomerase (32.5 kDa), β-lactoglobulin A (25.0 kDa), and lysozyme (16.5 kDa). The arrow indicates the position to which the C2 subunit migrated on the gel.

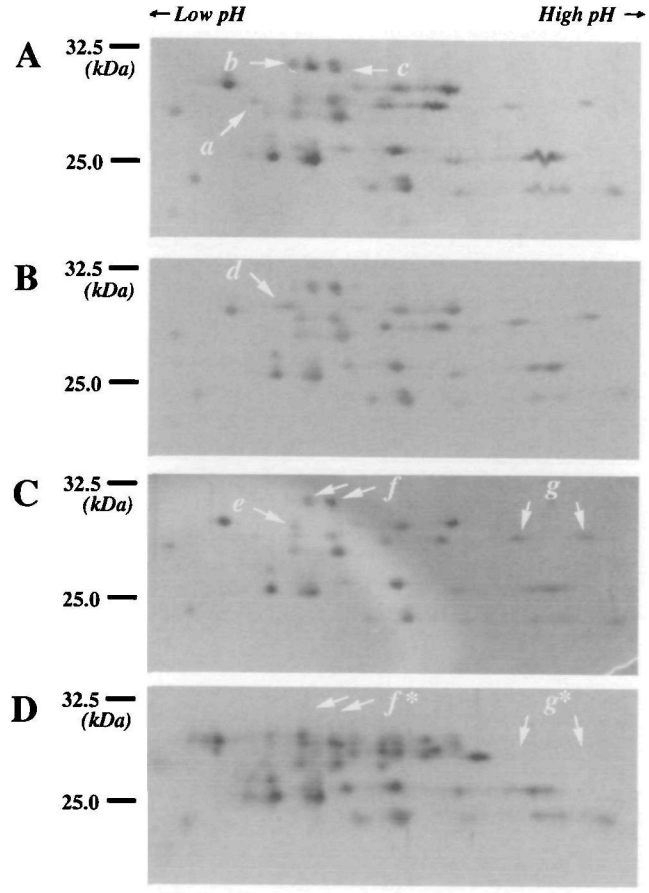

Fig. 3. **Two-dimensional PAGE of the purified proteases.** 2D-PAGE of the 20S proteasome (panel A), ERa proteasome (panel B), ERb proteasome (panel C), and ERb proteasome equivalent (tERb) isolated from trypsin-treated microsomes (panel D). Arrows indicate the specific spots for each protease. Spots a and b appeared for 20S, but not clearly for ERa and ERb, and spot c was observed predominantly for 20S and faintly for ERa and ERb. Spots d and e indicate distinct components of ERa and ERb, respectively. Spots f and g for ERb were missing for tERb (region f* and g*).

Figure 2C shows that while binding of anti-HC2 antibodies was detected on ERa (lane 1) and 20S (lane 2), the reactivity of these antibodies was rather weak for ERb (lane 3) and no reactivity was detected for tERb (lane 4). This constitutes suggestive evidence that the missing subunit in tERb was the C2 subunit, and that the antigenic property of the C2 subunit of ERb was different from those of 20S and ERa.

Structural Properties of the Purified Proteases—To determine whether or not ERa and ERb have different structural features from 20S, we performed 2D-PAGE of these proteases (Fig. 3). The 2D-PAGE patterns were very similar among these proteases. However, some minor differences were detected. Spots a, b, and c found for 20S (Fig. 3A) were faint or not detected for ERa and ERb. On the other hand, spots d and e were only detected for ERa (Fig. 3B) and ERb (Fig. 3C), respectively. These results were confirmed by immuno-blot analysis using specific antisera against each proteasome (data not shown), suggesting that ERa and ERb, purified from different cholate extracts of microsomes, have a subunit structure slightly different from that of the cytosolic 20S.

