Isolation and Characterization of Recombinant Human Cathepsin E Expressed in Chinese Hamster Ovary Cells*

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Takayuki Tsukuba†, Hitoshi Hori§, Takeshi Azuma§, Tanami Takahashi§, R. Thomas Taggart¶, Akifumi Akamine**, Mitsue Ezaki†, Hiroshi Nakani§, Hideaki Saka§, and Kenji Yamamoto§§

From the †Department of Pharmacology, Kyushu University Faculty of Dentistry, Fukuoka 812, the §Biotechnology Research Laboratory, Tosoh Co., Nagasaki 852, the ¶Department of Preventive Medicine, Kyotan Prefectural University, Kyoto 602, the **Department of Conservative Dentistry I, Kyushu University, Faculty of Dentistry, Fukuoka 812, Japan, the ¶¶Department of Pharmacology, Nagasaki University School of Dentistry, Nagasaki 852, and the §§Department of Molecular Biology and Genetics, Wayne State University School of Medicine, Detroit, Michigan 48201

The cDNA sequence encoding precursor forms of human cathepsin E (CE), an intracellular aspartic proteinase, was expressed in Chinese hamster ovary cells using an SV40 promoter-driven expression vector. By immunoelectron microscopic studies using an anti-human CE antibody and by Percoll density gradient fractionation, the expressed CE was found to be in two different intracellular fractions; the cytosolic compartment and the vacuolar system. The CEs in both the cytosolic and the vacuolar fractions were highly purified by a simple method involving Percoll density gradient fractionation, chromatography on concanavalin A-Sepharose, Mono Q, and TSK-Gel G2000SW, and termed s-CE and v-CE, respectively. The v-CE was further separated into a major (v-CE1) and a minor (v-CE2) form by Mono Q chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting revealed that the s-CE and v-CE1 consists of two polypeptides of 90 and 84 kDa, whereas v-CE2 is composed of 84- and 82-kDa polypeptides. The NH2-terminal amino acid sequence analyses showed that the 90- and 84-kDa proteins from both s-CE and v-CE started with Ser3 and Lys86 of the sequence of human gastric CE predicted from its cDNA sequence, respectively, and that the NH2 terminus of the 82-kDa protein of v-CE2 is the Ile37. Upon acid treatment at pH 3.5 and 37 °C for 5 min, the 90- and 84-kDa forms are rapidly converted to the 82-kDa form, indicating that the 90-, 84- and 82-kDa proteins are the pro-CE, the intermediate form, and the mature CE, respectively. All the forms of CE are N-glycosylated with high-mannose-type oligosaccharides. The catalytic properties of s-CE and v-CE are comparable to those of natural human CE. These results suggest that the recombinant CE is initially synthesized on membrane-bound ribosomes as a N-glycosylated preproenzyme and that, after cleavage of the signal segment, the 90-kDa proenzyme is proteolytically processed to the intermediate (84 kDa) and mature (82 kDa) forms by the transport system.

Cathepsin E (CE)† (EC 3.4.23.34) is an intracellular aspartic proteinase consisting of two identical subunits with a molecular mass of about 42 kDa (1–4). In contrast to cathepsin D (CD) (EC 3.4.23.5), a lysosomal aspartic proteinase that is present in almost all the mammalian cells, CE has a limited distribution in certain cell types such as lymphoid tissues, gastrointestinal tracts, and urinary organs (5, 6). Recent immunoelectron microscopic studies have revealed that the intracellular localization of CE also is different from that of CD in various mammalian cells (7, 8). In these studies, it has been shown that CE is not found in lysosomes of any cell types. The membranous localization of CE was found in intracellular canaliculi of gastric parietal cells (human and rat), renal proximal tubule cells (rat), and bile canaliculi of hepatic cells (rat), while its localization in the cytosolic compartment was demonstrated in various cells, such as gastric cells (human and rat) and neutrophils (rat). The localization in the cisternae of rough endoplasmic reticulum was also shown in gastric cells (human and rat). From these findings, it has been suggested that the physiological role of CE is different from that of CD, and that the possible involvement of CE in extralysosomal proteolysis is related to specialized functions of certain cell types, such as absorption and secretion. However, the precise role of this enzyme in intracellular proteolysis still remains to be elucidated.

