Cathepsin C Propeptide Interacts with Intestinal Alkaline Phosphatase and Heat Shock Cognate Protein 70 in Human Caco-2 Cells

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Abstract: The oligomeric structure and the residual propeptide are distinct characteristics of cathepsin C from other members in the papain superfamily. In this study, we examined the physiological role of the cathepsin C propeptide. The stable overexpression of cathepsin C propeptide significantly decreased the activities of intestinal alkaline phosphatase (IAP) and sucrase in human Caco-2 intestinal epithelial cells, whereas it did not change the proliferation and cathepsin C activity. The overexpression of cathepsin C propeptide significantly decreased the amounts of IAP protein in differentiated Caco-2 cells, compared with the transfection of mock vector, whereas the amounts of IAP transcripts were not changed. Pulse-chase analysis confirmed that the reduction in IAP activity was due to an increase in IAP degradation, but not a decrease in IAP expression. For the mechanism of the enhanced IAP degradation, we identified proteins interacting with cathepsin C propeptide in Caco-2 cells by immunoprecipitation and mass spectrometry. Cathepsin C propeptide interacted with proteins with a molecular mass of approximately 70 kDa, including IAP and heat shock cognate protein 70. Our present results suggest that the propeptide of cathepsin C may stimulate the sorting to the lysosome, at least in part, contributing to the degradation of IAP in Caco-2 cells.

Key words: cathepsin C propeptide, HSC-70, human Caco-2 cells, IAP, sucrase.

Cathepsin C (dipeptidyl peptidase I or cathepsin J) is capable of sequentially removing dipeptides from the amino termini of various protein substrates [1–3] and serves as an essential factor in the activation of several granular serine peptidases involved in cell-mediated apoptosis, inflammation, and connective tissue remodeling [4–6]. Cathepsin C is a lysosomal cysteine protease that belongs to the papain superfamily of cysteine enzymes, consisting of propeptide and the catalytic domains of heavy and light chains [1, 7]. A recent analysis of its crystal structure revealed that the propeptide of cathepsin C forms a β-barrel and stabilizes the oligomeric formation of cathepsin C [8–10]. However, the biological function of the propeptide is still unknown.

In the present study, we overexpressed the propeptide of cathepsin C in human adenocarcinoma Caco-2 cells, which possess characteristics of small intestinal epithelial cells [11], to elucidate the physiological function of the propeptide. Cathepsin C is abundantly expressed in epithelial cells of the intestinal tract [7], suggesting that it plays an important role in the differentiation/development of intestinal epithelium. Therefore we hypothesized that an overexpression of the propeptide of cathepsin C would induce abnormalities in the differentiation of Caco-2 cells. It is interesting that the activity and amount of intestinal alkaline phosphatase (IAP) and the activity of sucrase significantly decreased in Caco-2 cells overexpressing cathepsin C propeptide. Furthermore, the propeptide interacted with heat shock cognate protein 70 (HSC70) and IAP, and enhanced IAP degradation. Our present results suggest that the propeptide functions as a component modifying the sorting and lysosomal degradation of several interacting proteins.

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Abbreviations: ANOVA, analysis of variance; CMA, chaperone-mediated autophagy; DMEM, Dulbecco’s Modified Eagle’s Medium; GFP, green fluorescence protein; HSC70, heat shock cognate protein 70; HSP 70, heat shock protein 70; IAP, intestinal alkaline phosphatase; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RT-PCR, reverse transcription and polymerase chain reaction.

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MATERIALS AND METHODS

Cell culture and electroporation. A plasmid containing rat cathepsin C cDNA was prepared as described previously [7]. We amplified cDNA for cathepsin C prepropeptide by polymerase chain reaction (PCR) with the following primers: 5'-CCGGGATCCGCTGTTAGTGG-3' and 5'-TTAAACCTCAGGCTTCTTAG-3'. The EcoRI/BamHI fragments of the PCR products were reconstructed into the plasmid expression vector pE-GFP-N2 with GFP at the C-terminal site (Clontech Lab., Palo Alto, CA) to express cathepsin C prepropeptide-GFP chimera protein. The reconstructed plasmid was named pE-CCP-GFP.

