Involvement of Carboxy-Terminal Amino Acids in Secretion of Human Lysosomal Protease Cathepsin L

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ABSTRACT: Cathepsin L, a lysosomal cysteine protease, is overexpressed and secreted by malignantly transformed cells. However, the reason for secretion of this man 6-phosphate-containing lysosomal protease into the extracellular medium is not clear. We wished to determine whether there is a region within the primary sequence of the proenzyme form of cathepsin L which affects its subcellular and extracellular localization. High-level transient expression of human procathepsin L in mouse NIH 3T3 cells results in the secretion of most of this protein into the extracellular medium. At the same time, the endogenous mouse procathepsin L in these nontransformed cells is found in its usual location in lysosomes. Mutants of human procathepsin L with carboxy-terminus deletions involving the last 11 amino acids are not secreted into the medium. Deletion of as little as two amino acids, Thr and Val, from the carboxy terminus, blocked the secretion of the protein but did not affect its enzyme activity, posttranslational processing, or subcellular distribution. Replacement of Thr-Val by two bulky amino acids Tyr-Asn allowed secretion of the procathepsin L, but the replacement of these two amino acids by nonbulky alanines prevented its secretion. Single alanine substitutions of the last six amino acids (ASYPTV) indicated that substitution by alanine of Y or T does not affect the secretion of hproCAT L, but alanine substitutions of S, P, or V completely blocked its secretion into the culture medium. We therefore conclude that the carboxy terminus of procathepsin L contains a sequence essential for its secretion.

Cathepsin L is a lysosomal cysteine protease expressed in a wide variety of tissues. The primary function of cathepsin L in normal cells is thought to be intracellular protein degradation and turnover (*1*). However, secreted cathepsin L has also been implicated in antigen presentation (*2*), sperm maturation (*3*), bone resorption (*4*, *5*), renal pathology (*6*, *7*), and tumor invasion and metastasis (*8*−*10*). Elevated expression of cathepsin L has been reported in a variety of human tumors (*11*−*13*). Cathepsin L (CAT L) is synthesized as a pre-proenzyme and processed in human cells to a 42 kDa proenzyme (hproCAT L) which is targeted to the lysosomes by the man 6-phosphate recognition marker (*14*, *15*). In lysosomes, it is further processed to lower molecular weight mature forms (34 and 26 kDa in the human) (*16*, *17*). In malignantly transformed cells, proCAT L is synthesized at elevated levels and is secreted into the medium. Overexpression of proCAT L is sufficient to result in secretion (*18*). Human cathepsin L is secreted in its 42 kDa unprocessed form which carries man 6-phosphate and should be targeted to the lysosomes.

We were interested in exploring if there was a protein determinant in hproCAT L that plays a role in its secretion into the medium. Protein sorting signals have been found to direct sorting into the regulated secretory pathway (*19*), sorting into vacuoles and lysosomes (*20*−*22*), exit from the endoplasmic reticulum (*23*), and retention in the Golgi (*24*).

To look for a sorting signal in hproCAT L, we expressed recombinant hproCAT L at high-levels using the transient expression system described by Labow et al. (*25*). As previously shown after stable transfection, expression of wild-type hproCAT L in NIH 3T3 cells resulted in the secretion of the majority of the protease into the medium as occurs in transformed cells. By expressing a series of deletion mutants, we found that an intact carboxy terminus of hproCAT L is essential for its secretion into the medium.

EXPERIMENTAL PROCEDURES

Cells. NIH 3T3 cells obtained from C. Scher, University of Pennsylvania, were grown in Dulbecco's minimal essential medium (DMEM) containing 10% calf serum (Colorado Serum Company, Denver, CO), glutamine (0.5 mg/mL), penicillin (50 U/mL) and streptomycin (50 U/mL). KB-3-1 cells were obtained from American Type Culture Collection and subcloned and grown in the laboratory as described earlier (*26*).

Plasmids. pLAP and pL14 CAT plasmids were gifts from Arnold J. Levine, Department of Biology, Princeton University, Princeton, NJ. pLAP encodes a chimeric lac

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repressor protein in which the lac repressor coding region includes a nuclear localization signal from SV40 large T antigen and a transcription activation domain from Herpes simplex virus type 1 virion protein 16. The gene for this chimeric protein, LAP (lac activator protein) is cloned under the control of human β-actin promoter. The pL14 CAT plasmid contains a chloramphenicol acetyl transferase gene under the control of the SV40 early promoter. Upstream to the promoter are 14 repeats of the lac operator sequences separated from each other by small distances. Wild-type or mutant procathepsin L cDNA containing an 85 bp synthetic SV40 polyadenylation signal was cloned in place of the CAT gene in pL14 CAT in the *Stu*I−*Bam*HI site.

Mutagenesis. All deletion or replacement mutants were constructed using the polymerase chain reaction (PCR). A 5′ 36 bp oligonucleotide primer from 454 to 486 bp in the cDNA (containing 3 bp extra introduced to generate an *Xho*I site) was used as a common sense primer for generating all carboxy-terminal deletion mutants. The 3′ antisense oligonucleotides varied with each mutant and are given in Table 1. This primer contained the desired deletions/replacements and an *Nsi*I site downstream from the translation termination codon. After PCR, the amplified fragment was gel purified and digested with *Bgl*II (650 bp in the cDNA) and *Nsi*I (1349 bp in the cDNA). For constructing the mutants, this *Bgl*II−*Nsi*I fragment was ligated in place of the corresponding wild-type fragment in the expression vector. Various deletion or replacement mutants used in the present study are given in Tables 2 and 3, respectively. All reagents for PCR were purchased from Perkin-Elmer Cetus and used according to the manufacturer's directions. A typical PCR reaction contained 50 ng of plasmid DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% Triton × 100, 0.2 mM of each dNTP, 2.0 units of Taq DNA polymerase, and 100 pmol of each primer in a final volume of 100 μL. Denaturation, annealing, and extension were performed in a Perkin-Elmer Cetus thermal cycler (Cetus Corporation) initially at 94 °C for 1 min, 42 °C for 2 min, and 72 °C for 3 min, respectively. The temperature of the annealing was varied empirically to maximize the yield of the products. Oligonucleotides were synthesized by using an Applied Biosystem 380B DNA synthesizer. All the mutant plasmids were sequenced by the dideoxy chain termination method (*27*) using a Genesis-2000 automated sequencer (Du Pont) to confirm their mutations.

Cell Transfections. All transfections were done by the calcium phosphate coprecipitation method described by Gorman et al. (*28*). NIH 3T3 cells (3.5×10^5) were plated in 10 cm tissue culture dishes 1 day before transfection. The cells were fed with fresh medium 2 h before transfection. Ten micrograms of the desired plasmid DNA was coprecipitated with the calcium phosphate with constant mixing by an air stream. The precipitate was allowed to stand at room temperature for 20 min and then gently dropped on the cells evenly. The cells were incubated overnight in a CO_2 incubator and the next morning washed three times with serum-free medium and fed with fresh medium. Thirty-six to forty-eight hours after the incubation, the cells were either harvested or metabolically labeled and processed for immunoprecipitation.

Expression of Lac Activator Protein (LAP) in Mouse Fibroblast Cells. NIH 3T3 cells were cotransfected with pLAP and pSV2 Neo, and G418 resistant colonies were picked individually and grown in medium containing G418. Individual clones were transiently transfected with pL14 CAT, and CAT activity was used as a measure of LAP expression. The CAT activity (*29*) in one of the clones was observed to be at least 50−60-fold higher than that in NIH 3T3 cells. This clone was named NIH LAP and used for all subsequent hproCAT L expression experiments.

