Processing of human cathepsin D is independent of its catalytic function and auto-activation: involvement of cathepsins L and B.

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ABSTRACT

The current mechanism proposed for the processing and activation of the 52 kDa lysosomal aspartic protease cathepsin D (cath-D) is a combination of partial auto-activation generating a 51 kDa pseudo-cath-D, followed by enzyme-assisted maturation involving cysteine and/or aspartic proteases and yielding successively a 48 kDa intermediate and then 34 + 14 kDa cath-D mature species. Here we have investigated the in vivo processing of human cath-D in a cath-D-deficient fibroblast cell line in order to determine whether its maturation occurs through already active cath-D and/or other proteases. We demonstrate that cellular cath-D is processed in a manner independent of its catalytic function and that auto-activation is not a required step. Moreover, the cysteine protease inhibitor E-64 partially blocks processing, leading to accumulation of 52-48 kDa cath-D intermediates. Furthermore, two inhibitors, CLICK148 and CA-074Met, specific for the lysosomal cath-L and cath-B cysteine proteases induce accumulation of 48 kDa intermediate cath-D. Finally, maturation of endocytosed pro-cath-D is also independent of its catalytic function and requires cysteine proteases. We therefore conclude that the mechanism of cath-D maturation involves a fully-assisted processing similar to that of pro-renin.
INTRODUCTION

Cathepsin D (cath-D) is a major intracellular aspartic protease of endosomes and lysosomes and is related to the other aspartic proteases such as renin, pepsin and yeast protease A [1]. All these enzymes are synthesized as inactive precursors, which are then processed either auto-catalytically (e.g. pepsin) [1] or by other enzymes (e.g. renin) to remove an N-terminal pro-peptide [2]. For cath-D, an intermediate mechanism had been proposed, namely, a partial auto-activation followed by a final processing by other enzymes [3-5].

Cath-D is synthesized on the rough endoplasmic reticulum (RER) as a pre-pro-enzyme that undergoes several proteolytic cleavages during biosynthesis to produce the mature form [3, 6, 7]. Following the initial co-translational removal of the signal peptide to yield pro-cath-D, sugars are attached at two N-linked glycosylation sites and the pro-enzyme is transported to Golgi stacks. The 52 kDa pro-cath-D is marked for mannose-6-phosphate (M6P) receptors and targeted to lysosomes [8, 9], where 44 amino acid residues are ultimately removed from the amino terminus, yielding an active intermediate 48 kDa single-chain molecule. This main proteolytic activation event most likely depends on the action of cysteine lysosomal and/or aspartic proteases [10-12]. Depending on the cell type, cath-D may also be targeted to lysosomes in an M6P-independent manner [12, 13]. The intermediate 48 kDa single-chain species is then cleaved in lysosomes into a mature two-chain enzyme consisting of a light 14 kDa amino-terminal domain and a heavy 34 kDa carboxyl-terminal domain. It is proposed that this proteolytic cleavage is accomplished by cysteine proteases in dense lysosomes, since processing was partially inhibited by leupeptin [14]. Accompanying the conversion to the two-chain species, 7 amino acid residues between the light and heavy chains are removed [15]. Several amino acids are also removed from the carboxyl terminus of heavy chains [16]. Pro-cath-D over-expressed by cancer cells is also secreted in excess and can be endocytosed by both cancer cells and by fibroblasts via M6P-receptors and other unknown receptor(s) [17]. Endocytosed pro-cath-D undergoes maturation successively into 48 kDa intermediate and 34 + 14 kDa mature forms [17, 18]. In addition, secreted pro-cath-D, like pepsinogen, is capable of acid-dependent auto-activation in vitro [19], resulting in a catalytically active pseudo-cath-D, an enzyme species that retains 18 residues (27-44) of the pro-segment.
A major question remains as to whether cath-D requires its proteolytic function and its auto-activation into pseudo-cath-D to be matured in cells by subsequent proteolytic cleavage by cysteine proteases, or pseudo-cath-D is only an in vitro artifact [7, 11]. Certain groups have attempted to elucidate the role of cath-D proteolytic activity and the relevance of pseudo-cath-D for the activation mechanism of pro-cath-D maturation, but no reported investigation has convincingly demonstrated that the activation of pro-cath-D is initiated in the absence of contaminating active cath-D [7, 20]. In the present study, we highlight the fact that the proteolytic activity and the auto-activation of cath-D were not required either for its processing in cells or after endocytosis. We were able to obtain this evidence since we previously engineered a catalytically-inactive D231N mutated cath-D expressed in cath-D-deficient fibroblasts [21]. We have also defined which cysteine proteases were implicated in cath-D processing in cells and after endocytosis.