In addition, 2D-PAGE revealed that some spots for ERb (*e.g.* Fig. 3C, spots f and g) are missing for tERb (Fig. 3D, spots f* and g*), suggesting that these components are digested during the trypsin treatment of microsomes, and some new or shifted spots are detectable (Fig. 3D). The spots of the highest molecular mass (spots b, c, and f for 20S, ERa, and ERb) reacted with anti-HC2 antibodies (data not shown), reconfirming that the C2 subunit of ERb is sensitive as to the trypsin digestion of microsomes. Furthermore, tERb was shown to be different from ERb by immuno-blot analysis using anti-20S and anti-ERb antisera (Fig. 4). However, no band that reacted with anti-20S antibodies was observed on Western blotting analysis of 2.5% sodium cholate extracts of trypsin-treated microsomes in the presence of Triton X-100 (data not shown). These results suggest that ERb is bound to the outer surface of the ER membrane or partially embedded in the ER membrane.

Enzymatic Properties of the ERa, ERb, and 20S Proteasomes—To characterize and compare the enzymatic properties of ERa, ERb, and 20S, we examined their substrate specificities using several peptidyl MCA substrates, and the effects of pH, SDS, phospholipids, and various inhibitors were also investigated.

Using LLVY, LRR, LLE, and LLL as substrates, only minor differences were detected with respect to substrate specificity and pH dependency (data not shown). The sensitivities to SDS and phospholipids, however, differed among ERa, ERb, and 20S (Fig. 5). SDS, a typical activator of the cytosolic 20S proteasome, enhanced the LLVY hydrolyzing activity of 20S by 21-fold (Fig. 5A). However, ERb and ERa were more sensitive to SDS than 20S, being activated by 50- and 36-fold, respectively (Fig. 5A). The sensitivities to phospholipids were also different among these proteases (Fig. 5B). A phospholipid mixture composed of PC:PE:PS = 60:20:5, a phospholipid composition which mimics that of the ER membrane, enhanced the LLVY hydrolyzing activity of ERb, ERa, and 20S by 2.5-, 2.0-, and 1.4-fold, respectively, in the presence of SDS. These results indicate that the basic amidolytic activities are very similar but the sensitivities to SDS and phospholipids are significantly different among these three proteasomes.

We next examined the effects of various inhibitors on the peptidyl MCA substrate hydrolyzing activities of ERa, ERb and 20S (Fig. 6). Lactacystin, MG132, and MG115, specific inhibitors for the cytosolic proteasome, caused almost complete inhibition of the three proteases, except for weak inhibition of the LLE hydrolyzing activities by lactacystin. TLCK inhibited the LLVY, LLL, LRR, and LLE hydrolyzing activities, although the residual activities were virtually the same for ERb, ERa, and 20S. Chymostatin also inhibited the LLVY, LLL, and LRR hydrolyzing activities of the three proteasomes, but the effect on the LRR hydrolyzing activity was slightly different between 20S and ERa or ERb. TPCK inhibited the LLVY and LLL hydrolyzing activities of 20S, ERa and ERb in that order. Surprisingly, the LRR hydrolyzing activities of these proteasomes were not inhibited by TPCK, but rather enhanced by this inhibitor. The degree of enhancement was approximately 6-fold for 20S, which was much higher than those for ERa (2.5-fold) and ERb (1.5-fold). The LLE hydrolyzing activities of these proteasomes were not affected by TPCK.

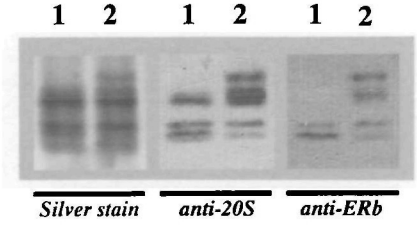

Fig. 4. **Western blot analysis of the ERb protease equivalent (tERb) isolated from trypsin-treated microsomes.** Western blot analysis of tERb and ERb using anti-20S antisera and anti-ERb antisera. Lanes 1, tERb; lanes 2, ERb. SDS-PAGE, Western blot analysis with anti-HC2 monoclonal antibodies and 2D-PAGE of purified tERb are also shown in Figs. 2A (lane 4), 2C (lane 4), and 3D, respectively.