Cell Labeling and Immunoprecipitation. The transfected cells were incubated with 100 μCi/mL (^{35}S) translabel (1187 Ci/mmol ICN) in 4 mL of cysteine- and methionine-free DMEM medium (NIH Media Unit), 48 h after the transfection, for 3−5 h. The medium was saved, and cell monolayers were washed twice with PBS and lysed in SDS buffer A (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, and 0.05% SDS). The lysate was centrifuged (for 10 min at 14000*g* at 4 °C) to remove cell debris, and the supernatant was designated cell extract. Cell extract (0.25 mL, 10^6 cpm) or 1.0 mL (5×10^5 cpm) of the medium was used in immunoprecipitations, performed with rabbit antihuman proCAT L polyclonal antibody (hproCAT L antibody) as described by Smith and Gottesman (*30*). Immunoprecipitates were run on 10% or 12% polyacrylamide gels (*31*). Gels were incubated at room temperature in acetic acid containing 3% PPO. After 30 min, the gels were rinsed three times with water, dried, and exposed to X-Omat AR film (Kodak) at −70 °.

Pulse-Chase Analysis. For pulse-chase labeling, 2×10^6 cells were plated in 100 mm tissue culture dishes. The next day, the cells were washed twice with methionine-free, cysteine-free medium containing 2 mM glutamine and preincubated for 2 h in the same medium before labeling for 30 min in the presence of (^{35}S) translabel (200 μCi/mL). After that the label was removed, and cells from one dish were washed twice with ice-cold normal saline and lysed by SDS buffer A to prepare cell extracts. The cells of the remaining dishes were washed and fed with regular medium. After 15, 30, 60, 120, and 240 min, the culture media were collected. The cells were washed with ice-cold PBS and lysed. Immunoprecipitations were performed on each cell extract and medium sample, using volumes the same as those containing 2×10^6 TCA precipitable counts of protein in the 30 min pulse cell extract and equivalent volumes of medium. Immunoprecipitates were subjected to SDS−PAGE and autoradiography. Quantitation of the 42 kDa hproCAT L band was performed by a PhosphorImager 425 (Molecular Dynamics, Sunnyvale, CA).

Cell Fractionation on Percoll Gradients. NIH LAP cells stably expressing wild-type or hproCAT L ΔTV were grown to 95% confluence in 150 mm dishes. The cell monolayers (1×10^7 cells) were washed twice with ice-cold phosphate-buffered saline, scraped and resuspended in 4.0 mL of 0.25 M sucrose solution, and processed for cell disruption and fractionation on Percoll gradients as described by Roff et al. (*32*). One milliliter fractions were collected from the bottom of the gradients using a Beckman Universal Fraction Recovery System (Beckman Instruments, Inc., Palo Alto, CA). These fractions were assayed for the activity of lysosomal and Golgi marker enzymes. The lysosomal marker enzyme β-hexosaminidase was assayed using umbelliferyl substrate by the procedure of Rome et al. (*33*).

Table 1: Oligonucleotides Used to Construct the Mutants of Human Cathepsin L

Sense oligonucleotide (SSC-47): Common oligonucleotide used for all mutants

XhoI

ATG AAG ATG AAT CTC GAG CTG CAC AAT CAG GAA TAC

Antisense oligonucleotides: specific for mutants, all contain Nsi I site (ATG CAT) downstream (upstream in antisense oligonucleotide) to translation termination codon TGA (TCA in antisense oligonucleotide)

1. hproCAT L Δ C16 (SSC-41):

NsiI

5' TGG TTT CTC CGG ATG CAT TCA TCT TTG GCC ATC 3'

2. hproCAT L Δ C12 (SSC-52A):

5' GGC AAT TCC ACA ATG CAT TCA ATG GTT TCT CCG GTC 3'

3. hproCAT L-- last 4 amino acids replaced by 26 amino acids (SSC-54):

5' TCA CAC AGT GGG GTA TAC GTA AGT GCT GGC TGC TGA GGC 3'

4. hproCAT L Δ C8 (SSC-73):

5' GCT GGC TGC TGA ATG CAT TCA GGC AAT TCC ACA ATG GTT TCT CCG 3'

5. hproCAT L Δ C4 (SSC-74):

5' CAC AGT GGG GTA ATG CAT TCA GCT GGG TGC TGA GGC AAT TCC 3'

6. hproCAT L Δ TV (SSC-68):

5' CCG TCC ACC AGC CAT TCA GGG GTA GCT GGC TGC 3'

7. hproCAT L Δ YP (SSC-69):

5' CCG TCC ACC AGC ATG CAT TCA CAC AGT GCT GGC TGC TGA GGC 3'

8. hproCAT L Δ AS (SSC-70):

5' CCG TCC ACC AGC ATG CAT TCA CAC AGT GGG GTA TGC TGA GGC AAT TCC 3'

9. hproCAT L Δ SA (SSC-88):

5' CCG TCC ACC AGC ATG CAT TCA CAC AGT GGG GTA GCT GGC GGC AAT TCC ACA ATG 3'

10. hproCAT L Δ IA (SSC-90):

5' CCG TCC ACC AGC ATG CAT TCA CAC AGT GGG GTA GCT GGC TGC TGA TCC ACA ATG GTT TCT C 3'

11. hproCAT L Δ GI (SSC-91):

5' CCG TCC ACC AGC ATG CAT TCA CAC AGT GGG GTA GCT GGC TGC TGA GGC ACA ATG GTT TCT CCG 3'

12. hproCAT L Δ V (SSC-87):

5' CCG TCC ACC AGC ATG CAT TCA AGT GGG GTA GCT GGC 3'

13. hproCAT L ΔT (SSC-89):

5' CCG TCC ACC AGC ATG CAT TCA CAC GGG GTA GCT GGC TGC 3'

14. hproCAT L TV → VT (SSC-92):

5' CCG TCC ACC AGC ATG CAT TCA AGT CAC GGG GTA GCT GGC TGC TGA GGC 3'

15. hproCAT L TV → YN (SSC-93):

5' CCG TCC ACC AGC ATG CAT TCA GTT GTA GGG GTA GCT GGC TGC TGA GGC 3'

16. hproCAT L TV → AA (SSC-104):

5' CCG TCC ACC AGC ATG CAT TCA TGC GGC GGG GTA GCT GGC TGC 3'

17. hproCAT L YP → AA (SSC-105):

5' CCG TCC ACC AGC ATG CAT TCA CAC AGT TGC GGC GCT GGC TGC TGA GGC3'

18. hproCAT L AS → AA (SSC-106):

5' CCG TCC ACC AGC ATG CAT TCA CAC AGT GGG GTA TGC GGC TGC TGA GGC AAT TCC 3'

19. hproCATL SA → AA (SSC-109):

5' CCG TCC ACC AGC ATG CAT TCA CAC AGT GGG GTA GCT GGC TGC TGC GGC AAT TCC ACA ATG 3'

20. hproCAT L V → A (SSC-110):

5' CCG TCC ACC AGC ATG CAT TCA CGC AGT GGG GTA G 3'

21. hproCAT L T → A (SCC-111):

5' CCG TCC ACC AGC ATG CAT TCA CAC AGC GGG GTA GCT GGC 3'

22. hproCAT L P → A (SSC-112):

5' CCG TCC ACC AGC ATG CAT TCA CAC AGT GGC GTA GCT GGC TGC 3'

23. hproCAT L Y → A (SSC-113):

5' CCG TCC ACC AGC ATG CAT TCA CAC AGT GGG GGC GCT GGC TGC TGA 3'