RESULTS

Catalytic function and auto-activation of human cath-D are not required for its maturation.

To determine whether human cath-D requires its catalytic activity for maturation, we investigated the processing of a mutated \^D_{231}N\ cath-D devoid of proteolytic activity expressed in cath-D-deficient CD55-/- fibroblasts [21]. As expected, cellular 52 kDa wild-type pro-cath-D underwent successive maturation into a 48 kDa intermediate and then a 34 kDa mature species in CD55-/-cath-D transfected cells (Figure 1a, left panel). Processing of the D231N mutated pro-cath-D precursor into intermediate and mature species was not prevented by the D231N mutation in CD55-/-D231N cath-D cells (Figure 1a, right panel). These results indicate that maturation of cath-D in cells occurs in a manner independent of its catalytic activity. However, the precursor, intermediate and mature heavy chain \^D_{231}N\ cath-D all showed an increased electrophoretic mobility, corresponding to a shift of about 1 kDa in the apparent molecular mass, resulting in 51 kDa, 47 kDa and 33 kDa \^D_{231}N\ cath-D species, respectively (Figure 1a, right panel).

As human cath-D carries two-N-linked glycosylation sites at asparagine residues 70 and 199 with about 2 kDa molecular mass, we first examined whether the differences in the electrophoretic mobility between the wild-type and D231N cath-D were due to altered glycosylation. CD55-/-cath-D and CD55-/-D231N cath-D cells...
were treated with increasing concentrations of tunicamycin, which inhibits N-linked glycosylation. It has been previously published that tunicamycin treatment of pulse-labelled cells results in a 5 kDa decrease in molecular mass of the cath-D pro-enzyme with no subsequent pro-peptide cleavage [22]. As can be seen in Figure 1b, treatment with tunicamycin induced accumulation of 47 kDa and 46 kDa bands respectively in CD55-/-cath-D and CD55-/-D231N cath-D cells, which were likely to be the non-glycosylated pro-enzymes. No single-chain enzymes were apparent in CD55-/-cath-D and CD55-/-D231N cath-D cells as previously described [22]. The 34 kDa and 33 kDa mature forms of wild-type and D231N cath-D detected were apparently synthesized prior to addition of the tunicamycin but were sufficiently stable to be detected by Western blotting. We conclude that the 1 kDa shift in the molecular masses of cellular wild-type and D231N cath-D forms was not due to a difference in glycosylation. We also observed the same shift of 1 kDa in the molecular mass of wild-type and D231N pro-cath-D secreted in the conditioned media by CD55-/-cath-D and CD55-/-D231N cath-D cells (Figure 1c). Treatment with endoglycosidase H revealed the presence of the mono-glycosylated and de-glycosylated pro-cath-D as previously described [23] (Figure 1c). A similar pattern of de-glycosylation was observed for D231N pro-cath-D, indicating that both wild-type and D231N pro-cath-D carries two N-linked glycosylation sites (Figure 1c). However, de-glycosylation of D231N pro-cath-D revealed predominantly the mono-glycosylated form and only a trace of de-glycosylated protein (Figure 1C). These results suggest that the mutation of cath-D in the D231N catalytic site may result in a conformational change which affects its recognition by endoglycosidase H and therefore its full de-glycosylation. Collectively, these findings demonstrate that the higher electrophoretic mobility of the mutated D231N cath-D was not due to any alteration of D231N cath-D glycosylation, since neither tunicamycin (Figure 1b) nor endoglycosidase H (Figure 1c) could restore the same molecular weights of cellular or secreted non-glycosylated D231N cath-D as compared to non-glycosylated cath-D. Since the shift was observed with both the precursor, intermediate and carboxyl-terminal heavy mature chain of D231N cath-D, we propose that human D231N cath-D might have been cleaved after synthesis at its carboxyl-terminus. Our results confirm a previous report that suggested a similar carboxyl-terminal truncation for the 45 kDa single-chain form of D293N mutated mouse cath-D transiently expressed in HEK-293 cells and for precursor, intermediate
and mature forms of endogenously expressed mutant cath-D in CONCL sheep fibroblasts [24].

