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The Role of the Cathepsin E Propeptide in Correct Folding, Maturation and Sorting to the Endosome

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Cathepsin E (CE) is an endosomal aspartic proteinase of the A1 family that is highly homologous to the lysosomal aspartic proteinase cathepsin D (CD). Newly synthesized CE undergoes several proteolytic processing events to yield mature CE, from which the N-terminal propeptide usually comprising 39 amino acids is removed. To define the role of the propeptide of CE in its biosynthesis and processing, we constructed two fusion proteins using chimeric DNAs encoding the CE propeptide fused to the mature CD tagged with HA at the COOH terminus (termed ED-HA) and encoding the CD propeptide fused to the mature CE (termed DE). Pulse-chase analysis revealed that wild-type CE expressed in human embryonic kidney cells is autoproteolytically processed into mature CE within a 12-h chase, whereas the chimeric DE failed to be converted into mature CE even after a 24-h chase. The DE chimera was nevertheless capable of acid-dependent autoactivation *in vitro* to yield a catalytically active form, although its specificity constants (k_{cat}/K_m) were considerably high but less (35%) than those of the wild-type CE. By contrast, the chimeric ED-HA expressed in HeLa cells underwent neither processing into a catalytically active enzyme nor acid-dependent autoactivation *in vitro*. The ED-HA protein was less stable than wt-CD-HA, as determined on pulse-chase analysis and on trypsin digestion. These data indicate that the propeptide of CE is essential for the correct folding, maturation, and targeting of this protein to its final destination.

Key words: aspartic proteinase, cathepsin E, cathepsin D, chimeric proteins, propeptide.

Abbreviations: CD, cathepsin D; CE, cathepsin E; DMEM, Dulbecco's modified Eagle's medium; Dnp, 2,4-dinitrophenyl; Endo H, endoglycosidase H; ER, endoplasmic reticulum; HEK, human embryonic kidney; MOCAc, (7-methoxycoumarine-4-yl) acetyl; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; wt, wild-type.

Cathepsin E (CE), like cathepsin D (CD), is a major endolysosomal aspartic proteinse of the A1 family (for reviews, see Refs. *1–3*), and is implicated in a wide range of physiological and pathological processes (*4–6*). Both enzymes exhibit a high degree of similarity that involves their enzymatic activities (*1–3*) and primary (*1–3*) and three-dimensional (*7*, *8*) structures. Despite this overall similarity, substantial differences exist between the two enzymes in such as the tissue distribution (*9*, *10*), cellular localization (*11*, *12*), and immunological properties (*13*), and consequently biological functions (*4–6*). While CD is widely distributed in almost all mammalian cells as a representative lysosomal enzyme, CE is predominantly expressed in cells of the immune system, including antigen-presenting cells, lymphoid tissues, and gastric cells. Different from CD, CE is localized in various cellular compartments such as the endosome (*12*, *14*), plasma membrane (*15–17*), Golgi complex and endoplasmic reticulumn (ER) (*11*, *12*, *18*). However, newly synthesized CE, like CD, undergoes several processing events to target it to the proper destination. The complete activation of pro-CE is accomplished through proteolytic removal of the N-terminal propeptide, which is usually 39 amino acids long.

Available evidence suggests that the propeptides of aspartic proteinases participate in folding and cellular sorting events (*19–21*). Indeed, human CD propeptide has been shown to be necessary for sorting of this protein to the lysosome through mannose-6-phosphate receptor–independent transport (*22*, *23*). The propeptide deletion mutant of human CD expressed in human HepG2 cells was rapidly degraded, but the chimeric protein between the CD propeptide and the secretory protein α-lactalbumin was correctly transported to the lysosomes (*19*). The propeptide deletion mutant of rat CD expressed in COS1 cells was also not transported to the lysosomes, although it was stably retained in the ER (*24*). It is thus considered that the CD propeptide plays an important role in the sorting of this protein to the lysosomes and in correct folding in the ER (*22*). Moreover, the propeptide from human CD has been shown to exhibit broad inhibitory activity toward pig pepsin, human gastricsin, and calf chymosin, like CD (*25*), and to exhibit mitogenic activity toward various human cell lines (*26*). Despite such

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extensive studies on the functions of the CD propeptides, however, the physiological role of the propeptide of CE has not been completely elucidated yet.

The unique structural characteristics of the propeptide of CE are conserved in all species (*27–30*). The propeptide is composed of 40 amino acid residues in all species and is highly positively charged. It is thus speculated that the propeptide is bound to active CE mainly through electrostatic interactions. The processing events for pro-CE include the removal of the propeptide, modification of oligosaccharide chains, and formation of a disulfide bond by the cysteine residue at position 7 of the amino-terminal portion of mature CE to yield the homodimer. Like other aspartic proteinases, the pro-CE is capable of acid-dependent autoactivation to generate mature CE via an intermediate (*18*, *28*). It is also known that pro-CD is capable of acid-dependent autoactivation *in vitro* to yield a catalytically active intermediate form (pseudo-CD) but that complete activation to mature CD is accomplished by lysosomal cysteine cathepsins (*31*, *32*). Precursor forms of most aspartic proteinases are known to be catalytically inactive because their N-terminal propeptide folds across the active site. In the case of CD, the propeptide is no longer bound to the enzyme after cleavage from the mature enzyme. More recently, the crystal structure of the CE/propeptide complex revealed that, different from those of other aspartic proteinases, the pro-sequence of CE remains stably associated with the catalytic domain after cleavage from the mature enzyme (*8*). In view of structural characteristics and variations in the cellular localization of CE, we considered that the role of CE propeptide in its biosynthesis, processing, sorting mechanism, and delivery to different cellular compartments is especially important for understanding its extralysosomal significance.

In this study, we constructed two fusion proteins using chimeric cDNAs encoding the CE propeptide fused to mature CD tagged with HA at the COOH terminus (termed ED-HA), and encoding the CD propeptide and mature CE (termed DE). The fusion proteins were expressed in human embryonic kidney cell line 293T (HEK-293T) and Hela cells. We report here that the CE propeptide is essential for the sorting of this protein to the endosomes in HEK-293T cells, and is also required for correct folding and maturation of pro-CE, although it can be partially replaced by the CD propeptide in the latter events.