Human adenocarcinoma Caco-2 cells were purchased from Dai-Nippon Pharmaceutical Co. (Osaka, Japan). Caco-2 cells were maintained and proliferated with Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum, 100 µg/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a 5% CO2 incubator. Proliferating Caco-2 cells (2 x 10^6/sample) were resuspended in 1 ml of the desired concentration of phosphate-buffered saline (PBS) with linearized plasmid DNA (10 µg/ml), including pE-CCP-GFP and pE-GFP-N2. Electroporation was then carried out according to published procedures [12]. The electrical pulse was supplied by a Gene Pulser II with a capacitance extender (Bio-Rad Japan, Tokyo) at 2,000 V. The solution containing the cells was held at 0°C for 10 min after electroporation. We then selected cells highly expressing the chimera protein or GFP with 1.5 mg/ml G418 and obtained three independent clones, which permanently expressed cathepsin C prepropeptide-GFP chimera protein (until at most the 10th passage) (Fig. 1). To confirm the high expression of cathepsin C prepropeptide in the transfected cells, we performed real time reverse transcription (RT–PCR) with SYBR Green dye and antisense primers for human/rat cathepsin C prepropeptide (5'-AACCTGCTCGGTTATGGGACC-3' and 5'-ACCCACCCAGTCATTGTCTC-3', respectively). Sense and antisense primers for human/rat cathepsin C prepropeptide were 5'-AACCTGCTCGGTTATGGGACC-3' and 5'-ACCCACCCAGTCATTGTCTC-3', respectively.

Western blotting. Whole-cell extracts (40 µg protein/lane) from Caco-2 cells were subjected to SDS–8%-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane at 35 mA for 6 h at 4°C. The membrane was blocked with 3% skim milk and then incubated for 1 h at 25°C in PBS with a 1:500 dilution of an antibody against rat IAP, a kind gift from Dr. D.H. Alpers, Washington University School of Medicine, St. Louis, MO; mouse HSC70; mouse heat shock protein 70 (HSP70), kind gifts from Dr. K. Rokutan, The University of Tokushima Graduate School, Tokushima, Japan; and GFP (Rockland Inc). The bound antibodies were detected by using the enhanced chemiluminescence system (Amersham, Little Chalfont, England, UK).

Immunoprecipitation. Whole-cell extracts or tissue homogenates in 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 1% Triton X-100, 1 tablet/25 ml protease inhibitor cocktails (Roche Diagnostics), and 10 µM epoxomycin, were centrifuged at 30,000 x g for 30 min at 4°C. The supernatants were stocked as soluble fractions at 4°C. The Journal of Physiological Sciences Vol. 58, No. 2, 2008
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-80°C until analysis. An antibody against a target protein preadsorbed on protein G–Sepharose was incubated with the supernatant overnight at 4°C, as described previously [14]. The immunoprecipitates obtained by centrifugation at 5,000 × g for 1 min at 4°C were washed four times in the same buffer and subjected to SDS-PAGE.

Ion-trap MS. A piece of gel containing target proteins was digested by 0.025% trypsin at 37°C overnight. After centrifugation, the supernatant was subjected to MS, which was performed using an ion-trap mass spectrometer Esquire 3000plus with enhanced ion trap optics (Bruker Daltonik, Bremen, Germany), according to the standard protocols [15]. An automation of data processing has been achieved by postprocessing with DataAnalysis (Version 3.0; Bruker Daltonik), transfer to the software package BioTools (Version 2.1; Bruker Daltonik), and the MASCOT-database (Matrix Science, London, UK) search via Visual Basic Scripts.

Immunohistochemistry. The transfected Caco-2 cells were fixed with methanol for 6 h at room temperature. After blocking nonspecific binding sites with rabbit sera (Vector Lab., Burlingame, CA) for 20 min in a humidified chamber, the cells were incubated in a 1:40 dilution of an antibody against GFP (Rockland Immunocchemicals, Philadelphia, PA) or LimpII/lgp85 (Affinity Bioreagents. Com., Golden, CO) for 1 h at room temperature. The antibodies bound to GFP and LimpII/lgp85 were detected by fluorescence microscopy with appropriate secondary antibodies labeled with Alexa 488 and Alexa 568, respectively (Molecular Probe, Eugene, OR).