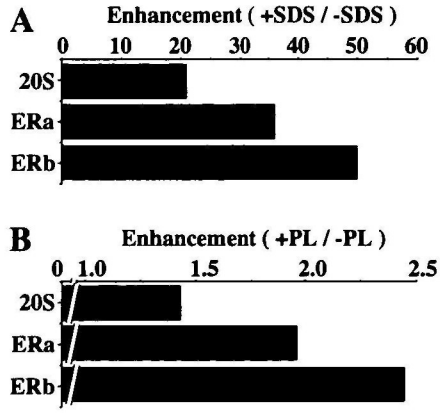

Fig. 5. **Enhancement of the LLVY hydrolyzing activities of the purified proteases with SDS and phospholipids.** A: Enhancement of the LLVY hydrolyzing activity in the presence of SDS. Enhancement was calculated from the ratio of the activities detected in the absence and presence of 0.025% SDS. B: Enhancement of the LLVY hydrolyzing activity in the presence of phospholipids. A phospholipid mixture (PC:PE:PS = 60:20:5) which mimics the composition of the ER membrane was used. Enhancement was calculated from difference in the activities in the presence and absence of phospholipids.

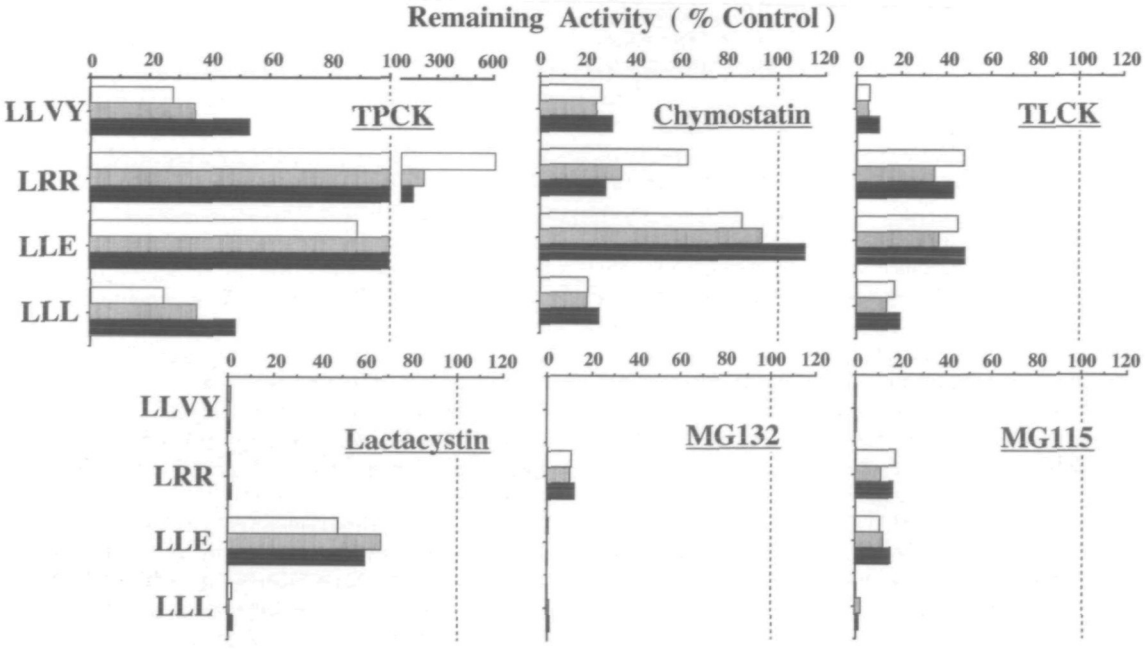

Fig. 6. **Effects of various inhibitors on the activities of ERb, ERa, and 20S.** TPCK (200 μM), chymostatin (100 μM), TLCK (100 μM), lactacystin (50 μM), MG132 (100 μM), and MG115 (100 μM) were used as inhibitors of the LLVY, LRR, LLE, and LLL hydrolyzing activities of the purified proteases. Assays were performed in the presence of 0.025% SDS, except for the LRR hydrolyzing activity. Other conditions were as described under "EXPERIMENTAL PROCEDURES." Open bars, 20S; dotted bars, ERa; closed bars, ERb. LLVY was hydrolyzed by the chymotrypsin-like activity, LRR by the trypsin-like activity, and LLE by the peptidyl glutamyl-peptide hydrolyzing activity of the proteasome.