EXPERIMENTAL PROCEDURES

Materials—[^{35}S]Methionine/cysteine, [^{32}P]orthophosphate, and Protein A–Sepharose were purchased from Amersham-Pharmacia Biothech, Inc. Pansorbin was from Calbiochem. The pcDNA 3.0/Amp plasmid was from Invitrogen. Dulbecco's modified Eagle's medium (DMEM), methionine-free DMEM, and Opti-Mem were from Gibco-Life Technologies, Inc. Endoglycosidase H (endo-H) and *N*-glycosidase-F were from Boehringer Mannheim. The two fluorogenic substrates, MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 specific for CD and CE (*33*), and MOCAc-Gly-Ser-Pro-Ala-Phe-Leu-Ala-Lys(Dnp)-D-Arg-NH_2 specific for CE (*34*), were synthesized as described previously.

Plasmid Construction and Mutagenesis—The pBluescript II SK-plasmid containing a full-length wild-type rat CE cDNA was used as described previously (*35*, *36*). The pUC-118 plasmid containing rat CD was kindly donated by Dr. H. Fujita (Kyushu University Graduate School of Pharmaceutical Sciences) (*37*). The pcDNA encoding full length rat CE cDNA was used as the wild-type (wt) CE expression vector (*36*). The pBluescript plasmid containing rat CE cDNA was digested with *Sma*I and *Xba*I, and then subcloned into the *Sma*I and *Xba*I sites of the pUC 118-CD plasmid. After insertion, the DE chimera was generated by site-directed mutagenesis according to the method described (*36*), digested with *Eco*RI and *Xba*I, and then subcloned into the same sites of pcDNA3. The primer used in the mutagenesis reaction was as follows: 5′-AGTCATCTCCTAGGACCAAGAGCGAGTCCTGTAATGTGGA-3′. The CD tagged with HA (wt-CD-HA) was constructed by a polymerase chain reaction method. The CD transcript was amplified from pUC-118 CD using primers 5′-GTATGGGTAACCGAGTGTGGCAGCCTTG-3′ and 5′-ATATAGAATTCATGCAGACCCCCGGC-3′, and then subcloned into the *Eco*RI and *Bst* EII sites of HA-pBluescript. It was then digested with *Eco*RI and *Xho*I, and cloned into the same site of pcDNA3. The CE/pBluescript was digested with *Xho*I and *Eco*RI, and then subcloned in frame into the *Xho*I and *Eco*RI sites of the pUC 118-CD. ED was also generated by site-directed mutagenesis according to the method described (*36*). The primer used for the mutagenesis reaction was as follows: 5′-ACTTGGACATGATCGAATTCGAGCCAGTGTCAGAGTTACT-3′. To construct ED tagged with HA (ED-HA), an ED fragment was generated using primers 5′-ATATCCCGGGATGAAACCTCTCTTC-3′ and 5′-ATATAGAATTCATGCAGACCCCCGGC-3′, and then subcloned into the *Eco*RI and *Sma*I sites of HA-pBluescript. It was then digested with *Eco*RI and *Xho*I, and then cloned into same sites of pcDNA3. All constructs were verified to be correct by DNA sequence analysis.

Tissue Culture and Transfection—HEK 293T cells and HeLa cells were maintained in DMEM supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in a 37°C incubator with 5% CO_2. Wild-type CE (wt-CE) or wt-CD-HA, or their chimeras were transfected into HEK-293T cells or HeLa cells with Lipofectin reagent (Life Technologies Co. Ltd.) using 10 μg of expression plasmid in 100-mm tissue culture plates. Cells were grown to approximately 90% confluency by overnight incubation in complete DMEM.

Preparation of Culture Media and Cell Lysates of Transfected Cells—The culture media of transfected cells were collected 48 h after transfection and then centrifuged at 16,000 × *g* for 20 min. The culture media were concentrated to give 500 μl/plate using Centriprep-30 and Microcon-30 concentrators (Amicon, Inc.). On the other hand, the cells were washed twice with phosphate-buffered saline (PBS) and removed from the plates with a rubber scraper, and then subjected to centrifugation at 300 × *g* for 5 min. The precipitated cells were suspended in PBS containing 0.1% Triton X-100 and then subjected to sonication for 1 min at 4°C, followed by centrifugation at 100,000 × *g* for 1 h. The resultant supernatant is referred to as the cell lysate.

Pulse-Chase Experiments—The transfected cells were preincubated for 1 h at 37°C in DMEM lacking methionine but supplemented with 10% dialyzed fetal bovine serum. The cells were pulse-labeled for 30 or 60 min with

[^{35}S]methionine/cysteine (100 μCi/ml/dish), and then chased in fresh serum-free Opti-MEM (1.5 ml/plate). At the indicated times, the cells were separated from the medium, washed twice with PBS, and then subjected to lysis in PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.02% sodium azide, and a proteinase inhibitor cocktail [antipain (50 μg/ml), chymostatin (50 μg/ml), leupeptin (50 μg/ml), pepstatin (50 μg/ml), and phenylmethylsulfonyl fluoride (0.1 mM)], followed by sonication and centrifugation at 6,500 × *g* for 10 min to yield the cell extract.

Immunoprecipitation of Pulse-Labeled CE—The cell extract and culture media were mixed with 40 μl of Pansorbin for 1 h at 4°C to prevent nonspecific binding to IgG–protein A complexes, and then centrifuged at 6,500 × *g* for 30 min. The culture media were incubated with 15 μl of anti-rat CE IgG, anti–rat CD IgG, or anti-HA IgG at 37°C for 10 min, and then stored at 4°C for 16 h. The immunocomplexes were reacted with 40 μl of Protein A–Sepharose beads (50% gel suspension) for 3 h at 4°C with gentle agitation. The beads were washed 3 times with 0.1% SDS/0.1% Triton X-100/200 mM EDTA/10 mm Tris-HCl (pH 7.5), followed by washing 3 times with the same buffer containing 1 M NaCl and 0.1% sodium lauryl sarcosinate, and twice with 5 mM Tris-HCl (pH 7.0). The sedimented beads were boiled for 5 min at 100°C with 50 μl of 0.1% SDS/0.5 mM EDTA/5% sucrose/5 mM Tris-HCl (pH 8.0) containing 1 mM 2-mercaptoethanol.

SDS-PAGE and Immunoblotting—The samples were subjected to electrophoresis on 7.5% SDS polyacrylamide gels. Immunoblotting was performed as described previously (*38*).