Concerning processing of cellular cath-D, it had been proposed that the 51 kDa pseudo-cath-D detected *in vitro* in the extracellular medium and generated by auto-activation at an acidic pH might be a necessary intermediate for subsequent maturation [19]. We verified that wild-type pro-cath-D secreted by CD55-/cath-D cells was capable of acid-dependent auto-activation yielding 51 kDa pseudo-cath-D, and that the aspartic protease inhibitor pepstatin A prevented its auto-activation (Figure 1d). As expected, because of its lack of proteolytic activity, the D231N pro-cath-D 51 kDa precursor secreted by CD55-/D231Ncath-D cells was not auto-activated into a shorter D231N pseudo-cath-D at acidic pH. Since D231N pro-cath-D underwent maturation in CD55-/D231Ncath-D cells (Figure 1a), we conclude that pseudo-cath-D generated by auto-activation is not a necessary species for cath-D maturation.

Together, our results demonstrate that maturation of cath-D occurs in cells in a manner independent of its catalytic function and auto-activation. This finding supports a fully-assisted mechanism of maturation mediated by other proteases.

**Requirement of cysteine proteases for cath-D processing.**

To investigate whether cysteine proteases were implicated in cath-D maturation in our model, CD55-/cath-D and CD55-/D231Ncath-D cells were first treated with E-64, a broad spectrum irreversible specific inhibitor of cysteine proteases. E-64 blocked processing leading to accumulation of 52-48 kDa cath-D intermediates, with hardly detectable 34 kDa mature form in CD55-/cath-D cells (Figure 2a, left panel). These processing intermediates were also observed in CD55-/D231Ncath-D cells (Figure 2a, right panel). Dose-response studies performed with CD55-/cath-D fibroblasts revealed that treatment with E-64 at a concentration of 100 µM leads to an accumulation of these processing intermediates (Figure 2b). Our various attempts to prevent the appearance of these processing intermediates by the concomitant use of E-64 plus serine, metallo-, or aspartic protease inhibitors were unsuccessful (data not shown). Analysis by 2D gel electrophoresis then highlighted the fact that these 52-48 kDa processing intermediates were not a single species but rather were composed of different processing intermediates (Figure 2c).

Having shown that both active and inactive cath-D required cysteine proteases for processing, we next attempted to identify which enzymes were involved. The
effects of two synthetic cell permeable cysteine protease inhibitors specific for the lysosomal proteases cath-L and cath-B were examined in CD55-/-cath-D transfected fibroblasts. CLICK-148 [26], a cath-L inhibitor and CA-074Met [25], a cath-B inhibitor, caused accumulation of single 48 kDa cath-D in CD55-/-cath-D fibroblasts (Figure 2d-e). Used together, these inhibitors had a cumulative effect, suggesting an additive role of cath-L and cath-B for cath-D processing (Figure 2e, left panel). Similar qualitative effects of these two inhibitors were observed for D231N-cath-D maturation in CD55-/-D231N-cath-D fibroblasts (Figure 2d-e, right panels). However, CLICK-148 and CA-074Met inhibitors tested alone or in combination were less efficient in preventing D231N-cath-D maturation as compared to wild-type cath-D. These results suggest that the D231N mutation may result in a cath-D conformational change which affects its recognition by specific inhibitors. Our data together suggest that cath-L and cath-B can partially process the 48 kDa intermediate into 34 kDa mature cath-D. Collectively, our results indicate that cath-D is successively matured into various processing intermediates by complex mechanisms involving not only the cysteine proteases but also unidentified protease(s) acting at initial steps. Figure 3 summarizes the identified steps of cath-D processing incorporating our new data.

Processing of endocytosed pro-cath-D.