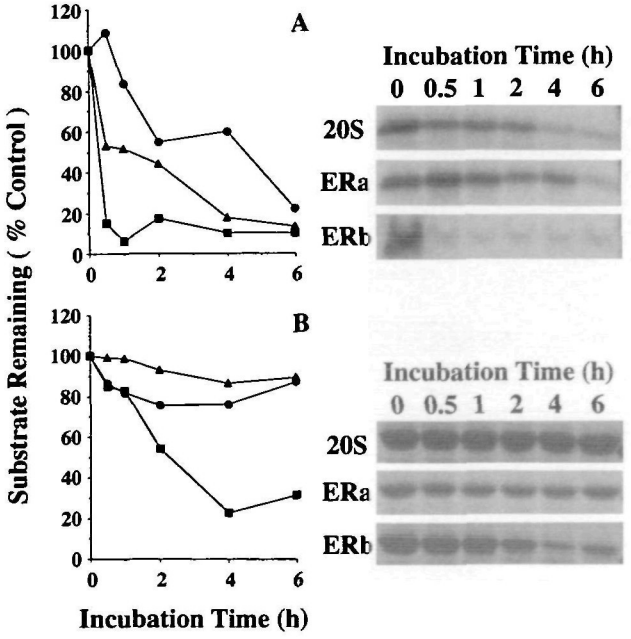

Fig. 7. **Degradation of protein substrates by ERb, ERa, and 20S.** A: Degradation of RCM-lysozyme. B: Degradation of β-casein. ■, ERb; ●, ERa; ▲, 20S. Taking the intensity of protein bands at 0 time incubation as 100%, the relative remaining substrate intensities are shown.

Since the specificities to synthetic peptide substrates reveal only a limited aspect of the enzymatic properties of the three proteasomes, we compared the proteolytic activities of ERa, ERb, and 20S, using BSA, lysozyme, β-casein, and reduced and *S*-carboxymethylated (RCM)-lysozyme as protein substrates. BSA and lysozyme were resistant to degradation by ERa and ERb, as well as 20S (data not shown). In contrast, RCM-lysozyme was degraded by all three proteases and, in the case of degradation by ERb, the original protein band disappeared within 30 min, while the degradation by ERa and 20S appeared to proceed in a time-dependent manner (Fig. 7A). Intact β-casein was more resistant to the degradation by these proteasomes. However, approximately 80% of the original protein band had disappeared after 4-h digestion with ERb, whereas only a small difference in protein bands was observed after 6-h digestion with ERa and 20S (Fig. 7B). The degradations of β-casein and RCM-lysozyme was inhibited in the presence of MG132 (data not shown).

DISCUSSION

ER-associated protein degradation was formerly thought to occur in the ER lumen through a cysteine protease(s) (*15, 36-38*), but more recent evidence strongly suggests that substrate proteins are degraded in the cytosol by the proteasome after retrograde translocation (*17-19*). On the other hand, Wu *et al.* demonstrated that apolipoprotein B (apoB) was degraded both in the ER lumen through an acethyl-leucyl-leucyl-norleucinal (ALLN)-resistant but DTT-sensitive protease pathway, and in the cytosol through an ALLN-sensitive protease pathway in HepG2 cells (*39*). Since the pore of the Sec61 translocon complex channel is too narrow for large proteins to pass through, it would be necessary for large proteins to be broken down

into intermediates for retrograde transport (*35*).

In this study, we purified ER-associated and -bound proteasomes, ERa and ERb, respectively, from cholate extracts of rat liver microsomes. On native PAGE, the purified ERa and ERb each migrated as a single band with different electrophoretic features from that of the cytosolic 20S proteasome. However, they migrated as multiple bands on SDS-PAGE and multiple spots on 2D-PAGE, which basically resembled those in the case of the 20S preparation. These observations indicate that both ERa and ERb belong to the proteasome family. Among the 2D-PAGE spots, however, some differences were detected between ERb, ERa, and 20S, suggesting that some of the subunits of these proteasomes are different from each other.

The purification of the proteases from trypsin-treated microsomes failed to produce an ERa equivalent, while a protease that is equivalent to ERb was isolated and designated as tERb. These results suggest that ERa and ERb were not contaminating components from the cytosol, but rather ERa was weakly associated with the ER membrane and ERb was tightly bound to, or partially embedded in, the ER membrane, resulting in the trypsin-resistance.