Pulse-chase analysis. Caco-2 cells transfected with pEGFP-N2 or pE-CCP-GFP were cultured until the complete differentiation and subjected to pulse-chase analysis as described previously [16]. The fully differentiated Caco-2 cells were cultured with methionine-free RPMI containing 10% dialyzed FCS for 4 h and labeled with 100 µCi of [35S]methionine (1 mCi/mmol) for 3 h at 37°C. The labeling medium was replaced with DMEM containing 10% FCS and incubated for the indicated times. At the end of the chase period, the cells were rinsed three times with PBS and lysed in 50 µl of Tris-HCl buffer, pH 7.2, containing 2 mM EDTA, 0.5% SDS, 1 mM phenylmethylsulfonylfluoride, 50 µg/ml leupeptin, 1 µM E-64, and 0.1 ng/ml aprotinin. Samples (100 µg protein) were immunoprecipitated by using an anti-IAP antibody, and the immunoprecipitates were subject to SDS–8%-PAGE, as described above.

Northern blotting. Total RNA (20 µg/lane) was separated in a denatured 1% agarose gel, blotted and ultraviolet–cross-linked to a nylon membrane (Amersham). After prehybridization, the hybridization of the membrane was performed in a high-speed buffer with an isotope-labeled IAP cDNA probe, as described previously [17]. The membranes were washed and exposed to Kodak X-ray films at -80°C for the appropriate times, and the films were then developed. Each mRNA level was standardized to that of 18S rRNA.

RESULTS

Stable expression of cathepsin C propeptide in Caco-2 cells

We established three clones of Caco-2 cells stably expressing cathepsin C propeptide–green fluorescence protein (GFP) with the G418 selection method. Western blot analysis showed that all tested clones of the transfected Caco-2 cells dominantly contained cathepsin C propeptide–GFP (Fig. 1A). Real time RT-PCR analysis revealed that at the mRNA level, the expression of cathepsin C propeptide in transfected Caco-2 cells was approximately fourfold higher than that of cathepsin C propeptide in nontransfected Caco-2 cells. The microsequencing confirmed that the N-terminal amino acids of cathepsin C propeptide were processed to the propeptide form (data not shown).

We also examined intracellular localization of the overexpressed propeptide in Caco-2 cells. The location of the fused protein, cathepsin C propeptide–GFP, was similar to that of a lysosomal marker protein limpiI/lgp85 in fixed Caco-2 cells, and GFP was located in the cytosol (Fig. 1B). The fluorescence of cathepsin C propeptide–GFP chimera protein in unfixed (intact) Caco-2 cells showed a pattern similar to the location of the fused protein in fixed Caco-2 cells (data not shown). These results are consistent with the previous reports that the propeptide of lysosomal cathepsins has a sorting signal to the lysosome [21].

Effects of overexpressed cathepsin C propeptide on IAP and sucrase activities in Caco-2 cells

Consistent with the previous reports [11, 22], GFP-transfected (control) Caco-2 cells reached 100% confluence 3 or 4 days after seeding and spontaneously started differentiating into small intestinal epithelial cells, which expressed IAP and sucrase (Fig. 2, A, C, and D). Caco-2 cells before confluence had constant activity of cathepsin C, and the activity decreased after starting differentiation (Fig. 2B). It is interesting that an overexpression of cathepsin C propeptide depressed the activity of cathepsin C to the same extent as that observed in the control cells. Conversely, the IAP activity increased with differentiation of Caco-2 cells (Fig. 2, B and D). The results suggest that the IAP activity is regulated independently of the activity of cathepsin C. The simultaneous expression of IAP and cathepsin C may possibly be a mechanism to maintain the balance of the activities of these enzymes in the cells.
psin C propeptide significantly decreased the activities of IAP and sucrase in Caco-2 cells, especially after confluence (Fig. 2, C and D), whereas the proliferation of Caco-2 cells was not changed by an overexpression of cathepsin C propeptide (Fig. 2A). An overexpression of cathepsin C propeptide did not affect cathepsin C activity in Caco-2 cells (Fig. 2B) because the propeptide itself did not have the catalytic domain of cathepsin C.

**Decrease in levels of IAP protein in Caco-2 cells overexpressing cathepsin C propeptide**

The overexpression of cathepsin C propeptide significantly decreased the amounts of IAP protein in completely differentiated Caco-2 cells, compared with an overexpression of GFP (Fig. 3A). In contrast, the amounts of IAP transcripts in all tested clones of the transfected Caco-2 cells were not changed by the overexpression of cathepsin C propeptide (Fig. 3B). We observed similar results in the measured values from all clones of Caco-2 cells expressing cathepsin C propeptide (Fig. 3). These findings indicate that a reduction in IAP activity caused by an overexpression of cathepsin C propeptide is due to an increase in IAP degradation, but not to a decrease in IAP expression.