Endoglycosidase Digestion—The immunoprecipitated or secreted proteins were denatured by boiling for 5 min at 100°C with 5 mM Tris-HCl (pH 8.0) containing 0.2% SDS. The samples were adjusted to a final concentration of 50 mM sodium acetate buffer (pH 6.0) containing 0.75% Triton X-100 and 100 μg/ml of the protease inhibitor cocktail. The mixtures were added to 10 mU of endo H or 1.0 U of *N*-glycosidase F, and then incubated at 37°C for 18 h. Reactions were stopped by boiling samples in the SDS solubilizing buffer.

Pepstatin A–Sepharose Binding—Aliquots of the cell lysate were brought to pH 3.5 by the addition of a 10% volume of 1 M sodium acetate buffer, pH 3.5, containing 0.5% Brij 35 and 10 M NaCl. Samples were then applied to a column of pepstatin A–Sepharose (*39*). The column was well washed with the same buffer and the bound protein was eluted with 0.1 M Tris-HCl, pH 8.6, containing 1 M NaCl and 0.05% Brij 35. The non-adsorbed (unbound) and adsorbed (bound) fractions were precipitated with 10% trichloroacetic acid. Samples were subjected to 7.5% SDS-PAGE, followed by immunoblotting.

Trypsin Treatment—Equal amounts of the cell extract were incubated with trypsin at final concentrations of 1, 50 and 100 ng/ml of 10 mM Tris-HCl (pH 7.5) at 25°C for 30 min. After incubation, a 2-fold excess as to trypsin of soybean trypsin inhibitor was added, followed by incubation at 25°C for 15 min to stop the reaction.

Enzyme Assays and Protein Determination—Acid proteinase activity was measured at pH 3.5 using 1.5% acid-denatured hemoglobin (*15*), MOCAc-Gly-Lys-Pro-Ile-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 (substrate I) (*33*), and MOCAc-Gly-Ser-Pro-Ala-Phe-Leu-Ala-Lys(Dnp)-D-Arg-NH_2 (substrate II) (*34*). Kinetic parameters for hydrolysis of the fluorogenic substrate, and inhibitor constants for interaction with pepstatin A and *Ascaris* pepsin inhibitor were determined as described previously (*36*). The amount of CE in each sample was determined by immunoblotting following the procedure recommended by the manufacturer for a Bio-Dot microfiltration apparatus.

RESULTS

Construction of Chimeric Proteins—Sequence alignment of the propeptides of rat CE (*29*) and CD (*37*) is shown in Fig. 1, where codes in white characters with black background represent the identical residues. The propeptide for CE starts at the 20th Gln and ends at the 58th Phe,

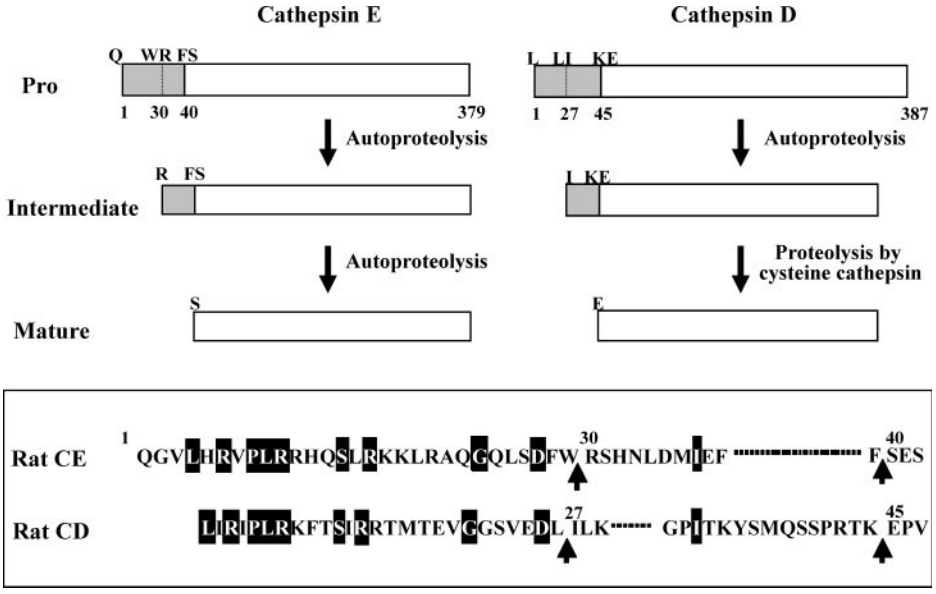

Fig. 1. **Schematic diagram of the processes of maturation of CE and CD and alignment of the amino acid sequences of propeptides of these enzymes.** The numbers of the residues are designated according to the respective proenzymes. The positions of cleavage sites are indicated. The conversion of procathepsin E into mature CE occurs very rapidly at low pH *via* a short-lived intermediate form and does not require other proteinases for complete removal of the propeptide, whereas procathepsin D is capable of only partial autoprocessing and requires lysosomal cysteine proteinases for its final activation. The identical amino acids in the propeptides of CE and CD are indicated by white characters with a black background.

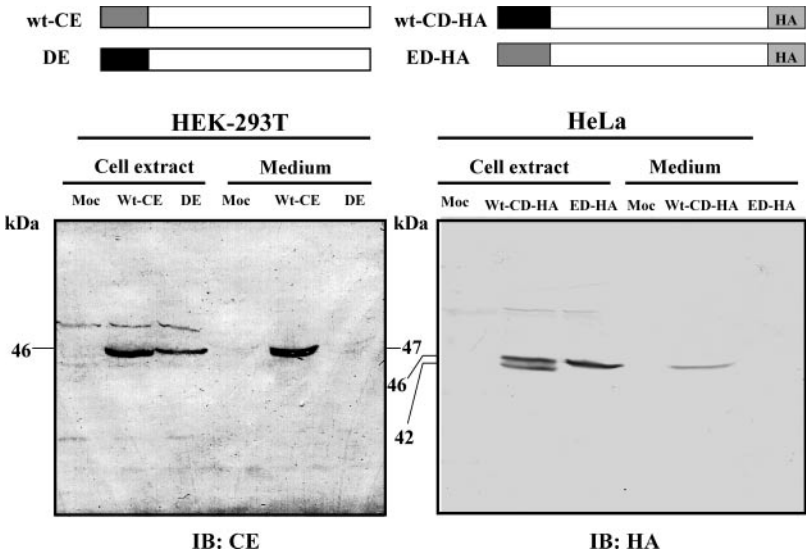

Fig. 2. **Expression of fusion proteins encoded by chimeric cDNAs in HEK-293T and HeLa cells.** Schematic diagram of construction of wt-CE, the DE chimera, the ED-HA chimera, and wt-CD-HA (upper). Cell lysates of HEK-293T cells containing wt-CE or the chimeric DE protein, and of HeLa cells containing wt-CD-HA or the chimeric ED-HA protein were analyzed by SDS-PAGE under reducing conditions, followed by immunoblotting with anti-CE IgG and anti-HA antibodies, respectively (lower). Moc indicates the cell lysate of each cell type transfected with pcDNA 3.0 without genes encoding the respective enzymes.