Table 2: Deletion Mutants of Human Cathepsin L

	sequence of carboxyl terminus		designation	result
1.	M A K D R R N H C G I	A S A A S Y P T V	Wild-type	Secreted
2.	M A K D		Δ C16	ER
3.	M A K D R R N H		Δ C12	ER
4.	M A K D R R N H C G I	A	Δ C8	Not secreted
5.	M A K D R R N H C G I	A S A A S	Δ C4	Not secreted
6.	M A K D R R N H C G I	A S A A S Y P	Δ TV	Not secreted
7.	M A K D R R N H C G I	A S A A S T V	Δ YP	Not secreted
8.	M A K D R R N H C G I	A S A Y P T V	Δ AS	Not secreted
9.	M A K D R R N H C G I	A A S Y P T V	Δ SA	Not secreted
10.	M A K D R R N H C G S	A A S Y P T V	Δ IA	Not detected
11.	M A K D R R N H C A S	A A S Y P T V	Δ GI	Not detected
12.	M A K D R R N H C G I	A S A A S Y P V	Δ T	Secreted
13.	M A K D R R N H C G I	A S A A S Y P T	Δ V	Secreted

Table 3: Replacement Mutants of Human Cathepsin L[a]

	sequence of carboxy terminus	result
1.	M A K D R R N H C G I A S A A S Y P T V	Secreted
2.	M A K D R R N H C G I A S A A S **T Y C M G G I H L Q S T S P R C V G Y T L E**	Not secreted
3.	M A K D R R N H C G I A S A A S Y P **V T**	Secreted
4.	M A K D R R N H C G I A S A A S Y P **Y N**	Secreted
5.	M A K D R R N H C G I A S A A S Y P **A A**	Not secreted
6.	M A K D R R N H C G I A S A A S **A A** T V	Not secreted
7.	M A K D R R N H C G I A S A A **A** Y P T V	Not secreted
8.	M A K D R R N H C G I A **A** A A S Y P T V	Secreted
9.	M A K D R R N H C G I A S A A S Y P T **A**	Not secreted
10.	M A K D R R N H C G I A S A A S Y P **A** V	Secreted
11.	M A K D R R N H C G I A S A A S Y **A** T V	Not secreted
12.	M A K D R R N H C G I A S A A S **A** P T V	Secreted

[a] The replaced amino acids are shown in bold-faced type.

Galactosyl transferase activity was measured by the procedure described by Roff et al. (*32*) with the only modification that trypsin inhibitor type III-O was used as an acceptor. A 100 μL aliquot from each fraction was taken and vortexed with 45 μL of 1 M Tris-HCl pH 6.8, 6.6% SDS, and incubated for 15 min at room temperature followed by centrifugation at 30000*g* for 15 min at 4 °C in a Beckman TL-100 ultracentrifuge. A 60 μL aliquot from the supernatant was subjected to SDS−PAGE and immunoblotting using antiserum to hproCAT L to detect mutant or wild-type hproCAT L in the Percoll fractions, as described by Gal et al. (*17*).

Estimation of Cathepsin L Activity. Cells (1×10^6) stably expressing hproCAT L or hproCAT L ΔTV were washed twice with ice-cold PBS and lysed by SDS−buffer A. The lysate was centrifuged (14000*g*) for 10 min at 4 °C in a microfuge to remove the cell debris. An aliquot of supernatant containing 6 mg of protein was subjected to immunoprecipitation and enzyme assay using Z-Phe-Arg-NH Mec (Sigma) as substrate as described earlier (*34*).

Immunocytochemistry. Cells were fixed in situ using 3.7% formaldehyde in PBS for 15 min at 23 °C. They were then incubated sequentially at 23 °C in the continuous presence of 0.1% saponin, 4 mg/mL normal goat globulin, in PBS (*1*) rabbit antihuman proCAT L (1:200 serum) for 30 min and (*2*) affinity purified goat antirabbit IgG conjugated to rhodamine (25 μg/mL) (Jackson Immunoresearch) for 30 min. The cells were then postfixed in formaldehyde and mounted in glycerol.

RESULTS

Overexpression of hproCAT L in NIH LAP Cells Results in Its Secretion into the Culture Medium. As described in Experimental Procedures, we created an NIH 3T3 cell line carrying the pLAP vector, as determined by its ability to support high-level expression of pL14 CAT (data not shown). This cell line was named NIH LAP. To express hproCAT L, the CAT gene in the pL14 CAT vector was replaced by hproCAT L cDNA and transiently transfected into NIH LAP cells. Transfected NIH LAP cells were metabolically labeled with ^{35}S-met and subjected to immunoprecipitation with anti-hproCAT L antibody followed by fluorography (Figure 1). Simultaneously, the same number of KB-3-1 cells (human epidermoid carcinoma cells that express moderate amounts of endogenous hproCAT L) were also labeled and processed similarly. In NIH LAP cells transiently transfected with pL14 hproCAT L, hproCAT L polyclonal antibody immunoprecipitated 42 and 34 kDa proteins (Figure 1, lane 3). These two proteins were also detected in KB-3-1 cells by hproCAT L antibody but in much smaller quantities (Figure 1, lane 1). Our laboratory has previously reported that hproCAT L is synthesized as a 42 kDa proenzyme form and subsequently processed into 34 and 26 kDa processed forms (*16*). None of these proteins could be detected by hproCAT L antibody in mock transfected NIH LAP cells (lane 2) or by preimmune serum in NIH LAP cells transfected with pL14 hproCAT L (not shown). The level of hproCAT L transient expression in NIH LAP cells was at least 10-fold higher on average than in KB cells (Figure 1, lanes 3 and 1, respectively); hproCAT L expression in NIH LAP cells did not affect the synthesis or processing of endogenous mouse proCAT L (Figure 1, lanes 4 and 5). As for KB-3-1 cells, the majority of the hproCAT L expressed in NIH LAP cells was secreted into the medium (Figure 1, lanes 7 and 9), as was the endogenous mouse pro CAT L (Figure 1, lanes 10, 11).

We compared the distribution of hproCAT L in NIH LAP cells stably expressing the hproCAT L cDNA and in KB-3-1 cells by immunocytochemical studies, and the results are given in Figure 2. No hproCAT L was detected in mock-transfected NIH LAP cells (Figure 2A). NIH LAP cells stably transfected with pL14 hproCATL displayed high-level expression of hproCAT L (Figure 2C).

The immunofluorescence pattern seen in KB cells was consistent with a predominant Golgi distribution; that seen for NIH LAP cells stably expressing hproCAT L was mainly lysosomal (Figure 2B,C). KNIH cells (NIH cells malignantly transformed with Kirsten sarcoma virus) have been reported to display an immunofluorescence pattern for mouse CAT L which is similar to the hproCAT L immunofluorescence pattern in KB cells (human epidermoid carcinoma cells). In contrast, the immunofluorescence pattern for mouse CAT L in nontransformed NIH cells and hproCAT L in NIH LAP cells was similar (*17*). Therefore, the Golgi immunofluorescence pattern for cathepsin L may be a characteristic of the transformed phenotype. Lysosomal distribution of hproCAT L in NIH LAP cells is consistent with the results of the cell fractionation experiment described later (Figure 8).