To examine whether endocytosed pro-cath-D requires its catalytic activity for maturation, we next studied the processing of [35S]methionine-labelled wild-type and D231N secreted pro-cath-D after its endocytosis by unlabelled cath-D-deficient recipient fibroblasts (Figure 4a). SDS-PAGE analysis of 35S-labelled proteins bound and taken up by CD55-/- fibroblasts revealed after anti-cath-D immunoprecipitation that both wild-type and D231N 35S-labelled pro-cath-D were transformed into 35S-labelled mature enzymes (Figure 4a). Our results reveal that endocytosed pro-cath-D is also processed independently of its catalytic activity. To identify which proteases were involved, internalization experiments were performed in the presence of E-64, CLICK-148 or CA-074Met. E-64 blocked processing, leading to accumulation of 52-48 kDa processing intermediates (Figure 4b), whereas CLICK-148 and CA-074Met caused accumulation of 48 kDa intermediate cath-D (Figure 4c-d), as previously observed in cath-D transfected fibroblasts (Figure 2). A synergic effect was observed when cells were treated with both CLICK-148 and CA-074Met inhibitors (Figure 4d). Overall, our data demonstrate that the processing of endocytosed pro-cath-D is
independent of its catalytic activity and involved the cysteine proteases with similar mechanisms to those involved in the maturation of cellular cath-D transiting via RER (Figure 3).

**DISCUSSION**

Mechanisms associated with the processing and activation of lysosomal proteases remain largely unknown [27]. In general, three types have been reported for the processing and activation of aspartic proteinases. The first mechanism, complete auto-activation, has been described for porcine pepsinogen [1]. The second mechanism is represented by fully-assisted activation of pro-renin [2]. The third mechanism, proposed for cath-D, is a combination of partial auto-activation and enzyme-assisted activation yielding mature enzyme [3-5].

The aim of the present work was to investigate the *in vivo* processing of human cath-D. We used a mouse cath-D-deficient cell line expressing a human catalytically-inactive D231N-cath-D mutant variant to determine whether processing of cath-D zymogen molecules could take place through already active cath-D and/or other proteases [21]. Here, we have demonstrated that the mechanism of cath-D maturation is independent of its catalytic function and involves a fully-assisted processing similar to that of pro-renin.

There has been much controversy over whether processing to pseudo-cath-D occurs in cells and is a required step for subsequent proteolytic cleavage by cysteine proteases, or pseudo-cath-D is only an *in vitro* artifact [7, 11]. Because aspartic protease family members share a high degree of sequence identity [28, 29], and pepsinogen, the most thoroughly studied aspartic protease, has been shown to undergo activation to pseudo-pepsinogen by an intra-molecular mechanism [30], a similar activation mechanism with pseudo-cath-D as an important intermediate has been proposed for pro-cath-D maturation [4]. However, auto-catalytic removal of the remainder of the pro-peptide has not been demonstrated for cath-D, as it has been for pepsinogen. The inability of pro-cath-D to generate the mature forms of the enzyme by auto-catalysis suggested that either another protease may be required or that autocalytic activation to pseudo-cath-D does not occur *in vivo*. Our work reveals that pseudo-cath-D generated by auto-activation *in vitro* is not a required intermediate for cath-D processing in cells.

Different groups have attempted to elucidate the *in vivo* relevance of auto-activated pseudo-cath-D and the role of cath-D proteolytic activity for its maturation
Human cath-D mutated at its auto-catalytic site in the pro-peptide was not able to process itself to form pseudo-cath-D \textit{in vitro}, but it was normally processed to the mature forms of the enzyme when transfected into mouse cells \cite{7}. This study indicates for the first time that auto-activation of pro-cath-D to the pseudo-form was not required for removal of the whole pro-peptide and suggested that pseudo-cath-D was not a normal intermediate of pro-cath-D processing \textit{in vivo}. However, the possibility remains that the mutant was not cleaved \textit{in vivo} at an alternative site by the endogenous mouse proteases, such as mouse cath-D, despite the \textit{in vitro} confirmation of its inability to activate. On the other hand, it was discovered that pro-cath-D mutated either in its auto-catalytic site in the pro-peptide or in its catalytic domain, resulting in a catalytically inactive enzyme, and expressed in a baculovirus system was cleaved at alternative sites in the pro-sequence by insect cath-D and/or additional and, as yet, unknown proteases \cite{20}. In the present study, we have been able to totally exclude any involvement of active cath-D in the maturation process by the use of a cath-D-deficient cell line expressing a proteolytically-inactive $^{\text{D231N}}$ cath-D. Moreover, we have observed that the cysteine protease inhibitor E-64 caused intracellular accumulation of 52-48 kDa processing intermediates in fibroblasts. Accumulation of large amounts of 50 kDa processing intermediate in fibroblasts treated with E-64 had already been suggested earlier \cite{11}. Our diverse attempts to totally prevent cath-D processing by the concomitant use of E-64 and serine, metallo- or aspartic protease inhibitors were unsuccessful (data not shown). Our data therefore argue in favour of the presence of different cleavage sites in the cath-D pro-part, generating \textit{in vivo} pseudo-cath-D by the action of unidentified proteases, as previously proposed \cite{20}, and not by auto-catalysis.