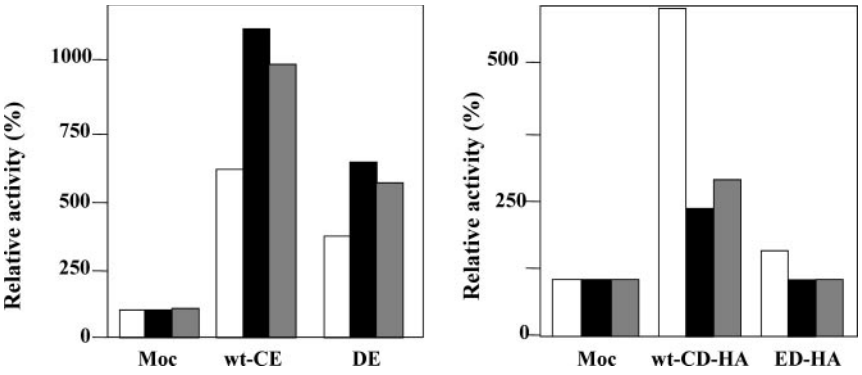

Fig. 3. **Comparison of the proteolytic activities of cell lysates of HEK-293T cells containing wt-CE or the DE chimera, and of HeLa cells containing wt-CD-HA or the ED-HA chimera.** The enzyme activities of cell lysates of transfected cells at the same protein concentration were measured using acid-denatured hemoglobin as a protein substrate (open), and MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 (black) and MOCAc-Gly-Ser-Pro-Ala-Phe-Leu-Ala-Lys(Dnp)-D-Arg-NH_2 (grey) as synthetic substrates. The values are expressed as percentages of the enzyme activities of the lysates of transfected with the empty vectors. Data shown are the representative results of three independent experiments.

whereas that for CD starts at the 21st Leu and ends at the 64th Lys. The identity between the mature domains of CE and CD is 52%, but that between their pro-parts is only 20%, indicating a significant structural difference between the CE and CD propeptides, which may result in the difference in their physiological roles. Although the roles of CD propeptides have been well documented, those of the propeptide of CE remain to be solved.

Therefore, to assess the role of the propeptide of CE, we constructed two fusion proteins using chimeric cDNAs encoding the CE propeptide fused to the mature CD tagged with HA at the COOH terminus, termed ED-HA, and encoding the CD propeptide fused to the mature CE, termed DE (Fig. 2). These fusion proteins were expressed in HEK-293T cells or HeLa cells. To distinguish between the endogenous human CD and transfected rat CD, both the wild-type rat CD and the ED mutant were constructed as proteins tagged with HA at the COOH-terminus termed CD-HA and ED-HA, respectively. Here, we used HEK-293T cells as recipient cells for CE expression, since these cells, unlike HeLa cells, exhibit no detectable endogenous CE expression, as shown by expression of the empty vector (Fig. 2), but exhibit the ability to mature the transfected pro-CE and to target it to the endosomal compartment (unpublished). On the other hand, we used HeLa cells as recipient cells for CD expression, since the expression level of CD-HA was higher in these cells than in HEK 293T cells (data not shown). SDS-PAGE and immunoblot analysis revealed that the chimeric DE protein in HEK-293T cells was predominantly expressed as a precursor form with an apparent molecular mass of 47 kDa under reducing conditions but was not detectable in the culture medium, whereas wt-CE was present mainly as a 46-kDa form both intracellularly and extracellularly (Fig. 2). On the other hand, wt-CD-HA existed as two major proteins with apparent molecular masses of 46 and 42 kDa in HeLa cells, and a single protein with an apparent molecular mass of 46 kDa in the culture medium, as demonstrated by immunoblotting with anti-HA antibodies. The chimeric ED-HA protein was expressed only as the 46-kDa form within the cells.

Catalytic Activities of Various Chimeic Proteins—To determine whether or not the fusion proteins expressed in heterologous cells have the ability to degrade various substrates, we measured the catalytic activities in their cell lysates using acid-denatured hemoglobin as a protein

substrate, and MOCAc-Gly-Lys-Pro-Ile-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 and MOCAc-Gly-Ser-Pro-Ala-Phe-Leu-Ala-Lys(Dnp)-D-NH_2 as synthetic fluorogenic substrates (Fig. 3). The cell lysate containing the chimeric DE protein exhibited a remarkable elevation of the enzyme activities toward all of the substrates, although the efficiency was lower than that of wt-CE (Fig. 3). By contrast, the cell lysate containing the ED-HA protein exhibited increased activity toward none of the substrates, although that containing wt-CD-HA showed marked elevation of the enzyme activities toward these substrates. These data indicate that the propeptide of CE is required for correct folding and activation of this protein but that it can be partially replaced by that of CD, whereas the propeptide of CD can not be replaced by that of CE for correct folding and activation of CD. The data also indicate that the addition of a HA-tag to CD does not affect the proteolytic activity of CD chimeras.

Kinetic parameters for the chimeric protein-expressing HEK-293T cells were determined using the same synthetic substrates as before. Table 1 shows the values for the DE chimera and compares them with those for wt-CE. The k_{cat}/K_m values of wt-CE with both substrates were in good agreement with the data available for the native enzyme from other mammalian sources (*33–36*, *40*). The k_{cat}/K_m values observed for the DE chimera were considerably high but less (35%) than those for wt-CE. The DE chimera was further examined to determine its sensitivity to two aspartic proteinase inhibitors (Table 2). Pepstatin A, a non-specific aspartic proteinase inhibitor first isolated from culture filtrates of various species of *Streptomyces*, potently inhibited the DE chimera, as well as wt-CE, the K_i values being of nM order. *Ascaris* pepsin inhibitor (API), a specific inhibitor of cathepsin E and pepsin, also strongly inhibited both DE chimera and wt-CE. The observed K_i values indicate that there are no significant differences in apparent binding affinities to the inhibitors between DE chimera and wt-CE.