**Enhanced degradation of IAP protein in Caco-2 cells overexpressing cathepsin C propeptide**

To elucidate the hypothesis described above, we examined time-dependent changes and the half-life of IAP in Caco-2 cells overexpressing cathepsin C propeptide or

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**Fig. 2.** Proliferation rate and activities of cathepsin C, IAP, and sucrase in cathepsin C propeptide–expressing Caco-2 cells. The cell number (A) and activities of cathepsin C (B), IAP (C), and sucrase (D) in Caco-2 cells expressing cathepsin C propeptide (●) or only GFP (○) were measured on the indicated days after confluence, as described in MATERIALS AND METHODS. Other two clones of Caco-2 cells expressing cathepsin C propeptide (see Fig. 1 legend) had similar characteristics. Data are means ± SD, n = 6. * Indicates significant difference, compared with the values in Caco-2 cells expressing GFP (P < 0.05).

**Fig. 3.** Expression of IAP in cathepsin C propeptide–expressing Caco-2 cells. Caco-2 cells expressing cathepsin C propeptide (CC-Pro-GFP, three independent clones) or only GFP were completely differentiated by the culturing for 10 days after confluence. Proteins (40 µg/lane) and total RNA (20 µg/lane) prepared from these cells and adult rat small intestine were subjected to Western (A) and Northern blottings (B) for IAP, respectively, as described in MATERIALS AND METHODS. β-Actin and 18S ribosome were used as internal controls for these analyses.
GFP (control). IAP protein was accumulated in parallel to the differentiation of control Caco-2 cells into small intestinal absorptive epithelial cells, whereas the overexpression of cathepsin C propeptide decreased the amounts of intact IAP protein (Fig. 4A). We found it interesting that a degradation intermediate of IAP (indicated by ΔIAP) was detected in cathepsin C propeptide–expressing Caco-2 cells within 1 wk after confluence. The molecular mass of intact IAP was shifted from 70 kDa to 66 kDa. This finding is consistent with the previous report that newly synthesized IAP undergoes posttranslational processing and glycosylation [23].

A pulse-chase analysis also revealed that the overexpression of cathepsin C propeptide accelerated the degradation rate of IAP. In control Caco-2 cells, the amounts of the newly synthesized IAP protein were almost constant until 18 h after labeling (Fig. 4B). Two kinds of IAP degradation intermediates were observed, and their amounts were constant during the same period. In contrast, the amount of intact IAP decreased in a time-dependent manner in cathepsin C propeptide–expressing Caco-2 cells. Furthermore, the IAP degradation intermediates, especially 45-kDa protein, were gradually accumulated from 2 h after labeling. The 45-kDa protein corresponded to the IAP on Western blotting. In our pulse-chase experiment, Caco-2 cells were labeled with [35S]methionine for 3 h. Prior to the labeling, they were precultured with methionine-free media for 4 h. These operations may tentatively stimulate the degradation of IAP because the amount of possibly degraded products at time 0 in control cells was similar to that in propeptide-overexpressed cells. It was quite likely that the bands reactive to an anti-IAP antibody at time 0 were products artificially degraded for the labeling period.

Identification of proteins interacting with cathepsin C propeptide in Caco-2 cells

For the mechanism of IAP degradation stimulated by cathepsin C propeptide, we tried to identify proteins interacting with cathepsin C propeptide in Caco-2 cells. Immunoprecipitation analysis using an anti-GFP antibody revealed that cathepsin C propeptide interacted with several proteins (Fig. 5A). Especially, the amount of the protein with a molecular mass of approximately 70 kDa was most abundant among proteins possibly binding to cathepsin propeptide (Fig. 5A). We then subjected it to ion-trap mass spectrometry (MS) and Western blotting. The MS and its data processing revealed that these proteins might
Fig. 5. Interaction of cathepsin C propeptide with IAP and HSC70. Caco-2 cells expressing cathepsin C propeptide (CC-Pro-GFP) or only GFP were completely differentiated by the culturing for 7 days after confluence. (A) Lysates (100 µg protein/lane) prepared from these Caco-2 cells were immunoprecipitated with an anti-GFP antibody. Half of the immunoprecipitates were subjected to SDS–8%–PAGE and silver-staining. Another half of immunoprecipitates were analyzed by Western blotting using an anti-GFP protein. Arrows indicate proteins possibly interacting with cathepsin C propeptide. (B) Whole-cell lysates (100 µg protein/lane) were immunoprecipitated with an anti-IAP or nonimmunized antibody, immunoprecipitates or whole-cell homogenates were subjected to Western blotting of GFP. (C) Lysates (100 µg protein/lane) from the transfected or untransfected Caco-2 cells or homogenates (100 µg protein/lane) from rat liver were immunoprecipitated with an anti-GFP, anticathepsin B (CB), anticathepsin C (CC), or nonimmunized antibody. The immunoprecipitates were then subjected to Western blotting for HSC70. A positive control sample for HSC70 was lane) from rat liver were immunoprecipitated with an anti-GFP, anticathepsin B (CB), anticathepsin C (CC), or nonimmunized antibody. A positive control sample for HSC70 was