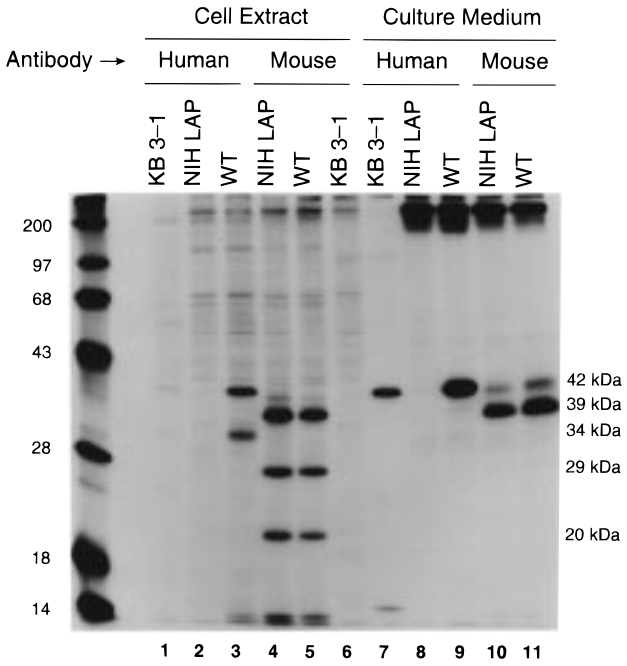

FIGURE 1: Expression of cathepsin L in NIH LAP cells. The CAT gene from pL14 CAT was replaced with human cathepsin L cDNA containing 85 bp synthetic SV40 polyadenylation sequences. The construct was transfected into NIH LAP cells. After 48 h of transfection, the cells were incubated in methionine-free medium for 2 h, followed by labeling with ^{35}S methionine in fresh methionine-free medium for 4 h. At the end of the labeling period, medium was removed, and the cells were washed 3× with PBS and lysed. The cell lysate was spun down to remove the debris. Similarly, KB3-1 and mock transfected NIH LAP cells were labeled with ^{35}S methionine and processed for the preparation of detergent-solubilized cell extracts. Radiolabeled cathepsin L in detergent-solubilized cell extracts (Cell Extract) and culture media was immunoprecipitated using rabbit antihuman procathepsin L (human antibody) or rabbit antimouse procathepsin L (mouse antibody) antiserum as indicated in the figure. Immunoprecipitates were run on SDS−polyacrylamide gels and subjected to autoradiography: KB3-1, human epidermoid carcinoma cells (a Hela variant expressing moderate levels of endogenous hproCAT L) were used as positive controls; NIH LAP, mock transfected NIH LAP cells; WT, NIH LAP cells transiently transfected with pL14 hproCAT L vector. These cells expressed wild-type hproCAT L at a high level.

Some Carboxy-Terminal Amino Acid Deletions and Substitutions of hproCAT L Block Their Secretion into the Medium. The secreted form of cathepsin L is always unprocessed and is identical to intracellular proCAT L which gets delivered to lysosomes. This suggests either that secretion is via a default pathway for protein which somehow escapes binding to the man 6-phosphate receptor or that there is information in the protein which facilitates its secretion. To test this latter hypothesis, we expressed many deletion mutants transiently in NIH LAP cells and found that carboxy-terminal deletion mutants affected hproCAT L secretion. Tables 2 and 3 describe the various hproCAT L carboxy-terminal deletion and substitution mutants which were tested.

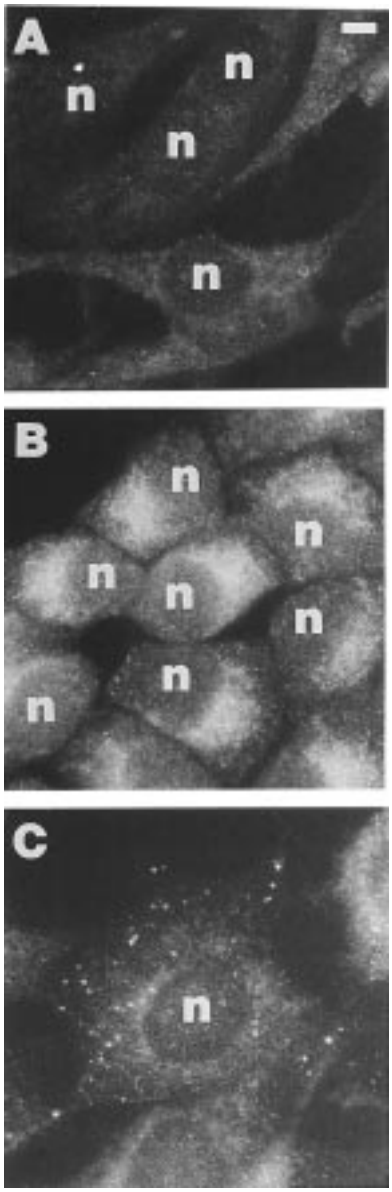

FIGURE 2: Immunohistochemical localization of hproCAT L in KB cells and NIH LAP cells stably expressing wild-type hproCAT L. NIH LAP cells were cotransfected with pL14 hproCAT L with pHyg (gene encoding a hygromycin phosphotransferase B gene cloned under the control of the HSV promoter) in a ratio of 9:1. The transfectants were selected by growth in the presence of G418 (750 μg/mL) and hygromycin B (200 μg/mL). Individual clones were picked, grown, and screened for the expression of hproCAT L. Cells stably expressing hproCAT L were processed for immunocytochemistry as described in the Experimental Procedures: A, NIH LAP cells; B, KB-3-1 cells; C, NIH LAP cells stably expressing hproCAT L; n, nucleus.

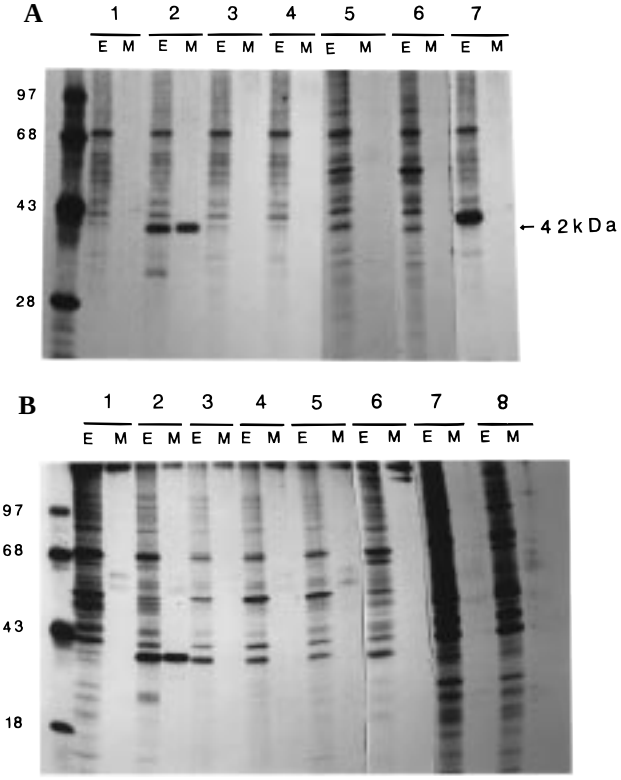

FIGURE 3: Expression of carboxy-terminal deletion mutants in NIH LAP cells. NIH LAP cells (3.5×10^5) were transfected with vectors encoding various deletion mutants of hproCAT L and labeled with ^{35}S methionine for 3 h in methionine-free media. Radiolabeled cathepsin L in cell extracts and media was immunoprecipitated: E = cell extract; M = medium. (Panel A) Lane 1, mock transfected; lane 2, wild-type human cathepsin L (hproCAT L); lane 3, hproCAT L Δ318−333 amino acids (aa) (Table 2, line 2); lane 4, hproCAT L Δ322−333 aa (Table 2, line 3); lane 5, hproCAT L Δ326−333 aa (Table 2, line 4); lane 6, hproCAT L Δ330−333 aa (Table 2, line 5); lane 7, hproCAT L 330−333 aa replaced by 26 nonspecific aa (Table 3, line 2). This figure is a composite of two experiments: lanes 1−4 and 7 have been taken from one experiment and lanes 5 and 6 from another experiment. Additional bands of sizes between 43 and 68 kDa, visible in lanes 5 and 6, were also immunoprecipitated from the radiolabeled cell extracts of mock transfected cells of the corresponding experiment; therefore, they are nonspecific. (Panel B) Lane 1, mock transfected; lane 2, hproCAT L; lane 3, hproCAT L ΔTV (Table 2, line 6); lane 4, hproCAT L ΔYP (Table 2, line 7); lane 5, hproCAT L ΔAS (Table 2, line 8); lane 6, hproCAT L ΔSA (Table 2, line 9); lane 7, hproCAT LΔ IA (Table 2, line 10); lane 8, hproCAT L ΔGI (Table 2 line 11); Δ = deleted.