Previous reports have shown that cysteine and aspartic protease inhibitors can partially prevent the processing of pro-cath-D to 48 kDa intermediate form of the enzyme \cite{10-12} and that the proteolytic cleavage of 48 kDa to 34 kDa was accomplished by cysteine proteases in dense lysosomes, since processing was partially inhibited by leupeptin \cite{14}. By the use of two synthetic cell-permeable cysteine protease inhibitors CLICK-148 and CA-074Met, we proposed the participation of cath-L and cath-B, two lysosomal cysteine proteases widely implicated in carcinogenesis for the processing of intermediate 48 kDa to mature 34 kDa cath-D \cite{31}. It is important to note that Mach and colleagues have questioned the selectivity of CA-074Met and suggested its inhibitory effect on both cath-B and
cath-L [32]. Since we obtained additive inhibitory effects when both inhibitors were used, we argue that cath-B might also be implicated in cath-D processing. Interestingly, accumulation of intermediate cath-D has been detected in the brains of cath-B/-L double knock-out mice [33] as well as in cath-L-deficient A549 cells and in lung tissue extracts of cath-L (-/-) mice [34]. Our results together with the reports using cath-B/-L and cath-L knock-out cells strongly suggest the involvement of cath-L and cath-B in cath-D processing in addition to other still unidentified proteases [32, 33].

As far as the mechanisms of cath-D maturation following its endocytosis are concerned, nothing has so far been described [35]. We have shown that the processing of endocytosed pro-cath-D is also independent of its catalytic function and requires the cysteine proteases, as expected, since the endocytosed cath-D would reach the same compartment as that synthesized inside the cells.

Taken together, our results indicate that pro-cath-D is successively processed, in a manner independent of its catalytic function and auto-activation, into different intermediate processing species by complex mechanisms involving not only the cysteine proteases but also as yet unidentified proteases. However, since we used a heterogenous expression system, we cannot totally exclude the possibility that human cath-D maturation might somehow slightly differ in human cells. Our main conclusion is that cath-D undergoes maturation by a fully-assisted mechanism similar to that of renin maturation.

MATERIALS AND METHODS

Materials.
E-64, CA-074Met, and pepstatin A were purchased from Sigma. Stock solutions of 10 mM CA-074Met and CLICK-148 were prepared in DMSO. DMSO vehicle was used as internal control in the studies with CA-074Met and CLICK-148. A stock solution of 100 mM E-64 was prepared in DMEM.

Cell lines.
CD55/- cath-D-deficient immortalized mouse fibroblasts were kindly provided by Dr Christoph Peters (University of Freiburg, Germany) and Dr Paul Saftig (Christian-Albrechts-University, Germany) [36]. CD55/-cath-D and CD55/-D231N cath-D cell lines were generated by stable transfection of wild-type or D231N mutated human cath-D
into CD55-/− fibroblast cells as described earlier [21]. These cells were cultured in DMEM medium with 10% fetal calf serum (FCS, GibcoBRL).

**Cath-D immunoblotting.**
Cells grown to 80% confluency in DMEM medium supplemented with 10% FCS were either untreated or treated with 10 mM E-64, 50 µM CA-074Met or 5-20 µM CLICK-148. Cells remained viable under these conditions and were lysed after 48 h in lysis buffer (20 mM Tris-Hcl [pH 7.4], 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.5% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, 1 mM vanadate, 10% glycerol, and 100 kallikrein units/ml Trasylol). After gentle shaking for 20 min at 4°C, cell extracts were obtained by centrifugation in a microfuge at 13,000 rpm for 15 min. Equal amounts of protein (100 µg for CD55-/− cath-D cells and 300 µg for CD55-/−D231Ncath-D cells) from cell extracts, quantitated by the Bradford assay, or equal volumes (80 µl) of conditioned media, were separated on a 12 or 15% gel by SDS-PAGE. Proteins were electro-transferred to Hybond-C extra membranes (Amersham Biosciences) and incubated with 1 µg/ml anti-human cath-D monoclonal antibody (#610800, BD Biosciences) that recognizes 52, 48 and 34 KDa cath-D forms. Proteins were then visualized with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (ECL Amersham) followed by the Renaissance chemiluminescence system (Perkin Life Sciences).