CE is known as a glycoprotein (1–3, 9) and considered to have oligosaccharide chains of both the high mannose type and the complex type (10). On the other hand, there is suggestive evidence that CE is synthesized as a high molecular mass precursor and subsequently processed to the mature form by autocatalytic release of the NH2-terminal prosegment (11). These findings suggest that CE is initially synthesized on the membrane-bound ribosomes and then transported to the Golgi complex in a manner similar to lysosomal enzymes. However, the precise mechanism of the biosynthesis and intracellular targeting of CE has not yet been studied. In particular, it is not known how CE is sorted from lysosomal proteins and secretory proteins and targeted to its final location, and where the proteolytic processing occurs in cells. The major problems for these studies are the difficulty in purification of the enzyme due to its low cellular concentration and the lack of a specific substrate that is discriminated only by CE.

To study the molecular basis of the segregation and matu-

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† To whom correspondence should be addressed: Tel.: 81-92-641-1151 (ext. 4201); Fax: 81-92-641-3770.

1 The abbreviations used are: CE, cathepsin E; CD, cathepsin D; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ConA, concanavalin A; dhfr, dihydrofolate reductase.
ration of CE, we decided to prepare heterologous cells expressing of the human enzyme at a high rate. We report here the expression of human CE cDNA in Chinese hamster ovary (CHO) cells and the intracellular localization and processing of the recombinant enzymes. We also demonstrate the purification and biochemical properties of recombinant CEs, as compared with the natural human CE.

**EXPERIMENTAL PROCEDURES**

**Materials**—The anti-serum against CE purified from human erythrocytes (12) was raised in rabbits and purified by affinity chromatography as described previously (8). The two chromogenic synthetic substrates, Pro-Pro-Thr-Ile-Phe-Phe(4-NOz)-Arg-Leu and Lys-Pro-Pro-Thr-Ile-Phe-Phe(4-NOz)-Arg-Leu were kind gifts of Dr. J. Kay (Department of Biochemistry, University of Wales, United Kingdom) and Dr. B. Dunn (Department of Biochemistry and Molecular Biology, University of Florida), respectively. Endoglycosidase H was purchased from Seikagaku Kogyo Co. (Tokyo). Protein A and benzoxycarbonylarginylarginine-4-methyl-7-coumarylamide were from the Protein Research Foundation (Osaka, Japan). All other chemicals were of reagent grade and were purchased from various commercial sources.

**Construction of Expression Plasmid**—The 1.7-kb EcoRI fragment (bp -36 to 1695) of the human CE cDNA in clone AGS 412 (13) was digested with Smal and EcoRV, and the resulting 1.8-kb fragment containing the sequence from the translation initiation site of human CE to the poly(A) additional signal was inserted into the Smal site of the pCE-dhfr (14) to obtain the expression plasmid pCE-dhfr.

**Transfection of Chinese Hamster Ovary Cells and Selection of the Transfectants**—The dihydrofolate reductase-deficient CHO cell line DXB11 (15) was routinely maintained in Ham's F-12 medium supplemented with penicillin (100 μg/ml) and streptomycin (100 μg/ml), α-minimal essential medium nonessential amino acids (100 μg/ml), L-glutamine (2 mM) containing 10% dialyzed fetal calf serum at 37 °C in a humidified 5% CO2 incubator. The expression plasmid, pCE-dhfr, was transfected into DXB11 by the calcium phosphate method (16) with glycerol treatment (17). After transfection, the cells were subcloned into a CHO dhfr sup cell-selective medium (Dulbecco's modified Eagle's medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), α minimal essential medium nonessential amino acids (0.1 mM) and L-glutamine (2 mM) containing 10% diazylated fetal calf serum) to generate single colonies of dhfr-positive CHO cells. The single colony-forming dhfr sup CHO cells were picked up by trypsin treatment in cloning cylinders, maintained in culture dishes, and subsequently screened for expression of human cathepsin E by Western blot analysis (18) using the anti-human CE antibody (6). To obtain samples for the analysis, the cytoplasmatic extract was prepared from the cells by treatment with 0.5% Nonidet P-40 followed by centrifugation (15,000 × g, 20 min).