Mutants lacking 16 or 12 carboxy-terminal amino acids were not detected in cell extracts or cell culture medium by immunoprecipitation (Table 2, lines 2 and 3; Figure 3A, lanes 3 and 4). However, immunocytochemical analysis of cells expressing these mutants exhibited very intense immunofluorescence consistent with the ER pattern suggesting the retention of these mutants in the ER (data not shown). It is known that Cys322 and Cys269 form a disulfide bridge which is essential for enzyme activity (*30*, *35*). Our results suggest that deletion of carboxy-terminal amino acids including the Cys322 residue results in the retention of the protease in the endoplasmic reticulum, presumably because of its improper folding. Shortening the carboxy terminus by eight or four amino acids (Table 2, lines 4 and 5) resulted in mutant proteins which were found only in the cell extract, but not in the medium, suggesting the involvement of the carboxy terminal amino acids of hproCAT L in its trafficking (Figure 3A, lanes 5 and 6). To determine whether simply shortening the carboxy terminus was responsible for the decreased secretion, we also constructed a mutant of hproCAT L in which the last four amino acids were replaced by a stretch of 26 random amino acids generated by a read-through into the untranslated region of hproCAT L (Table 3, line 2). Replacement of the last four amino acids by these 26 random amino acids resulted in the retention of this higher molecular weight protease in the cell in amounts approximately equal to the sum of wild-type protease in cell and culture medium (Figure 3A, lane 7). In this experiment, the addition of two cysteines and a methionine residue in the extended stretch

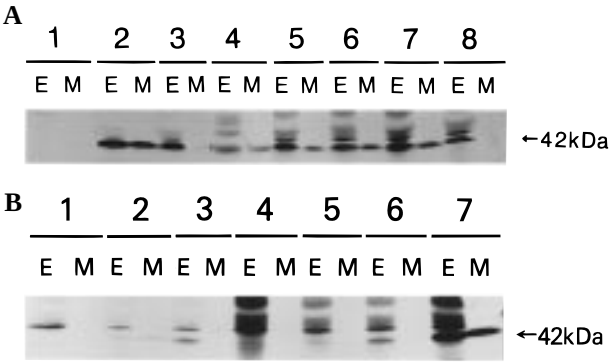

FIGURE 4: Expression of hproCAT L replacement mutants in NIH LAP cells. The carboxy-terminal amino acids of hproCAT L were deleted or replaced by the amino acids given below. NIH LAP cells were transfected with vectors encoding various mutants of hproCAT L, radiolabeled with ^{35}S methionine for 3 h, and processed for immunoprecipitation and autoradiography: E-cell extract; M-culture medium. (Panel A) Lane 1, mock transfected; lane 2, hproCAT L (Table 2, line 1); lane 3, hproCAT L ΔTV (Table 2, line 6); lane 4, hproCAT L ΔT (Table 2, line 12); lane 5, hproCAT L ΔV (Table 2, line 13); lane 6, hproCAT L TV-VT (Table 3, line 3); lane 7, hproCAT L TV-YN (Table 3, line 4); lane 8, hproCAT L TV-AA (Table 3, line 5). (Panel B) Lane 1, mock transfected; lane 2, hproCAT L (Table 3, line 1); lane 3, hproCAT L ΔTV (Table 2, line 6); lane 4, hproCAT L TV-AA (Table 3, line 5); lane 5, hproCAT L YP-AA (Table 3, line 6); lane 6, hproCAT L AS-AA (Table 3, line 7); lane 7, hproCAT L SA-AA (Table 3, line 8).

of amino acids may be partly responsible for the apparent increased level of the modified hproCAT L (Table 3, line 2; Figure 3A, lane 7).

To find out whether shorter deletions of the carboxy tail of hproCAT L would block its secretion into the medium, we sequentially deleted the last 11 amino acids, 2 amino acids at a time, and transiently expressed these proteins in NIH LAP cells (Table 2, lines 6−11). The results are shown in Figure 3B. Only 11 amino acids were analyzed because the 12th amino acid from the carboxy terminus is the cysteine essential for proper folding and enzyme activity. Deletion of any two contiguous amino acids of the last eight amino acids in the pairs TV, YP, AS, and SA resulted in the retention of hproCAT L in the cell (Figure 3B, lanes 3−6). However, even after several days longer exposure, no hproCAT L could be detected by fluorography of immunoprecipitates from cell extracts or medium when any two contiguous amino acids from the remaining three amino acids (GI or IA) were deleted (Figure 3B, lanes 7 and 8), suggesting that these three amino acids following Cys322 are needed to fold hproCAT L properly so as to obtain stable protein.

While deletion of the two amino acids Thr-Val at the carboxy terminus resulted in retention of mutant hproCAT L in the cell, deletion of either amino acid alone (Thr or Val) (Table 2, lines 12 and 13) slightly reduced the level of protease secretion into the medium (Figure 4A, lanes 4 and 5). Similarly, mutants in which their order (ThrVal → ValThr) was reversed (Table 3, line 3) or replaced by other bulky amino acids (ThrVal → TyrAsn) (Table 3, line 4) (*36*) were secreted into the medium albeit in marginally less amounts (Figure 4A, lanes 6 and 7). However, when these two bulky amino acids (Thr-Val) were substituted by alanine residues (Table 3, line 5), secretion of the hproCAT L was again blocked, and the mutant protease was only detected in the cell extract and not in the medium (Figure 4A, lane 8).

Sequential replacement of several different amino acids in pairs of two upstream to Thr-Val (Table 3, lines 6−8) by alanines also blocked the secretion of hproCAT L (Figure 4B, lanes 5 and 6). However, a Ser → Ala substitution at the Ser 8 amino acids from the carboxy terminus had no effect on the secretion of hproCAT L (Figure 4B, lane 7). These results suggest that carboxy-terminal amino acids ASYPTV play an important role in some aspect of hproCAT L secretion. To delineate the role of each one of these amino acids in the secretion of this protease, we replaced the last four amino acids one at a time by an alanine, and the results are given in Table 3, lines 9−12. Replacement of tyrosine or threonine by alanine does not affect the secretion of hproCAT L, whereas the replacement of Ser, Pro, and Val at the carboxy terminus blocked secretion of hproCAT L (Table 3, lines 9−12).