Table 1. **Kinetic parameters for the hydrolysis of two synthetic substrates by wt-CE and the chimetic DE protein.** All reactions were carried out at pH 4.0 in 50 mM sodium acetate buffer at 40°C for 10 min. Data shows are the representative results of three independent experiments.

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
(1) MOCAc-GKPILFFRLK(Dnp)-D-Arg-NH_2			
wt-CE	2.80	32.2	11.50
DE chimera	3.39	25.6	7.55
(2) MOCAc-GSPAFLAK(Dnp)-D-Arg-NH_2			
wt-CE	1.53	13.58	6.19
DE chimera	2.02	8.43	4.17

Table 2. **Inhibition constants (K_i) for the interaction of wt-CE and the chimeric DE protein with pepstatin A and *Ascaris* pepsin inhibitor.** All measurements were carried out using MOCAc-GSPA-FLAK(Dnp)-D-Arg-NH_2 as a substrate at pH 4.0 in 50 mM sodium acetate buffer at 40°C for 10 min. Data shown are the representative results of three independent experiments.

Enzyme	K_i (nM)	
	Pepstatin A	*Ascaris* pepsin inhibitor
wt-CE	3.1	13.5
DE chimera	8.9	15.1

Activation of Chimeric Proteins upon Acid Treatment—The activation of zymogens of aspartic proeinases is most likely initiated by dramatic conformational rearrangement of the pro-parts of the zymogens (*41*, *42*). This process is often triggered by acidic pH and results in the proteolytic removal of propeptides. To determine whether or not the chimeric DE and ED proteins, like CE and CD, are capable of acid-dependent autoactivation *in vitro* to yield the catalytically active enzymes, the cell lysates containing these proteins were treated at pH 3.5 for 5 min. The DE chimera, like wt-CE, was rapidly converted to a catalytically active form, and this process was completely inhibited in the presence of pepstatin A (Fig. 4A). The discrepancy in molecular masses between wt-CE and the DE chimera before and after acid treatment is mainly due to difference in the size of the propeptide between CE and CD. This was further substantiated by the observation that the acid-dependent auto-cleavage of the pro-part of DE chimera occurred at the Leu25-Ileu26 bond of the CD propeptide, thereby yielding an intermediate form, like pseudo-CD (data not shown). The conversion of the DE chimera to the intermediate was completed within 0.5 min, and the formed intermediate form was stably retained during the experimental period (Fig. 4B). These data were comparable to those obtained with wt-CE. By contrast, the ED-HA chimera was resistant to acid-dependent autoactivation, although wt-CD-HA appeared to be slowly converted into the intermediate form (corresponding to pseudo-CD) (Fig. 4C). These data were consistent with the results in Fig. 3, where the ED-HA chimera exhibited significant activity toward none of the substrates.

Folding of Chimeric Proteins—Accumulating evidence suggests that the pro-parts of aspartic proteinases function as an intramolecular chaperon during the proper folding of zymogens. To determine whether or not the chimeric proteins were properly folded, we examined their ability to bind to pepstatin A and their susceptibility to trypsin digestion. It is known that pepstatin A is directly associated with the active site of correctly folded aspartic proteinases (*32*, *43*) and that the improperly folded proteins are susceptible to trypsin digestion (*44*). Therefore, binding to pepstatin A–Sepharose beads and resistance to trypsin digestion are useful measures of proper protein folding. The cell lysates containing the chimeric proteins as well as the wt-proteins were incubated with pepstatin A–Sepharose beads as described under Experimental Procedures. Under the conditions used, wt-CE and wt-CD-HA, like natural CE and CD from various sources (*15*, *16*, *38*, *39*, *45*), specifically bound to the beads and were efficiently eluted at pH 8.6 (Fig. 5A). Similarly, the DE chimera was mostly bound to and efficiently eluted from the beads. By contrast, most of the ED-HA chimera was not bound to the beads. The DE chimera, like wt-CE and wt-CD-HA, showed strong resistance to trypsin digestion, whereas the ED-HA chimera was dose-dependently digested by trypsin (Fig. 5B). These results strongly suggest that the DE chimera, like wt-CE and wt-CD-HA, are properly folded in such a way to adopt its active conformation.

Processing of Chimeric Proteins—To determine whether or not the chimeric DE and ED-HA proteins, as

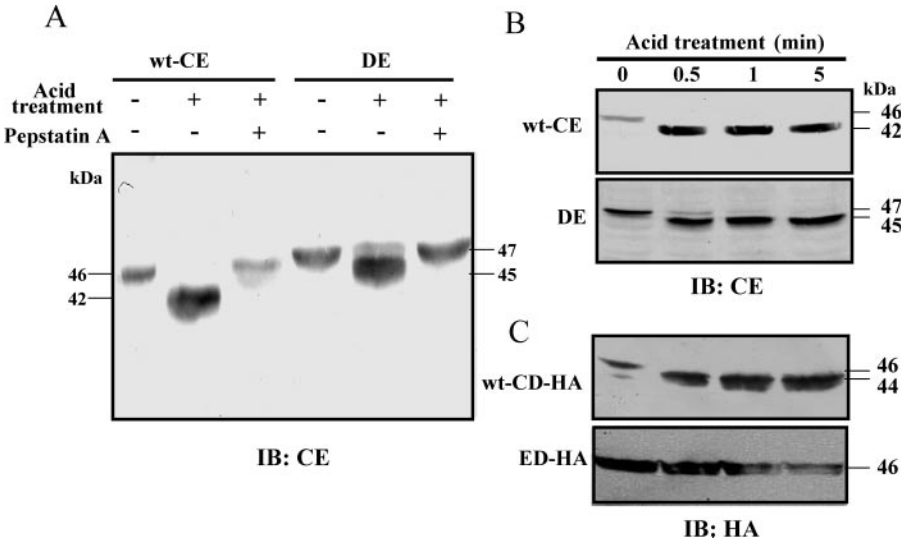

Fig. 4. **Autoproteolysis of the fusion proteins expressed in HEK-293T cells and HeLa cells.** (A) Cell lysates of HEK-293 cells containing wt-CE or the chimeric DE protein were incubated at 37°C for 10 min with 0.1 M sodium acetate buffer at pH 3.5 in the presence or absence of pepstatin A. Then, samples were added to 1 M Tris-HCl buffer (pH 9.0), boiled at 100°C for 5 min, and then subjected to SDS-PAGE followed by immunoblotting with anti-CE IgG. (B) The cell lysates of HEK-293T cells containing wt-CE or the DE chimera were incubated at 37°C in 0.1 M sodium acetate buffer at pH 3.5. At the indicated times, the reaction was terminated by the addition of 1 M Tris-HCl buffer (pH 9.0), followed by heating at 100°C for 5 min, and then samples were subjected to SDS-PAGE followed by immunoblotting with anti-CE IgG. (C) The cell lysates of HeLa cells containing wt-CD-HA or the ED-HA chimera were treated under the same conditions as in (B). Samples were analyzed by SDS-PAGE and immunoblotting with anti-HA antibodies.