**Determinations**—Acid proteinase activity was determined at pH 3.5 and 7.2. The matured hemoglobin is a substrate as described previously (12). The amounts of cathepsins E and D were determined by immunoprecipitation using antibodies specific for each enzyme, as described previously (6). Cathepsin B was assayed with benzoylcarbonylarginylarginine-4-methyl-7-coumarylamide as a substrate according to the method of Barrett and Kirschke (19), with some modifications (20). β-Glucuronidase was assayed with 4-methylumbelliferon-glucuronide as a substrate by the method of Towbin et al. (18) using the avidin-biotin-peroxidase complex (ABC) method by a Vectastain ABC kit (Vector Laboratories). The cells were incubated in 0.5 mM 3,3'-diaminobenzidine tetrahydrochloride and 0.005% H2O2 in 50 mM Tris-HCl buffer, pH 7.4, for 15 min at room temperature. The stained cells were then postfixed in 1% osmium tetroxide in 100 mM cacodylate buffer, pH 7.4, and embedded in Epon 812 after dehydration treatment. Thin sections were examined using a Hitachi-7000 electron microscope.

**Activation of Procathepsin E**—Activation of the recombinant procathepsin E was carried out at pH 3.5 and 37 °C. After incubation for 5 min in 10 mM sodium acetate buffer, pH 3.5, the activation was stopped by addition of 200 mM Tris-HCl buffer, pH 9.0, to give a final concentration of 20 mM. The samples were analyzed by SDS-PAGE followed by immunoblotting.

**RESULTS**

**Establishment of Cell Lines Producing Human Cathepsin E**—The expression plasmid of pCE-dhfr, which directs human CE expression in mammalian cells, is shown in Fig. 1. This plasmid contains the sequence of the SV40 promoters, the full length human CE coding region with poly(A) signal sequence, and the DHFR gene expression unit. After introducing the plasmid pCE-dhfr into dihydrofolate reductase-
deficient CHO cells (CHO dhfr–), the transfected cells selected for the dihydrofolate reductase-positive phenotype (CHO dhfr+) were cloned and subsequently screened for expressing human CE. Western blot analysis showed that the cloned CHO dhfr+ cells contained proteins reactive with anti-human CE antibodies (not shown).

**Distribution and Localization of the Recombinant Human Cathepsin E in the Transfected CHO Cells**—To examine the intracellular distribution of the recombinant CE in the transfected CHO cells, the postnuclear supernatant obtained by centrifugation of the disrupted cells at 400 × g for 8 min was subjected to Percoll density gradient centrifugation. As shown in Fig. 2, the CE was separated into two pools: the light and the high density pools equilibrated in the density range of 1.006–1.025 (g/ml) and 1.095–1.125 (g/ml), respectively. The same behavior was observed for β-glucuronidase which is associated with both lysosomes and endoplasmic reticulum. Another lysosome marker, cathepsin B was the most abundant in the density region of 1.105–1.125 g/ml (approximately 70% of the total activity). When the light density pool was collected and centrifuged at 105,000 × g for 1 h, more than 95% of the total CE and lactate dehydrogenase, a cytosol marker, were recovered in the supernatant, whereas about 60% of the total β-glucuronidase was found in the precipitate. The results indicate that at least 50% of CE found in the light density fraction results from the cytoplasmic matrix. Thus, the recombinant CE in the transfected cells appears to be localized in both the soluble cytosolic compartment and the endocytic vesicular fraction.