Characterization of hproCAT L ΔTV. Since deletion of only the two most carboxy-terminal amino acids blocked the secretion of hproCAT L into the medium, we focused on this simple mutant for further characterization. To confirm that this block in the secretion of hproCAT L resulting from deletion of Thr-Val was a cis-acting mutation only, we stably expressed either hproCAT L ΔTV or wild-type hproCAT L in NIH LAP cells. The cells expressing either protein exhibited normal secretion of endogenous mouse procathepsin L into the medium (data not shown), indicating that the cellular secretory pathway was intact. To characterize hproCAT L ΔTV further, we analyzed the posttranslational modifications, enzyme activity, and cellular distribution of mutant or wild-type hproCAT L. NIH LAP cells and KB-3-1 cells were used as negative and positive controls, respectively.

The results of a pulse-chase analysis revealed that the kinetics of intracellular synthesis and processing of mutant and wild-type hproCAT L are similar (Figure 5A). However, radiolabeled hproCAT L decays with a half-life of 100 minutes, whereas hproCAT L ΔTV has a longer half-life (130 min). The 34 kDa intracellular processed form of the proteases was detected in both cases (data not shown). By using the Phosphor Imager, we could detect hproCAT L in the culture media after 30 min of chase, and its quantity increased with the passage of time (Figure 5B). However, hproCAT L ΔTV was not detectable in the medium at any time during the chase. These results argue against the possibility of secretion and rapid degradation of hproCAT L ΔTV.

Glycosylation of hproCAT L ΔTV. To study the glycosylation of wild-type and mutant hproCAT L, we labeled stable cell lines with ^{35}S-methionine for 5 h in the presence or absence of tunicamycin and immunoprecipitated hproCAT L from cell extracts or cell culture medium. We detected the 42 kDa hproCAT L band in both cell extracts and medium of KB-3-1 cells and NIH LAP cells expressing wild-type hproCAT L (Figure 6, lanes 2 and 3). The size of this band was reduced by approximately 2 kDa when the cells were labeled in the presence of tunicamycin (Figure 6, lanes 2 and 3), and a higher percentage of hproCAT L was secreted in the presence of tunicamycin because the lysosomal recognition marker, man 6-phosphate, could not be added. A similar difference in the size of hproCAT L ΔTV was

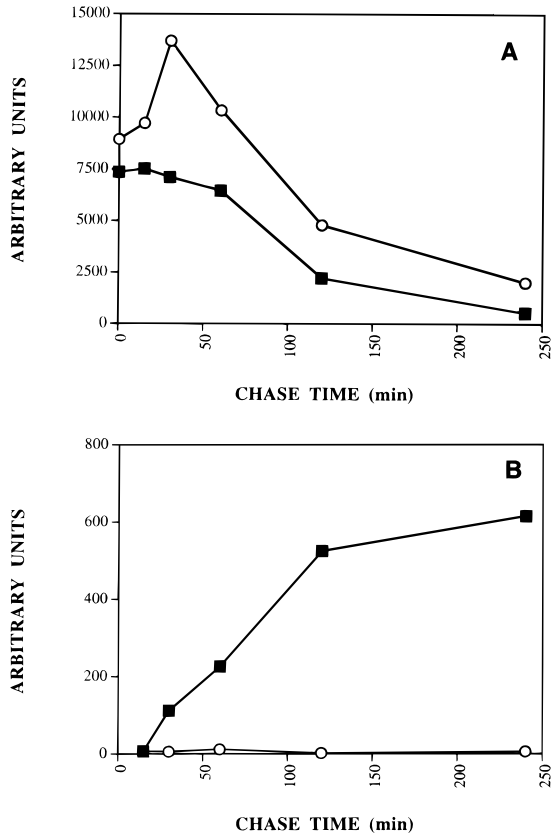

FIGURE 5: Kinetics of hproCAT L and hproCAT L ΔTV turnover and secretion into the medium. Cells (4×10^6) stably expressing hproCAT L or hproCAT L ΔTV were labeled with ^{35}S methionine in methionine-free medium. After 30 min the cells were washed and incubated in regular medium. At the indicated time intervals, the culture medium was saved and cell extracts were prepared. Immunoprecipitations were performed on each cell extract and medium as described in Experimental Procedures. The immunoprecipitates were subjected to SDS−PAGE and the gel was dried without enhancing and exposed to the storage phosphor screen overnight. The levels of unprocessed forms (42 kDa) of hproCAT L and hproCAT LΔTV in cell extract (A) and media (B) were quantitated as described in Experimental Procedures and plotted against the chase time: ■, hproCAT L; ○, hproCAT LΔTV.

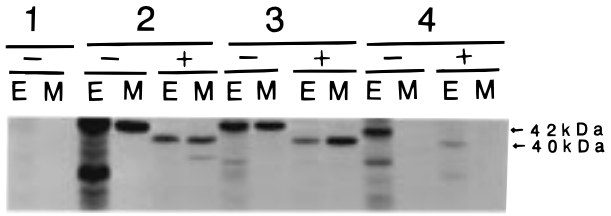

FIGURE 6: Glycosylation of hproCAT L and hproCAT L ΔTV. NIH LAP cells (1×10^6) stably expressing hproCAT L or hproCAT L ΔTV were labeled for 5 h with ^{35}S-methionine in the presence or absence of 15 μg/mL tunicamycin. The cells were washed with PBS and lysed with SDS buffer A. Human cathepsin L was immunoprecipitated from cell extracts and media. The immunoprecipitates were subjected to autoradiography after SDS−PAGE. Cathepsin L immunoprecipitated from KB cells was used as a positive control: E, cell extract; M, medium; -, cells labeled in the absence of tunicamycin; +, cells labeled in the presence of 15 μg/mL tunicamycin; lane 1, NIH LAP cells; lane 2, KB cells; lane 3, hproCAT L; lane 4, hproCAT L ΔTV.

observed in the absence and presence of tunicamycin in cell extracts (Figure 6, lane 4). However, we could not detect any hproCAT L in the media of cells expressing hproCAT L ΔTV in the presence or absence of tunicamycin (Figure 6, lane 4), suggesting that the secretory pathway was blocked for hproCAT L ΔTV even when man 6-phosphate was not present. We also conducted pulse-chase experiments in the presence of tunicamycin, and hproCAT L ΔTV still showed no secretion after a 6 h chase despite secretion of virtually all the wild-type hproCAT L under these conditions (data not shown). Our laboratory previously reported that glycosylation increases the size of hproCAT L by 2 kDa. Therefore, these results are in accordance with our previous studies on glycosylation (*30*) and suggest that deletion of carboxy-terminal Thr-Val did not affect carbohydrate addition on hproCAT L.

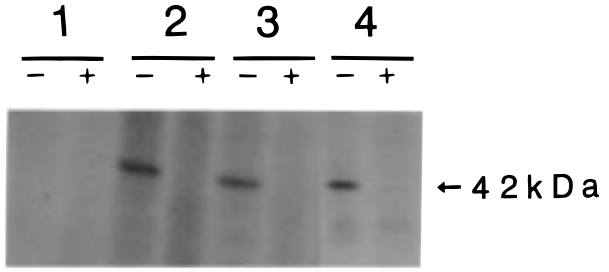

FIGURE 7: Phosphorylation of hproCAT L and hproCAT L ΔTV. NIH LAP cells (1×10^6) stably expressing hproCAT L or hproCAT L ΔTV were depleted of phosphate by incubating for 3 h in phosphate-free DMEM. These cells were then labeled for 3 h with [^{32}P] orthophosphate (0.5 mCi/mL) in fresh phosphate-free medium. After the labeling period, cell monolayers were washed with ice-cold PBS and lysed with SDS−buffer A. Human cathepsin L was immunoprecipitated and eluted from the *Staphylococcus aureus* pellets by incubating at 95 °C for 10 min in 10 mM sodium phosphate pH 5.5, containing 0.3% SDS and 10 mM pepstatin. The eluent was divided into two equal aliquots. One aliquot was incubated without endo H, and the other one was treated with endo H. For endo H treatment, this solution was diluted three times with the same buffer without SDS. Endo H (0.01 unit, Sigma Chemical Co, St. Louis, MO) was added to each aliquot and incubated at 37 °C for 16 h. NIH LAP and KB cells treated the same way served as negative and positive controls, respectively: -, no endo H treatment; +, treated with endo H; lane 1, NIH LAP cells; lane 2, KB cells; lane 3, hproCAT L; lane 4, hproCAT L ΔTV.