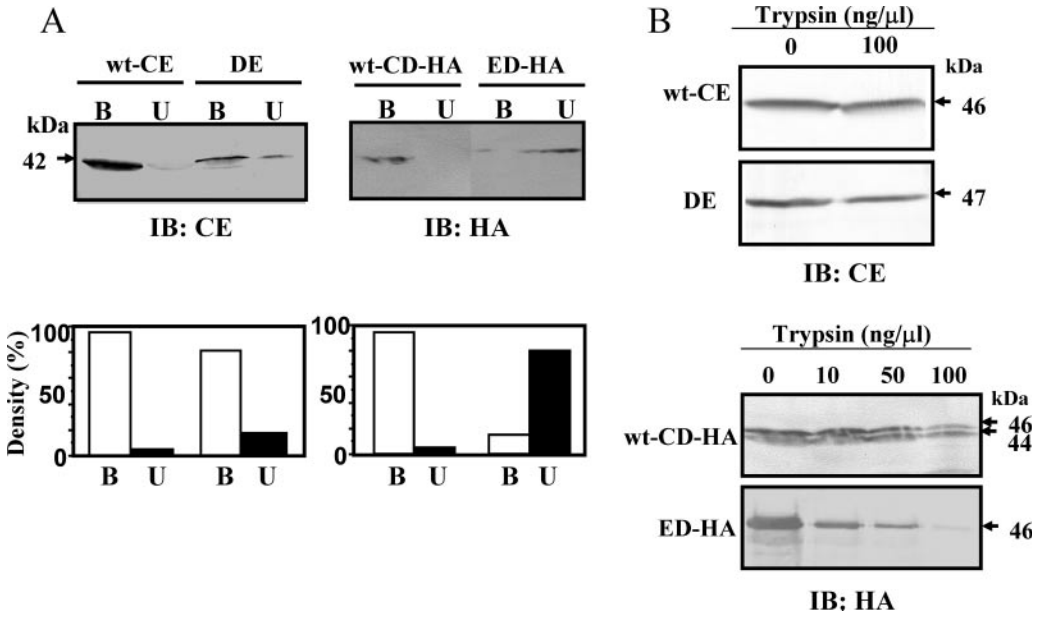

Fig. 5. **Pepstatin A binding and sensitivity to trypsin digestion of the fusion proteins.** (A) Cell lysates of HEK-293 cells containing wt-CE or the DE chimera, and of HeLa cells containing wt-CD-HA or the ED-HA chimera were adjusted to pH 3.5 by the addition of a one-tenth volume of 1 M sodium acetate buffer, pH 3.5, containing 10 M NaCl and 0.5% Brij 35. After centrifugation, the supernatants were applied to a column of pepstatin A–Sepahrose. The column was washed with same buffer, and the bound protein was eluted with 0.1 M Tris-HCl buffer, pH 8.6, containing 1 M NaCl and 0.05% Brij 35. Equal amounts of the non-adsorbed (U) and adsorbed (B) proteins were subjected to SDS-PAGE and immunoblotting. The distribution of the fusion proteins into the non-adsorbed and adsorbed fractions was determined by densitometric scaning of immunoblots by laser densitometory as the ratio of the amount of each protein to that of the total immunoreactive proteins. (B) Each cell lysate was incubated at 25°C for 30 min with or without various concentrations of trypsin at pH 7.5. The reaction was terminated by the addition of a 2-fold excess of soybean trypsin inhibitor. Then samples were analyzed by SDS-PAGE and immunoblotting with anti-CE IgG or anti-HA antibodies.

well as wt-CE and wt-CD-HA, were normally processed in HEK-293T and HeLa cells, respectively, pulse-chase experiments with [^{35}S]methionine were performed. After 60-min pulse labeling, the 46-kDa proform of wt-CE appeared in HEK-293T cells and remained in the cells after a 6 h chase (Fig. 6). Then, a part of the proform was released into the culture medium and accumulated during the chase. After a 12-h chase period, the 46-kDa proform was significantly processed to the 42-kDa mature CE. About 50% of the proform was processed to the mature form after a 24-h chase. By contrast, the 47-kDa proform of the DE chimeric protein appeared in the cells after 60-min pulse labeling and was retained in the cells for at least 24 h, and during this period it was neither processed nor degraded. Figure 7 shows the results with wt-CD-HA and the ED-HA chimera in HeLa cells. It was apparent that the 46-kDa proform of wt-CD-HA that appeared in the cells after 30-min pulse-labeling was processed to the 42-kDa mature CD during a 4-h chase period. A part of the proform was released into the culture medium after a 2-h chase. However, the newly synthesized ED-HA chimera remained in the 46-Da precursor form since it exhibited no change in size on the gel. This form was not released into the medium for at least 4 h. A half of this form appeared to be degraded in the cells after a 4-h chase period. The results indicate that the CE propeptide is essential for targeting of this protein to the endosomes but induces intracellular degradation of the ED-HA chimera, probably as a result of improper folding.