Fig. 3 shows the results of immunocytochemical localization of the recombinant CE in the transfected cells. The immunoreactivity for CE was abundant in the cytoplasmic matrix and endoplasmic reticulum of the transfected CHO cells, especially the oval-shaped cells which comprise a large part of the heterologous cells (Fig. 3B). In these cells, the immunostaining was reticular or punctate in the cytoplasmic matrix among plentiful mitochondria or endoplasmic reticulum, but barely detectable in any organelles such as nuclei and mitochondria. The immunolabeling was also found in the endocytic vacuoles of the cells, especially the triangular cells which represent a small population of the transfected cells (Fig. 3C). In these cells, staining was somewhat diffuse in these vacuoles and punctate in dilated perinuclear spaces, but barely detectable in mitochondria and nuclei. No immunostaining was observed with nonimmune rabbit IgG (Fig. 3A), and the nontransfected CHO cells did not stain with the anti-human CE antibody (not shown). We tentatively designated the enzyme in the soluble compartment and the vacuolar system as s-CE and v-CE, respectively.

**Purification of the Recombinant Cathepsin E**—The two enzyme active pools (fractions 1–4 and 8–13) obtained by Percoll density gradient centrifugation were centrifuged at 105,000 × g for 60 min to remove Percoll and then sonicated in the presence of 0.5% Triton X-100. After centrifugation at 105,000 × g for 60 min, each pool was separately applied to ConA-Sepharose columns (1.0 × 2.0 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 0.05% Brij 35 and 1 mM NaCl. Each column was washed with the same buffer, and the enzyme activity was eluted with 0.5 mM methyl-α-D-mannoside in the buffer. More than 95% of the total activity was absorbed on the column and eluted with the methyl-α-D-mannoside solution, indicating that the recombinant CE is apparently glycosylated. After concentration by ultracentrifugation on an Amicon Diaflo PM10 membrane, the enzyme solution was dialyzed against 10 mM sodium phosphate buffer, pH 7.0, and subjected to anion-exchange chromatography on a Mono Q column (0.5 × 5 cm) which had been equilibrated with the same buffer. As shown in Fig. 4, the enzyme from the first pooled fraction (fractions 1–4) (s-CE) was eluted 200 mM NaCl. The enzyme from the second pooled fraction (fractions 8–13) (v-CE) was separated into two fractions on the the Mono Q column; the major peak of activity that represented about 35% yield of the applied activity was eluted with 100 mM NaCl (v-CE1), and the minor peak that represented about 25% yield was eluted with 200 mM NaCl (v-CE2). The minor peak of activity was eluted slower than that from the first pooled fraction, although the same salt concentration was required for their elution. The pooled active fractions were each concentrated and run on a gel filtration column of TSK-Gel G2000SW in the fast protein liquid chromatography system, equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 100 mM Na2SO4.

The elution profiles of the three enzyme samples were similar, and the activity peaks corresponded to a molecular mass of about 90 kDa (Fig. 5). Fractions 66–74 were pooled, concentrated, and used as pools of purified CE. A summary of the purification is shown in Table I.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**—The final preparations of the recombinant CE were examined by SDS-PAGE (Fig. 6A). Under nonreducing conditions, the s-CE preparation revealed a major 90- and a minor 84-kDa polypeptide. Similarly, the v-CE1 preparation showed the 90- and 84-kDa polypeptides, although the relative amount of the 84-kDa form was higher than that in the s-CE
Fig. 3. Immunocytochemical staining of the recombinant CE in CHO cells. CHO cells contain numerous mitochondria (arrows), vacuolar structures, and the rough endoplasmic reticulum. Immunoreaction products for human CE are accumulated in the cytoplasmic matrix (B) and the vacuolar structures (C). No immunostaining was observed with nonimmune rabbit IgG (A). N, nucleus. Original magnifications: A, × 7000; B, × 10000; C, × 9000. Bars = 2 μm.