While the hproCAT L ΔTV mutant was not secreted, it did appear to be transported to lysosomes, as indicated by the presence of processed forms of the protein in cell extracts (Figure 6, lane 4). It is important to note that both hproCAT L and hproCAT L ΔTV exhibited an identical pattern of pro and processed forms of the protease (Figure 6, lanes 3E and 4E, tunicamycin), demonstrating similar intracellular processing of wild-type and secretion defective forms of human cathepsin L. These results suggest that hproCAT L ΔTV was folded and glycosylated properly for normal transit through the intracellular transport pathway. To analyze this point further, we carried out additional characterization of the mutant.

Phosphorylation of hproCAT L ΔTV. Lysosomal enzymes are known to carry the man 6-phosphate recognition marker which plays a vital role in their targeting to lysosomes (*15*, *37*). We were interested in determining whether deletion of the carboxy-terminal amino acids Thr-Val had any effect on the phosphorylation of hproCAT L. When KB-3-1 or NIH LAP cells stably expressing wild-type hproCAT L or hproCAT L ΔTV were labeled with [^{32}P]-orthophosphate, we detected a 42 kDa protein band upon immunoprecipitation and autoradiography (Figure 7, lanes 2, 3, and 4). This 42 kDa band was not detected in NIH LAP cells (Figure 7, lane

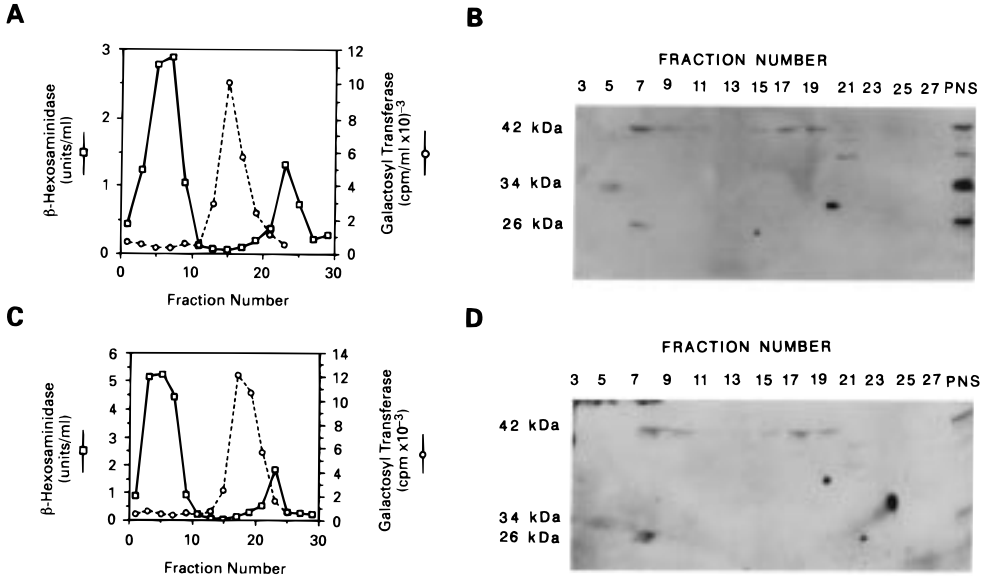

FIGURE 8: Subcellular distribution of hproCAT L and hproCAT L ΔTV in NIH cells. (Panels A and C) NIH LAP cells analyzed by Percoll density gradient fractionation. NIH LAP cells stably expressing hproCAT L(A) or hproCAT L ΔTV(C) were fractionated by Percoll gradient as described in Experimental Procedures. Lysosomes were localized by β-hexosaminidase activity and Golgi fractions by galactosyl transferase activity. (Panels B and D) Western blot localization of hproCAT L (B) or hproCAT L ΔTV (D) in various Percoll fractions.

1). When [^{32}P]-labeled immunoprecipitates were treated with endohexosaminidase H (endo H), no 42 kDa band was detected in cells expressing wild-type hproCAT L from KB-3-1 or NIH LAP cells stably expressing hproCAT L (Figure 7, lanes 2 and 3) or hproCAT L ΔTV (Figure 7, lane 4). Incubation of [^{32}P]-labeled immunoprecipitates under identical conditions in the absence of endo H did not result in the disappearance of the 42 kDa band (Figure 7, lanes 2, 3, and 4), precluding the possibility of nonspecific degradation of the protein or phosphatase activity in the extracts. These results suggest that both wild-type and mutant forms of hproCAT L were normally phosphorylated, and the phosphorylation was sugar-linked.

Acid Protease Activity in hproCAT L ΔTV. To determine if deleting the two carboxy-terminal amino acids, Thr-Val, had any effect on the catalytic function of hproCAT L, we performed assays for cathepsin L activity on immunoprecipitates of hproCAT L from cells expressing wild-type or hproCAT L ΔTV. This procedure has been previously used successfully in our laboratory to detect mouse cathepsin L activity in cell extracts (*34*). After normalization for the level of expression of hproCAT L, as determined by immunoprecipitation of ^{35}S-methionine labeled hproCAT L, the protease specific activities of wild-type hproCAT L and hproCAT L ΔTV in cell extracts were found to be comparable (data not shown). These results suggest that deletion of TV had no apparent effect on the enzyme activity of hproCAT L, again ruling out major effects on enzyme folding or structure.

Subcellular Distribution of hproCAT L ΔTV by Percoll Density Gradient Fractionation. The presence of processed forms of hproCAT L ΔTV in cell extracts of stable cell lines (Figure 6, lane 4) suggested that the mutant protein was capable of being transported to lysosomes. To confirm the subcellular distribution of wild-type and mutant hproCAT L, we performed self-forming Percoll gradient fractionation of cell extracts. Marker enzymes β-hexosaminidase (lysosomes) and galactosyl transferase (Golgi) were assayed in the various Percoll fractions to mark the fractions. In NIH LAP cells expressing either wild-type or mutant hproCAT L, we observed two regions of the Percoll gradient with β-hexosaminidase activity (Figure 8A,C). Fractions 5−7 contained the major peak of β-hexosaminidase activity and, therefore, were considered to contain lysosomes. The second peak of β-hexosaminidase, which represented approximately 5−8% of the total activity (fractions 20−25), has been reported to contain the material that does not enter the gradient (*38*). Galactosyl transferase activity was detected in fractions 13−21, and its distribution was identical in both wild-type and mutant hproCAT L expressing cells (Figure 8A,C).

Alternate Percoll gradient fractions were analyzed by immunoblotting with hproCAT L antiserum, and the results are given in Figure 8, parts B and D. Two major peaks of cathepsin L were detected, from fraction 3−11 and from 15 to 19 (Figure 8, parts B and D). Fraction 5, which contained the highest level of β-hexosaminidase activity, only contained the 34 kDa processed form of hproCAT L in both cell lines (Figure 8B,D). However, in fraction 7, which exhibited comparable β-hexosaminidase activity, both the 42 kDa pro form and a 26 kDa processed form of hproCAT L were detectable. Fractions 9−11 exhibited only the 42 kDa form of hproCAT L. All three forms of hproCAT L were detectable in postnuclear supernatants (PNS). Similarly, in fractions 15−17, which represent Golgi fractions, only the 42 kDa form was detectable. The distribution of wild-type and hproCAT L ΔTV was identical by this analysis (cf., Figure 8B,D). However, the total amount of hproCAT L ΔTV was lower when compared to hproCAT L. These data show that although hproCAT L ΔTV cannot be secreted, it can get to lysosomes or subcellular structures with the same density characteristics as lysosomes.