Although tERb was purified by the same procedure as for ERb, some bands and spots, especially those of high molecular mass components, were lost on SDS-PAGE and 2D-PAGE, respectively, when compared with the case of ERb. Western blot analysis with anti-HC2 antibodies suggested that the C2 (α6/PRE5) subunit was one of the components absent in tERb. These results suggest that ERb is not a resident protein in the ER lumen but bound to the outer site of the ER membrane. Moreover, anti-HC2 antibodies reacted with the band(s) for ERa and 20S, but only weakly with that for ERb. This suggests that the C2 subunit of ERb is structurally different from those of ERa and 20S.

Since ERb, ERa, and 20S were purified based on the LLVY hydrolyzing activity in the presence of 0.025% SDS, we expected that these proteases, having a distinct structure, may have similar enzymatic activities. In fact, we found little difference in the specificity towards peptidyl MCA substrates, pH optimum, and sensitivities to proteasome specific inhibitors. However, we detected distinct differences in the enhancement of LLVY hydrolyzing activities among these three proteasomes both in the presence of SDS, a typical activator of the cytosolic proteasomes, and the phospholipid mixture that mimics the ER membrane phospholipid composition. It should be pointed out that ERb, among the three, was most sensitive to these reagents. These differences in sensitivities to SDS and phospholipids may be due to differences within their subunit components. Moreover, in the protein substrate degradation experiment, β-casein was only degraded by ERb, and ERb degraded RCM-lysozyme much more effectively than ERa and 20S.

TPCK, an inhibitor of chymotryptic proteases, unexpectedly enhanced the LRR hydrolyzing activities of these proteasomes, although the sensitivity to TPCK was different among the three proteasomes. Furthermore, we detected an interaction between the C2 subunits of these three proteasomes and biotinyl alanyl-alanyl-phenylalanine chloromethyl ketone (biotinyl AAFck; SIGMA, St. Louis, MO), an inhibitor of chymotryptic proteases (data not shown). It is tempting to speculate that TPCK and AAFck, which bound irreversibly to the active site(s) of the proteasomal subunit with chymotrypsin-like activity, also interact with the proteasomal subunit(s) that contributes to a structural feature of proteasomes and influence the enhancement of the trypsin-like activities of these proteasomes. Further investigations are necessary to confirm this hypothesis.

Thus, in addition to a partial structural difference among ERb, ERa, and 20S, some distinctive differences in their sensitivities to SDS and phospholipids were revealed. However, there was little difference in their enzymatic properties, consistent with the fact that most components of their molecular structures comprise common subunits among the three proteasomes. This does not conflict with our observation that the regions of differences detected on 2D-PAGE among ERb, ERa, and 20S were present in the portion within their α subunits which constructs a structural feature of proteasomes. We also suggest that differences within their α subunits may regulate the interaction with the components of the ER membrane and influence the localization of individual proteasomes.

In the cytosol, proteasomes are known to be present mainly as a 26S proteasome, the PA700(19S)-20S-PA700-(19S) complex-type proteasome. In this study, however, we isolated ERa, ERb, and 20S in the absence of ATP. So, it is not clear whether or not ER membrane-associated or -bound 26S proteasome is present.

Collectively, our results suggest that ERb, the ER membrane-bound form of proteasome, readily degrades misfolded proteins and that its proteolytic activity may be enhanced when it is bound to the ER membrane, suggesting its physiological role in ER-associated protein degradation.

In this study, we isolated and characterized two new members of the proteasome family that are localized in the ER membrane. Since the function of these proteasomes may be modulated by the associating materials and may be regulated by some proteasomal regulators, further studies are required to establish which of these proteasomes, if any, are involved in the "quality control" mechanisms in the ER.

We wish to thank Dr. Keiji Tanaka (The Tokyo Metropolitan Institute of Medical Science) for providing the anti-human C2 (HC2) subunit monoclonal antibodies and Dr. Satoshi Omura (The Kitasato Institute) for providing the lactacystin. We also thank Dr. Walter Kisiel (University of New Mexico) for critically reading the manuscript, and Drs. Sadao Wakabayashi, Fuminori Tokunaga and Hitoshi Yagisawa of our department for the helpful discussions.

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