Fig. 4. Anion-exchange chromatography on a Mono Q column of the recombinant CE fractions (s-CE and v-CE) obtained by ConA-Sepharose affinity chromatography. The samples of s-CE (●) and v-CE (○), which were separated by Percoll density gradient centrifugation followed by ConA-Sepharose chromatography, each was applied to a Mono Q column equilibrated with 10 mM sodium phosphate buffer, pH 7.0. The flow rate was 0.5 ml/min and fractions of 2 ml were collected. Dashed line, NaCl concentration.

Fig. 5. Gel filtration on TSK-Gel G2000SW of the pooled enzyme fractions from Mono Q chromatography. The column was equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M Na2SO4. The flow rate was 1 ml/min, and fractions of 0.25 ml were collected. ●, s-CE; ○, v-CE1; △, v-CE2.

TABLE I

<table>
<thead>
<tr>
<th>Purification</th>
<th>Protein</th>
<th>Total activity mg</th>
<th>Units</th>
<th>Specific activity units/mg</th>
<th>Yield %</th>
<th>Purification -fold</th>
</tr>
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<tbody>
<tr>
<td>Homogenate</td>
<td>254</td>
<td>20,100</td>
<td>79</td>
<td>100</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Postnuclear supernatant</td>
<td>172</td>
<td>16,500</td>
<td>96</td>
<td>82</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Percoll fractionation s-CE</td>
<td>8,600</td>
<td>43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v-CE</td>
<td>7,100</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ConA-Sepharose s-CE</td>
<td>2.7</td>
<td>6,600</td>
<td>2,450</td>
<td>33</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>v-CE</td>
<td>4.7</td>
<td>8,300</td>
<td>1,770</td>
<td>41</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Mono Q s-CE</td>
<td>0.3</td>
<td>3,700</td>
<td>14,100</td>
<td>18</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>v-CE1</td>
<td>0.2</td>
<td>2,800</td>
<td>15,140</td>
<td>14</td>
<td>191</td>
<td></td>
</tr>
<tr>
<td>v-CE2</td>
<td>0.5</td>
<td>1,900</td>
<td>4,130</td>
<td>9</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

Ser9, of the sequence predicted from the cDNA sequence (13) and to be lacking residues Gln1-Gly2. The amino acid sequence was identical with the corresponding sequence of human gastric CE predicted from the cDNA sequence (Table II). The NH2-terminus of the 42-kDa polypeptide from the 84-kDa forms of both s-CE and v-CE1 was Lys, which corresponded to the NH2-terminal 30th residue of the amino acid sequence predicted from the cDNA sequence. For the 42-kDa polypeptides of v-CE2, 2 amino acid residues were found in the first several steps of sequencing; one species started with Ile7 of the sequence predicted from the cDNA sequence and the other started with the Lys20. The former had the NH2-terminal amino acid sequence predicted from its cDNA sequence.
(13), whereas the NH₂-terminal sequence of the latter was identical with that of the 84-kDa form. Thus, the results indicate that the 90-, 84-, and 82-kDa forms correspond to the pro-CE, the intermediate form, and the mature enzyme, respectively.

**Acid Treatment**—When s-CE and v-CE were incubated at 37 °C for 5 min in 10 mM sodium acetate buffer, pH 3.5, both samples of purified recombinant CE and human erythrocyte CE were analyzed by SDS-PAGE under nonreducing conditions.

**Fig. 6.** SDS-PAGE under nonreducing conditions (A) and immunoblotting (B) of the purified recombinant CE. SDS-PAGE was performed with 10% polyacrylamide gels in Tris-Cl buffer, pH 8.9, under nonreducing conditions. The final preparations of s-CE, v-CE1, and v-CE2 were subjected to SDS-PAGE after denaturation at 37 °C for 30 min in 1% SDS. Lane 1, s-CE; lane 2, v-CE1; lane 3, v-CE2; lane 4, human erythrocyte membrane CE. After electrophoresis, the polypeptides were visualized by the silver staining (A) or electroblotted on to nitrocellulose membranes followed by immunoreaction with the anti-human CE antibody (B).