Modification of Oligosaccharide Chains of Chimeric Proteins—Human (*27*) and rabbit (*46*) CE have a single potential *N*-glycosylation site at position 73 (Asn73-Phe-Thr75), which is conserved in all species, whereas rat (*29*) and guinea pig (*28*) CE have another potential *N*-glycosylation site at position 305 (Asn305-Val-Thr307) or 318 (Asn318-Val-Thr320). Mouse CE has three potential *N*-glycosylation sites at positions 73, 305, and 318 (*30*). The oligosaccharide chains of CE are also known to be *N*-glycosylated with either high mannose-type and/or complex-type oligosaccharide chains. The type of *N*-glycosylation appears to be dependent on the cell type or the cellular localization. CE from human erythrocyte membranes (*35*, *47*) and rat microglia (*12*) is *N*-glycosylated with complex-type oligosaccharaides, whereas the enzymes from rat spleen (*47*) and rat stomach (*48*) have high mannose-type oligosaccharide chains. To determine the nature of the oligosaccharide chains of wt-CE and chimeric DE, the immunoprecipitates of metabolically labeled HEK-293T cells after a 24-h chase were treated with endo H, as well as *N*-glycosidase F, and then analyzed by SDS-PAGE followed by fluorography (Fig. 8A). It was apparent that there was no significant difference in the

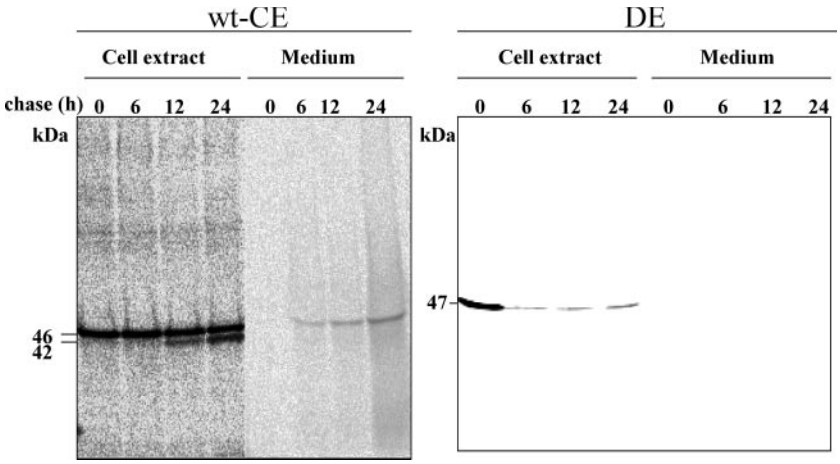

Fig. 6. **Biosynthesis of wt-CE and the DE chimera in HEK-293T cells.** HEK-293T cells expressing wt-CE and the DE chimera were pulse-labeled with [^{35}S]methionine/cysteine for 1 h and then chased in non-radiolabeled medium for the times indicated (h). After solubilization, aliquots of the cells and the culture medium were immunoprecipitated with anti-CE IgG, and then analyzed by SDS-PAGE followed by fluorography.

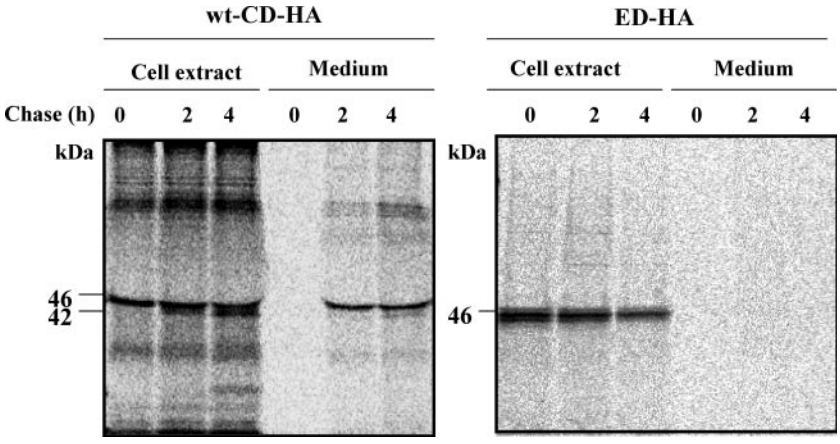

Fig. 7. **Biosynthesis of wt-CD-HA and the ED-HA chimera in HeLa cells.** HeLa cells expressing wt-CD-HA and the ED-HA chimera were pulse-labeled with [^{35}S]methionine/cysteine for 30 min, and then chased in non-radiolabeled medium for the times indicated (h). After solubilization, aliquots of the cells and the culture medium were immunoprecipitated with anti-CE IgG, and then analyzed by SDS-PAGE followed by fluorography.

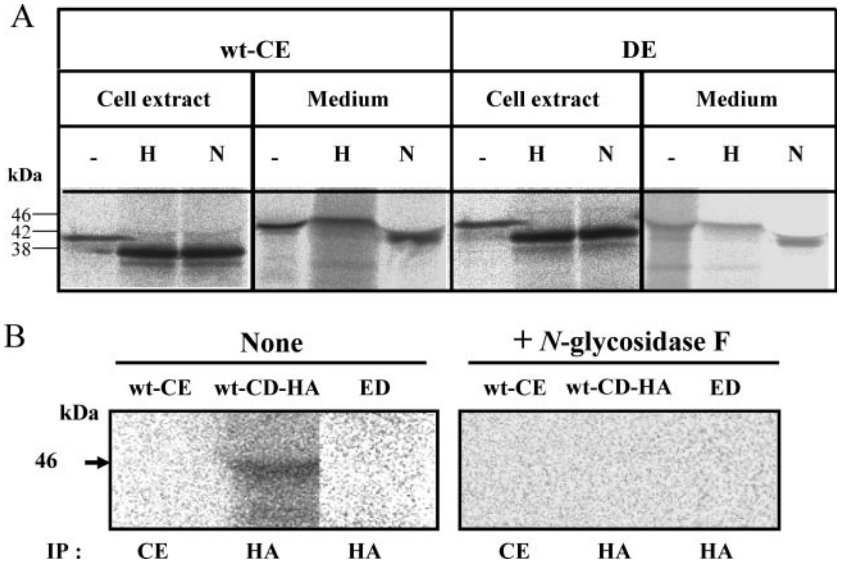

Fig. 8. **Treatment with endoglycosidase H and *N*-glycosidase F of the fusion proteins expressed in HEK-293T or HeLa cells.** (A) HEK-293T cells expressing wt-CE and the DE chimera were pulse-labeled with [^{35}S]methionine/cysteine for 1 h, and then chased in non-radiolabeled medium for 24 h. After solubilization, aliquots of the cells and the culture medium were immunoprecipitated with anti-CE IgG. The resulting immunoprecipitates were incubated at 37°C for 18 h with or without endo-H (H) or *N*-glycosidase F (N), and then analyzed by SDS-PAGE followed by fluorography. (B) HEK-293T cells expressing wt-CE and the DE chimera, and HeLa cells expressing wt-CD-HA were pulse-labeled with [^{32}P]orthophosphate for 6 h. After solubilization, aliquots of the cells were immunoprecipitated with anti-CE or anti-HA IgGs, and then analyzed by SDS-PAGE followed by fluorography.

nature of the oligosaccharide chains between wt-CE and the DE chimera. Both the 46-kDa proenzyme and the 42-kDa mature wt-CE in the cell lysate were completely sensitive to endo H, migrating faster on electrophoresis than the untreated proteins, whereas the 46-kDa proenzyme in the culture medium was resistant to endo H treatment. The same results were obtained with the DE chimera, indicating the removal of *N*-linked high mannose-type oligosaccharide chains. These results were in good agreement with the data obtained with recombinant human CE expressed in Chinese hamster ovary cells (*18*).

