

Mannose 6-Phosphate-independent Targeting of Lysosomal Enzymes in I-Cell Disease B Lymphoblasts

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Abstract. B lymphocytes from patients with I-cell disease (ICD) maintain normal cellular levels of lysosomal enzymes despite a deficiency of the enzyme UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase. We find that an ICD B lymphoblastoid cell line targets about 45% of the lysosomal protease cathepsin D to dense lysosomes. This targeting occurs in the absence of detectable mannose 6-phosphate residues on the cathepsin D and is not observed in ICD fibroblasts. The secretory protein pepsinogen, which is closely related to cathepsin D in both amino acid sequence and three-dimensional structure, is mostly excluded from dense lysosomes, indicating that the lymphoblast targeting

pathway is specific. Carbohydrate residues are not required for lysosomal targeting, since a non-glycosylated mutant cathepsin D is sorted with comparable efficiency to the wild type protein. Analysis of a number of cathepsin D/pepsinogen chimeric proteins indicates that an extensive polypeptide determinant in the cathepsin D carboxyl lobe can confer efficient lysosomal sorting when introduced into the pepsinogen sequence. This determinant overlaps but is not identical to the recognition marker for phosphotransferase. These results indicate that a specific protein recognition event underlies Man-6-P-independent lysosomal sorting in ICD lymphoblasts.

MOST lysosomal enzymes are soluble glycoproteins which, along with secretory proteins, are cotranslationally inserted into the lumen of the rough ER and glycosylated at selected asparagine residues by the en bloc transfer of a large preformed oligosaccharide (reviewed in Kornfeld and Mellman, 1989). In normal mammalian cells, the N-linked oligosaccharides of the lysosomal enzymes are subsequently modified by the concerted action of the enzymes UDP-GlcNAc: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (phosphotransferase) and N-acetylglucosamine-1-phosphate α -N-acetylglucosaminidase to generate an exposed mannose 6-phosphate (Man-6-P)¹ moiety. Lysosomal enzymes bearing phosphomannosyl residues bind to Man-6-P receptors present in the TGN and are then transported to an acidified prelysosomal compartment by means of clathrin-coated vesicles. Phosphotransferase selects these acid hydrolases for modification by recognizing a conformation-dependent protein determinant present on lysosomal enzymes but not on structurally related secretory proteins (Baranski, 1990, 1991).

I-cell disease (ICD) is an inherited human disorder in which an unknown mutation eliminates or severely reduces the activity of phosphotransferase (Reitman et al., 1981;

Hasilik et al., 1981). As a result, newly synthesized lysosomal enzymes do not acquire Man-6-P residues and are quantitatively secreted by many cell types. This results in gross cellular deficiencies of most lysosomal enzymes and severe clinical manifestations in the first few years of life (Nolan and Sly, 1989). However, certain tissues and cell types isolated from ICD patients, such as liver and spleen (Waheed et al., 1982; Owada and Neufeld, 1982) and B lymphocytes immortalized by Epstein-Barr virus (EBV) (Little et al., 1987; Okada et al., 1988; Tsuji et al., 1988), have cellular lysosomal enzyme levels within the normal range for those cell types despite virtually undetectable phosphotransferase activity. In B lymphocytes, these enzymes have been localized to a dense membrane compartment likely representing lysosomes (Miller, A. L., M. Liddle, V. Norton, and D. Wright. 1988. *J. Cell Biol.* 107:341a), and a recent report indicates that newly synthesized lysosomal enzymes are retained intracellularly in the absence of phosphorylation (DiCioccio and Miller, 1991).

The ability of these cells to retain intracellular lysosomal enzymes in the absence of phosphotransferase activity suggests that they may have a cell type-restricted, Man-6-P-independent mechanism of lysosomal enzyme targeting. We decided to investigate this possibility further by studying an established B lymphoblastoid cell line derived from an ICD patient (described in Little et al., 1987). We find that in these cells, both endogenous and transfected cathepsin D,

1. *Abbreviations used in this paper:* BLCL, B lymphoblastoid cell line; EBV, Epstein-Barr virus; endo H, endoglycosidase H; ICD, I-cell disease; Man-6-P, mannose 6-phosphate.

a lysosomal protease, are sorted directly to dense lysosomes without acquiring Man-6-P residues, consistent with previous reports on other lysosomal hydrolases. Furthermore, we find that transfected pepsinogen, the related nonlysosomal protease, is largely excluded from lysosomes, indicating that ICD lymphoblasts target lysosomal enzymes selectively. Finally, we present evidence that an extended protein determinant in the carboxyl lobe of cathepsin D directs its lysosomal sorting in these cells.

Materials and Methods

Materials

Rabbit antisera to human cathepsin D have been described previously (Baranski et al., 1990; Griffiths et al., 1988). Immobilized bovine Man-6-P/IGF-II receptor (MPR) was prepared as described previously (Varki and Kornfeld, 1983). Rabbit antisera to human pepsinogen were generated by injection of the purified protein (gift of Dr. I. Michael Samloff, University of California, Los Angeles, CA). EXPRE^{35S} protein-labeling mix was from DuPont/NEN (Wilmington, DE). All restriction and other DNA modifying enzymes were from New England Biolabs (Beverly, MA) or Promega Biotec (Madison, WI). Endoglycosidase H (Endo H) was from Boehringer Mannheim Biochemicals (Mountain View, CA). *Vibrio cholerae* neuraminidase and hygromycin B were from Calbiochem-Behring Corp. (San Diego, CA). Percoll was from Pharmacia Fine Chemicals (Piscataway, NJ). All other chemicals were from Sigma Immunochemicals (St. Louis, MO).