**Fig. 7.** Activation of procathepsin E at pH 3.5 and 37 °C analyzed by SDS-PAGE under nonreducing conditions. The samples of purified recombinant CE and human erythrocyte CE were incubated at 37 °C for 5 min at pH 7.0 (a) or pH 3.5 (b). After heating at 37 °C for 30 min in 1% SDS, each sample was analyzed by SDS-PAGE followed by immunoblotting as described under "Experimental Procedures." Lane 1, s-CE; lane 2, v-CE; lane 3, human erythrocyte CE after acid treatment.

**TABLE II**

Comparison of the NH₂-terminal amino acid sequences of recombinant CEs and human erythrocyte CE with the corresponding sequences of human gastric CE predicted from its cDNA sequence.

<table>
<thead>
<tr>
<th>s-CE (90 kDa)</th>
<th>v-CE1 (90 kDa)</th>
<th>cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>v-CEi (84 kDa)</td>
<td>v-CE2 (84 kDa)</td>
<td>cDNA</td>
</tr>
<tr>
<td>v-CE (82 kDa)</td>
<td>Human mature CE I</td>
<td>Human mature CE II</td>
</tr>
<tr>
<td>cDNA</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>

The numberings refer to the sequence of human gastric CE predicted from its cDNA sequence (13).
during acid-soluble products as described previously (12). Hydrolysis substrates, respectively. The values given are means for hemoglobin and mM for the synthetic substrates. The values expressed as percent for hemoglobin and mM for the synthetic substrates, respectively. The values given are means of three determinations.

**TABLE III**

Comparison of catalytic activity of recombinant CE and natural human erythrocyte CE

<table>
<thead>
<tr>
<th>Substrate</th>
<th>s-CE</th>
<th>v-CE1</th>
<th>v-CE2</th>
<th>erythrocyte CE</th>
<th>K_m values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>0.10</td>
<td>0.11</td>
<td>0.10</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Pro-Pro-Thr-Ile-Phe-Phe(4-NO_2)-Arg-Leu</td>
<td>0.11</td>
<td>0.10</td>
<td>0.10</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Lys-Pro-Ile-Glu-Phe-Phe(4-NO_2)-Arg-Leu</td>
<td>0.40</td>
<td>0.13</td>
<td>0.09</td>
<td>0.06</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

In this paper, we report the high level expression of human CE in heterologous CHO cells and describe the purification and biochemical properties of recombinant CE. Intracellular localization of CE in the transfected cells was investigated by immunoelectron microscopy using specific antibodies to human CE and by Percoll density gradient fractionation. The results showed that the recombinant CE is distributed into two cellular pools: the cytosolic compartment and the vacuolar system. Since all the forms of recombinant CE were shown to be glycoproteins, the localization of this protein in the soluble cytosolic compartment was seemingly curious. However, considering the fact that natural CE as a nonlysosomal glycoprotein also has been found in the soluble cytosolic compartment of various mammalian cells, such as rat neutrophils (7, 25), human and rat gastric cells (8, 26), rat lymphocytes (27), and murine Friend erythroleukemia cells (28), it is more likely that the localization of this protein in the soluble cytosolic compartment is a special property of the recombinant CE in the transfected cells. Although it is difficult to completely rule out the possibility that the cytosolic localization of CE may be artificially produced in the course of subcellular fractionation or during the preparation of specimens for the immunoelectron microscopy, the existence of CE in the cytosolic matrix may suggest a new pathway for delivery of proteins to the cytoplasm from the traditional secretory pathway. On the other hand, the vacuoles indicating CE antigenicity were also present in the cells. At this time, it is unclear what the functional relationship is between the vacuole-associated CE and the soluble protein. However, since the vacuole-associated CE is enriched in triangular-shaped cells rather than oval-shaped cells, the vacuolar association may be influenced by the functional state of the cells. The occurrence of the vacuole-associated form of CE has also been shown in murine Friend erythroleukemia cells (28) and rat osteoclasts.2