DISCUSSION

Human procathepsin L is overexpressed in a variety of human malignancies (*11*−*13*). In tissue culture, such overexpression of proCAT L results in secretion of the proenzyme

(*17*, *18*, *39*, *40*). In this study, we expressed hproCAT L in a mouse cell line at a level at least equivalent to that in transformed cells. This overexpression also resulted in secretion of the majority of the hproCAT L into the culture medium (Figure 1). In this way, we simulated the secretion of hproCAT L which is a characteristic of transformed cells (*39*) and other normal cells which synthesize large amounts of hproCAT L. Since hproCAT L antibodies do not recognize mouse procathepsin L, this system gave us the advantage of modifying hproCAT L while still being able to differentiate it from the endogenous mouse protein. Procathespsin L is secreted into the medium in a form which contains the man 6-phosphate lysosomal marker and is identical to intracellular proCAT L which is transported to lysosomes (*41*, *42*). The mechanism by which this lysosomal protein sometimes escapes normal transport to lysosomes is not entirely clear. Dong et al. (*43*) and Lazzarino and Gabel (*42*) have independently demonstrated a low affinity of mouse proCAT L for man 6-phosphate receptor. This apparently is due either to a low number of man 6-phosphate-containing carbohydrates (*44*) or to the presence of a protein determinant which interferes with receptor binding (*42*). In either case, the suggestion is that proCAT L secretion results from the preferential saturation of man 6-phosphate receptor when receptor levels become limiting in cells. However, recent data from one of our laboratories (*45*) indicate that addition of extra mannose 6-phosphorylated carbohydrates onto hproCAT L can allow transit of hproCAT L to lysosomes without eliminating its secretion. Results of the present study demonstrate that carboxy-terminal deletions as small as two amino acids reduced the secretion of hproCAT L into the medium, but the mutant hproCAT L was still able to get to lysosomes. One possible, if not unlikely, explanation for this result is that there is a positive signal for secretion of hproCAT L by NIH 3T3 cells, although a subtle defect in protein folding which does not affect enzyme activity but affects normal transit of hproCAT L cannot be ruled out. According to the published crystal structure of procathepsin L (*46*) and molecular modeling of cathepsin L, the C-terminal tail is largely buried within the folded structure of the protein, with only the last 2−3 residues appearing accessible for contact with other cellular factors. It seems possible that deletion of two residues from the carboxy terminus would have subtle effects on protein−protein interactions without affecting the overall folded structure of hproCAT L. Williams and Fukuda (*47*) have demonstrated that a tyrosine residue in the carboxy terminus of human lysosomal membrane protein is necessary for its targeting to lysosomes. Similarly, cytoplasmic tails have been shown to be responsible for lysosomal targeting of acid phosphatase, LIMP II, and lam-I (*20*, *21*, *48*, *49*).

Deletion of the last two amino acids of hproCAT L (Thr-Val) blocked the secretion of hproCAT L without affecting its intracellular distribution, phosphorylation, glycosylation, or catalytic activity (Figures 3B and 6−8 and data not given). As improper folding is known to alter enzymatic activity and glycosylation of lysozymes (*15*), these results suggest that gross folding of the protein was not affected by this small deletion. Proper conformation of lysosomal enzymes is important for acquiring the lysosomal recognition marker (*15*). Since hproCAT L ΔTV undergoes normal sugar-linked phosphorylation, it is unlikely that deletion of TV affects the conformation of hproCAT L in a way which alters its ability to receive man 6-phosphate (Figure 7). Analysis of radiolabeled immunoprecipitates of hproCAT L and hproCAT L ΔTV on SDS−PAGE followed by autoradiography demonstrated an identical pattern of pro and processed forms of cathepsin L suggesting their similar intracellular processing (Figure 6, lanes 3E and 4E, tunicamycin). However, treatment of cells stably expressing hproCAT L ΔTV with NH_4Cl did not result in the secretion of the mutant protease (data not shown). One possible, if unlikely, explanation for this result is that wild-type hproCAT L and hproCAT L ΔTV may be delivered to lysosomes by different mechanisms. Since we demonstrated the same subcellular distribution of hproCAT L and hproCAT L ΔTV (Figure 8), this difference in mechanism is not likely to result from grossly different subcellular compartmentalization. An alternative explanation of this result is that hproCAT L ΔTV cannot interact properly with a secretory apparatus, even in the presence of NH_4Cl.

Deletion of either Thr or Val only reduced the secretion of hproCAT L (Figure 4A, lanes 4 and 5). Surprisingly, exchanging the positions of these amino acids (Figure 4A, lane 6) or replacing them by the unconserved amino acids YN also resulted in the secretion of hproCAT L albeit at a marginally lower level (Figure 4A, lane 7). Both Thr and Val are bulky amino acids. Failure to secrete hproCAT L when they were replaced by two nonbulky amino acids (alanine) suggests that bulky amino acids on the carboxy terminal of hproCAT L are needed for secretion (Figure 4A, Lane 8). However, secretion of hproCAT L was blocked by the deletion and/or Ala replacement of two contiguous residues of the four amino acids upstream to Thr-Val, suggesting that the most carboxy-terminal two amino acids are not the only amino acids necessary for secretion or that residues upstream from Thr-Val indirectly affect orientation of Thr-Val (Figure 4B, lanes 5−7).

Deletion of any two amino acids upstream to the six carboxy-terminal amino acids shortens the length of the carboxy tail and blocks secretion of hproCAT L (Figure 3B). The replacement of these upstream amino acids by Ala maintains the length of the carboxy tail and allows secretion (Figure 4B, lane 7). Extension of the carboxy terminus by 22 amino acids with bulky amino acids (RV) on their carboxy terminus also blocked the secretion of hproCAT L, raising the possibility that there may be an optimal length for the carboxy tail (Table 3 and Figure 3A). The present study also demonstrates that the nature of several of the six carboxy-terminal amino acids also plays an important role in the secretion of hproCAT L. Of these six carboxy-terminal amino acids, replacement of one amino acid at a time revealed that Val, Pro, and Ser at positions −1, −3, and −5, respectively, are essential for the secretion of hproCAT L (Table 3 and data not shown). Therefore, our results suggest that the putative sequence needed for the secretion of hproCAT L is −S−X−P−X−V or another bulky amino acid substituted for V. Mouse cathepsin L which is also secreted into the medium upon overexpression contains a similar sequence (−S−X−P−X−V−N) at its carboxy terminus (*30*). This sequence is conserved in the cathepsin L from various species (*30*). The simplest model to explain these results is that the sequence described above alone or by interacting with some other part of hproCAT L

forms a complex signal needed for recognition by a putative secretory apparatus, or is essential for progression of hproCAT L into a compartment from which secretion can occur. We cannot rule out, for example, an interaction of the carboxy tail with a putative 9 amino acid amino-terminal lysosomal targeting sequence in procathepsin L which has been described by McIntyre et al. (*50*). It is also possible that the carboxy-terminal mutations produce a subtle defect in protein structure which indirectly affects compartmentalization of hproCAT L.

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