**Glycosylation and auto-activation of pro-cath-D.**
To inhibit N-linked glycosylation, cells were grown for 24 h in medium supplemented with 10% FCS containing increasing concentrations of tunicamycin (Sigma; stock solution: 10 mM in DMSO). Cell extracts (100 µg of proteins for CD55-/− cath-D cells and 300 µg for CD55-/−D231Ncath-D cells) were then analysed for cath-D expression by immunoblotting as described above. For digestion of N-linked oligosaccharides, culture medium conditioned for 4 days in the presence of 5% FCS was incubated with 50 µU/ml of endoglycosidase H (Sigma) at 37°C in a 50 mM citrate phosphate buffer [pH 5.4] containing a protease inhibitor cocktail (Boehringer Mannheim). To auto-activate in vitro the secreted pro-cath-D, culture medium conditioned for 48 h with 10% FCS was incubated at 37°C for various times at pH adjusted to 3.5 by the addition of 1 N HCl in the presence or absence of 1 mM pepstatin A. Samples were
then neutralized to pH 7.4 with 1 N NaOH. Conditioned media (80 µl) were then analysed for cath-D expression by immunoblotting as described above.

**Two-dimensional electrophoresis.**
First dimension electrofocusing gels contained 9.5 M urea, 2% ampholines, 2% NP-40 and 5% DTT with pH 3-10. Second dimension gels contained 10% acrylamide, 0.1% bis-acrylamide. Cath-D expression was then analyzed by immunoblotting as described above.

**Endocytosis of secreted pro-cath-D.**
Established 3Y1-Ad12 cancer cells stably transfected either with wild-type or D231N mutated cath-D [37] were labelled with 200 µCi/ml [³⁵S]methionine for 24 h in DMEM without methionine and FCS, and the labelled conditioned culture medium was used directly for internalization studies. Parental CD55-/- fibroblasts plated in 6-well plates were incubated in 1 ml of serum-free DMEM medium supplemented with the conditioned medium corresponding to 3 x 10⁶ cpm of total TCA-precipitable proteins with an excess of non-radioactive methionine (10 mM), in the presence or absence of E-64, CA074-Met or CLICK-148. After 18 h incubation, medium was discarded and cells were washed 3 times, then lysed with 700 µl of extraction buffer (10 mM NaPO₄ [pH 7.4], 10 mM EDTA, 10 mM NaCl, 1% NP-40, 1 µM Trasylol, 1 mM PMSF). Cell extracts were immunoprecipitated with the M1G8 anti-cath-D-antibody and protein A-Sepharose as described previously [17] and analyzed by 12% SDS-PAGE and fluorography.

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REFERENCES


**LEGEND TO FIGURES**

Figure 1. Maturation, glycosylation and activation of human wild-type and D231N mutated human cath-D transfected into cath-D-deficient CD55-/fibroblasts.
(a) Maturation of wild-type and D231N mutated human cath-D. Cells were cultured in DMEM medium with FCS and cell lysates were analyzed by immunoblotting with an antibody specific for human cath-D.

(b) Effects of tunicamycin on cellular wild-type and D231N mutated cath-D glycosylation. Cells were either untreated or treated with increasing concentrations of tunicamycin for 24 h and cell lysates were analysed for cath-D expression by Western blotting.

(c) Effects of endoglycosidase H on the glycosylation of wild-type and D231N secreted pro-cath-D. Conditioned media from CD55-/-cath-D and CD55-/D231Ncath-D cells were incubated for different times with or without endoglycosidase H and media were analysed for cath-D expression by Western blotting.

(d) Auto-activation of wild-type and D231N secreted pro-cath-D. Conditioned media from CD55-/cath-D and CD55-/D231Ncath-D cells were incubated for different times at pH 3.5 in the absence or presence of pepstatin A, and media were analysed for cath-D expression by Western blotting.

Black arrows indicate the migration of the 3 forms of wild-type cath-D; grey arrows indicate the migration of the 3 forms of D231Ncath-D; white arrows indicate the migration of non-glycosylated cellular cath-D. K= molecular mass in kDa.