The recombinant CEs were purified from both intracellular pools of the transfected cells. Affinity chromatography on ConA-Sepharose was the most straightforward step and facilitated the subsequent purification procedures. SDS-PAGE and immunoblotting revealed that s-CE from the cytosolic fraction is composed of the 90- and 84-kDa proteins, whereas v-CE from the vacuolar fraction contains the 82-kDa form, besides the 90- and 84-kDa forms. The NH_2-terminal amino acid residues of all the forms of CE were successfully identified. This is clearly different from the prediction recently made by Athauda et al. (11) who reported that the NH_2-terminal glutamine residue of human gastric procathepsin E might be blocked by reason of the failure to detect an NH_2 terminus by direct sequence analysis. However, it has been shown that the NH_2-terminal amino acid residue of pro-CE from human erythrocyte membranes may be identified directly by the protein sequencing and starts with Ser (29), which is consistent with the present results. Therefore, the modification of the NH_2 terminus of pro-CE appears to be cell-specific and species-specific. The reason for the deletion of residues Gin-Gly is not known at the moment. It is probably due to autodegradation of CE or the occurrence of limited proteolysis by the other proteases during preparation.

Of interest is the observation that acidification caused the clear conversion of the 90- and 84-kDa forms into the 82-kDa form by cleavage of the Met^{36}-Ile^{37} bond, as determined by NH_2-terminal sequence analysis. This cleavage site was the same as that of human gastric pro-CE (11) but apparently different from that of the prosegment of human cathepsin D (30) or from autocatalytic cleavage sites commonly present in other human gastric aspartic proteinases such as pepsinogens A (31). Since this conversion was rapid and complete within 5 min under the conditions used, the precursor forms of CE appeared to be activated by one-step conversion as described for natural human gastric CE (11). These results, together with those obtained by NH_2-terminal sequence analysis, strongly suggest that the 90-, 84-, and 82-kDa forms correspond to the pro-CE, the intermediate, and the mature CE, respectively. In addition, the occurrence of the intermediate form in a significant amount suggests the conversion of the pro-CE to the mature form via the intermediate form, although it is unclear whether the processing of pro-CE to the intermediate form proceeds autocatalytically.

The precursor forms of recombinant CE, as well as the mature form, showed high catalytic efficiency against hemoglobin and synthetic substrates, comparable to human erythrocyte CE (Table III). However, since the precursor forms were rapidly converted to the mature form under acidic assay conditions, it is difficult to determine whether they are catalytically active or not. The K_m values of s-CE and v-CE for the synthetic substrates were also similar to those reported for human gastric CE (4). In addition, no significant differences were observed between the recombinant CE and the natural human CE with respect to other enzymatic properties, such as optimal pH, substrate specificity, and susceptibility to various protease inhibitors (not shown). Therefore, we found that the recombinant CE expressed in CHO cells is identical to the natural human CE not only structurally but also catalytically. Considering the fact that all the forms of recombinant CE resulted in a reduction of about 2 kDa in molecular mass (in the reduced forms) on endoglycosidase H treatment (Fig. 8), they are glycosylated with high mannos...
type of oligosaccharide chain. Since the natural CE from human and rat stomachs has been shown to contain a oligosaccharide chain of the high mannos type (3, 10), it is likely that both the recombinant pre-CE and the natural CE undergo the same maturation of their oligosaccharide moiety. On the other hand, the present study shows that human erythrocyte CE is resistant to endoglycosidase H. Also, Yonezawa et al. (10) recently showed by gas-liquid chromatography that rat gastric CE has a significant amount of galactose and sialic acid. These observations suggest that natural CE exists in either as the high mannose type or the complex type and that the type of its oligosaccharide moiety may be cell-specific or may vary with the cellular location.

At the present time, the mechanism by which CE is sorted from other secretory proteins and targeted to the final location is not known. But, the present study suggests that CE is initially synthesized as glycosylated preproenzymes on membrane-bound ribosomes, transported to the Golgi complex, and then segregated from other proteins. During this process, the protein appears to undergo multiple proteolytic processing.

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REFERENCES