It is known that CD, like other soluble lysosomal enzymes, is phosphorylated in a portion of the oligosaccharide chains during passage through the Golgi complex (*49*, *50*). This event contributes to its recognition by mannose-6-phosphate receptors and its delivery to the prelysosomal compartments. We thus analyzed the ability of the propeptide of CE to induce the phosphorylation of the oligosaccharides of the ED chimera. The immunoprecipitates of HeLa cells pulse-labeled with [^{32}P]phosphoric acid for 6 h were treated with or without *N*-glycosidase F, and then analyzed by SDS-PAGE followed by fluorography (Fig. 8B). The cellular 46-kDa proform of wt-CD-HA was apparently phosphorylated. This phosphorylation was abolished on *N*-glycosidase F treatment, indicating that the phosphorylation occurred at the oligosaccharide side chains of this protein. By contrast, the ED chimera, like wt-CE, was not phosphorylated. These data indicate that the propeptide of CD is essential for the export of this protein from the ER to the Golgi complex, and that the propeptide of CE can not be replaced by that of CD.

DISCUSSION

To assess the role of the propeptide of CE in the correct folding, maturation, and targeting to the proper destination, of this protein, we constructed two fusion proteins using chimeric cDNAs encoding the CE propeptide fused to mature CD and encoding the CD propeptide fused to mature CE, and successfully expressed them in HeLa cells and HEK-293T cells, respectively. The data presented in this paper demonstrate that the propeptide of CE is essential for the targeting of this protein to its final destination in HEK-293T cells, where this protein is autocatalytically processed to mature CE. The data also demonstrate that the propeptide of CE is important for the correct folding of pro-CE in the ER and its activation in the post-Golgi compartment, although it appears that the propeptide of CE can be partially replaced by the propeptide of CD. Similarly, the propeptide of CD can not be replaced by the propeptide of CE regarding for the correct folding, maturation, and sorting of CD to the lysosomes. This conclusion is based on several lines of evidence. First, pulse-chase analysis revealed that, while wt-CE was initially synthesized as its zymogen and subsequently processed to mature CE within a 2-h chase, the DE chimera was not processed to the mature enzyme even after a 4-h chase period (Fig. 6). The inability of the DE chimera to be converted into the mature form in the cells indicates that the propeptide of CE is essential for the export of pro-CE from the ER to the Golgi compartment and that it can not be replaced by the propeptide of CD. Second, the DE chimera, like wt-CE, is capable of acid-dependent autoactivation *in vitro* to yield the catalytically active form (Fig. 4). However, the efficiency of hydrolysis of three different substrates (Fig. 3), and the specificity constants (k_{cat}/K_m) for two synthetic substrates (Table 1) were apparently lower than those for wt-CE. Consistently, the auto-activated DE chimera appeared not to be identical with the mature enzyme derived from wt-CE since the sequence surrounding the autocatalytic cleavage site was similar to the first cleavage site of pro-CE yielding pseudo-CD (data not shown). Therefore, it is most likely that the presence of the remaining portion of the CD propeptide may promote competition at the active site between exogenous substrates and the remaining propart, thereby resulting in the structural difference between mature CE and the autoactivated DE chimera.

Recent evidence suggests that the propeptide of CD is essential for proper folding and sorting of this protein to

the lysosomes. In this study, we provide unambiguous evidence supporting this observation. When expressed in HeLa cells, wt-CD-HA was normally processed to the mature enzyme and a small part of this protein was secreted into the medium as the proform (Fig. 7). By contrast, the chimeric ED-HA protein was not processed to the mature form and not detectable in the medium (Fig. 7). Although wt-CD-HA became autoactivated on a brief acid treatment to yield catalytically active pseudo-CD-HA, ED-HA was not converted to the catalytically active form with the same treatment (Fig. 4). About half of the wt-CD-HA expressed in HeLa cells was stably retained in the ER, whereas the ED-HA protein was less stable than wt-CD-HA, as determined on pulse-chase analysis (Fig. 7) and from its trypsin sensitivity (Fig. 5). In contrast to the DE chimera, the ED-HA protein exhibited no detectable enzyme activity (Fig. 3) and no binding activity toward pepstatin A–Sepharose beads (Fig. 5). The data thus indicate that the ED chimera was neither correctly folded nor matured. Accordingly, it is apparent that the propeptide of CD is essential for the correct folding, activation, and delivery of this protein to the lysosomes, and that it can not be replaced with the propeptide of CE.

The intracellular wt-CE and DE chimera contained high mannose-type oligosaccharide chains, as determined from their endo H sensitivity, whereas the extracellular proteins had endo-H resistant oligosaccharides (Fig. 8). Previous studies have shown that CE is localized as an active enzyme in the endosomal compartment of antigen-presenting cells such as microglia (*12*), whereas it is expressed on the plasma membrane of various cells as pro-CE (*17*, *35*, *47*). Regardless of their cellular localization, these CE molecules contained endo-H resistant, complex oligosaccharides. By contrast, human CE molecules expressed in CHO cells, which contained pro-CE (90 kDa), the intermediate form (84 kDa), and mature CE (82 kDa), all were *N*-glycosylated with high mannose-type oligosaccharide chains (*18*). Therefore, it appears to be difficult to explain the targeting of CE to the proper cellular compartments based on the modification of its oligosaccharide chains only. Moreover, the oligosaccharides of wt-CD-HA were efficiently phosphorylated, whereas those of ED-HA were not phosphorylated at all (Fig. 8). Taken together, these data indicate that the propeptides of CE and CD can not be replaced for the correct folding, maturation, and sorting of each enzyme.

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