have the partial sequences of human IAP, heat shock protein 70 (HSP70), and HSC70 (data not shown). Western blotting for IAP showed that IAP and cathepsin C propeptide were coimmunoprecipitated in extracts of cathepsin C propeptide–expressing Caco-2 cells, though IAP failed to interact with GFP in control Caco-2 cells (Fig. 5B). Furthermore, an anti-HSC70 antibody, but not an anti-HSP70 antibody, was reacted with the immunoprecipitates by an anti-GFP antibody (Fig. 5C and data not shown). Of interest is the interaction of HSC70 with cathepsin C was observed in samples from untransfected Caco-2 cells or rat liver. However, cathepsin B, which belongs to the family of papain-type cysteine proteases and did not have propeptide in the mature molecule, failed to interact with HSC70 (Fig. 5C).

DISCUSSION

In the present study, we succeeded in overexpressing the propeptide of cathepsin C fused with GFP, processing it to the propeptide, and sorting it to the lysosome in human Caco-2 cells fully differentiated into small epithelial cells. We noted that the overexpression of cathepsin C propeptide significantly decreased the activities of IAP and sucrase and the amounts of IAP protein in Caco-2 cells, compared with those in cells transfected with mock vector. There were two possibilities for these phenomena:

(i) disturbed differentiation of Caco-2 cells by overexpressed cathepsin C propeptide resulted in a decreased expression of IAP and sucrase, and (ii) an overexpression of cathepsin C propeptide stimulated a degradation of IAP and sucrase. The amounts of IAP transcripts were not affected by the overexpression. The pulse-chase analysis in this study showed that cathepsin C propeptide accelerated the degradation rate of IAP. Furthermore, the propeptide of cathepsin C interacted with HSC70, which is required for a step in chaperone-mediated lysosomal protein degradation, in rat liver and also in Caco-2 cells. Based on these findings, we suggest that the propeptide of cathepsin C can interact with HSC70 as well as substrates, stimulating their sorting to lysosomes.

In mammals, three main forms of autophagy have been described: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) [24]. CMA is the only autophagic pathway that allows a selective degradation of soluble proteins in lysosomes. Previous studies have implicated HSC70 in stimulating CMA: lysosomal HSC70 is required for a step in the degradation pathway before protein digestion within lysosomes, most likely for the import of substrate proteins [25, 26]. Moreover, lysosomes containing HSC70 have been reported to possess relative higher pH than those without it [27]. Therefore it is considered that lysosomes containing propeptide of cathepsin C that interacted with HSC70 also have a relative high pH.
This hypothesis is supported by the evidence that GFP fused to cathepsin C propeptide can be detected in unfixed cells because the intensity of GFP fluorescence is greatly reduced under low pH [28]. Besides HSC70, cathepsin C propeptide interacted with several unidentified proteins, as shown in Fig. 5 A. Thus cathepsin C, especially its propeptide, may function as a chaperone and contribute to CMA.

The expression and degradation of digestive enzymes in intestinal epithelial cells are strictly regulated by hormones and growth factors. The posttranslational regulation of digestive enzymes is still unknown, although the mechanism of their expression is well characterized. It is of interest that the present study suggests that the expression of cathepsin C propeptide is associated with the lysosomal degradation of digestive enzymes, such as IAP and sucrase. Cathepsin C is abundantly located at the epithelial cells of the intestinal tract [7]. Further, it has been reported that the expression of cathepsin C was regulated by hormones, such as cortisol and thyroid hormone [29]. Cathepsin C propeptide-associated lysosomal degradation may play a role, at least in part, in the hormone-mediated regulation of digestive enzymes in intestinal epithelial cells. Further examinations are necessary to verify this hypothesis.

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