Figure 2. Effects of cysteine protease inhibitors on the maturation of wild-type and D231N mutated cath-D in transfected fibroblasts.

(a) Effects of E-64 in fibroblasts. CD55-/-cath-D and CD55-/D231Ncath-D cells were either untreated or treated for 48 h with 10 mM E-64 and cellular cath-D was analysed by immunoblotting.

(b) Concentration dependency of the E-64 effects. CD55-/-cath-D cells were either untreated or treated for 48 h with increasing concentrations of E-64 and cellular cath-D was analysed by immunoblotting.

(c) Effects of E-64 using two-dimensional electrophoresis. CD55/- cath-D cells were either untreated or treated for 48 h with 10 mM E-64 and cellular cath-D was analysed by two-dimensional electrophoresis and immunoblotting.

(d) Effects of CA-074Met. CD55-/cath-D and CD55-/D231Ncath-D cells were untreated or treated for 48 h with 50 µM CA-074Met and cellular cath-D was analysed by immunoblotting. The effect of 10 mM E-64 is shown.
(e) Effects of CLICK-148 alone or in combination with CA-074Met. CD55-/cath-D and CD55-/D231Ncath-D cells were either untreated or treated for 48 h with 5 or 20 µM CLICK-148 alone or in combination with 50 µM CA-074Met. Cellular cath-D was analysed by immunoblotting.

Brackets indicate the 52-48 kDa processing intermediates detected in CD55-/cath-D cells and the 51-47 kDa processing intermediates detected in CD55-/D231Ncath-D cells.

**Figure 3. Schematic representation of human cath-D processing.**

Cath-D is synthesized on the RER as a pre-pro-enzyme that undergoes several proteolytic cleavages during biosynthesis to produce the mature form. Following the initial co-translational removal of the signal peptide to yield 52 kDa pro-cath-D, this pro-enzyme is then processed into numerous 52-48 kDa processing intermediates. These intermediates are not generated by auto-activation, but by the action of unknown proteases whose activities are only partially inhibited by the cysteine protease inhibitor E64, but are not affected by the cath-B or cath-L inhibitors. The intermediate 48 kDa single-chain species is then cleaved into a mature two-chain enzyme consisting of a light 14 kDa amino-terminal domain and a heavy 34 kDa carboxyl-terminal domain by the cysteine proteases cath-B or cath-L. Accompanying this conversion to the two-chain species, 7 amino acid residues between the 14 KDa light and 34 kDa heavy chains are removed [15]. Several more amino acids are removed from the carboxyl terminus of the 34 kDa heavy chain [16].

**Figure 4. Processing of endocytosed wild-type and D231N mutated human cath-D.**

(a) Processing of endocytosed human \textsuperscript{D231N}pro-cath-D. Parental cath-D-deficient CD55-/ fibroblasts were incubated for 18 h with \textsuperscript{35}S-labelled conditioned medium containing the secreted wild-type or D231N pro-cath-D. After washing, cell lysates containing endocytosed \textsuperscript{[S\textsuperscript{35}]}methionine-labelled cath-D were analyzed by SDS-PAGE after immunoprecipitation. Immunoprecipitation was performed in triplicate.

(b) Effects of E-64. CD55-/ fibroblasts were incubated for 18 h with \textsuperscript{35}S-labelled conditioned medium containing the wild-type pro-cath-D in the absence or presence
of 10 mM E-64. Endocytosed $[^{35}\text{S}]$methionine-labelled cath-D was analyzed by SDS-PAGE, as described in panel a.

(c) Effects of CA-074Met. CD55-/− fibroblasts were incubated for 18 h with $^{35}\text{S}$-labelled conditioned medium containing pro-cath-D in the absence or presence of 25 µM CA-074Met. Endocytosed $[^{35}\text{S}]$methionine-labelled cath-D was analyzed by SDS-PAGE.

(d) Effects of CLICK-148 alone or in combination with CA-074Met. CD55-/− fibroblasts were incubated for 18 h with the $^{35}\text{S}$-labelled conditioned medium containing pro-cath-D in the absence or presence of 20 µM CLICK-148 alone or in combination with 25 µM CA-074Met. Endocytosed $[^{35}\text{S}]$methionine-labelled cath-D was analyzed by SDS-PAGE.