Cell Culture and Transfection

A B lymphoblastoid cell line (BLCL) derived from an ICD patient was kindly provided by Dr. A. L. Miller (University of California, San Diego, CA) and has been described (Little et al., 1987). CGM-1, a BLCL derived from a normal individual, was provided by Dr. S. Korsmeyer (Washington University, St. Louis, MO). The cells were maintained in RPMI 1640, 10% bovine calf serum (Hyclone, Logan, UT), 100 U/ml penicillin, 100 U/ml streptomycin at a density of $3-5 \times 10^5$ /ml in a humidified 5% CO₂ atmosphere. Skin fibroblasts from normal individuals and ICD patients, obtained from the Human Genetics Mutant Cell Repository (Bethesda, MD), were maintained in MEM- α , 10% FCS (Hazleton Biologics, Lexena, KS), 100 U/ml penicillin, 100 U/ml streptomycin.

ICD lymphoblasts were electroporated essentially as in Margolskee et al. (1988). All procedures were carried out under aseptic conditions. 5×10^7 cells in logarithmic growth phase were pelleted, washed once with PBS, and resuspended in 500 μ l HEPES buffered saline containing 100–150 μ g plasmid DNA and 300 μ g sheared salmon sperm DNA. Mock transfections received 500 μ g salmon sperm DNA only. Cells were transferred to sterile, washed plastic cuvettes (Fisher Scientific Co., Pittsburgh, PA), electroporated at room temperature with a BTX electroporator using a capacitor discharge of 150 V, 1,050 μ F (pulse length 6–9 ms). Electroporated cells were maintained at 5×10^5 /ml for 48 h in the absence of selection, and then in media containing 100 U/ml hygromycin B for 2 d, 150 U/ml for 3 d, and 200 U/ml thereafter. During selection, cells were maintained at a density of 5×10^5 /ml in a 50%/50% mixture of fresh and ICD BLCL-conditioned media and were fed every 2 to 3 d. Transfected cell lines were maintained in complete RPMI 1640 medium containing 200 U/ml hygromycin B.

Plasmid Constructions

All recombinant DNA techniques used standard protocols (Maniatis et al., 1989). The cDNA for human cathepsin D (Faust et al., 1985), subcloned into pSP64, was excised by digestion with HindIII and NaeI. The generation of the nonglycosylated mutant cathepsin D cDNA has been described previously (Cantor and Kornfeld, 1992) and was excised similarly. The cDNA for human glycopepsinogen, subcloned into pSP64 (Baranski et al., 1990), was digested with EcoRI, blunt ended with Klenow fragment, and excised with HindIII. Insert fragments were gel purified and ligated into the plasmid EBO pLPP (a modified version of plasmid EBO pcD; Margolskee et al., 1988; gift of P. Kavathas, Yale University, New Haven, CT), which had been cut with XbaI (blunted) and HindIII. Ligation mixes were used to transform *Escherichia coli* DH5 α , plasmids were isolated and the constructs confirmed by restriction mapping. The chimeric constructs CP2,

CP3, CP4, CP5, CP6, CP17, CP51, CP52, CP55, CP56, CP65, and CP69 have been described previously (Baranski et al., 1990, 1991, 1992). The constructs CP71, CP72, and CP73 were assembled from restriction fragments of previously published constructs using shared HinfI, PvuII, and BamHI restriction sites. Plasmid constructs were confirmed by restriction mapping and DNA sequencing where appropriate. The chimeric cDNAs were subcloned into EBO pLPP after subcloning in Bluescript SK (Stratagene Corp., La Jolla, CA) or pGEM3Zf(-) (Promega Biotec) to acquire flanking restriction sites.

Metabolic Labeling and Immunoprecipitation

$1-1.5 \times 10^6$ cells in logarithmic growth phase were washed twice in methionine-free, glutamine-free RPMI 1640, 10% dialyzed FCS, 25 mM Hepes-NaOH, pH 7.4, 100 U/ml penicillin, 100 U/ml streptomycin, and resuspended in this media at $\sim 5 \times 10^6$ cells/ml. EXPRE^{35S} labeling mix was added to 0.25 mCi/ml (methionine), and cells were incubated as described in the Results section. Chase was initiated by the addition of cold methionine to 5 mM and continued as described in Results. In some experiments, cells were incubated for 1 h before labeling in methionine-free media containing 200 μ M leupeptin, 200 μ M pepstatin A.

At the end of the chase, cells were harvested by centrifugation for 1 minute in a microfuge at 4°C. The media was decanted and saved, and the cells were washed twice with 1 ml ice-cold PBS plus 2 mM methionine and lysed in 200 μ l PBS, 10 mM EDTA, 0.5% Triton X-100, pH 7.4 supplemented with 1 mM PMSF, 0.25 TTU/ml aprotinin, and 1 \times of a protease inhibitor cocktail containing 2 μ g/ml (final) antipain, chymostatin, leupeptin, and pepstatin A. The lysate was sonicated on ice with 3 10-s bursts at 80 W using a probe sonicator (Branson Ultrasonics Corp., Danbury, CT), incubated on ice for 15–30 minutes, and spun for 30 min at 40,000 g, 4°C in a Sorvall SS34 rotor. The supernatant was decanted and used for immunoprecipitation.

Cell and media samples were adjusted to 0.1 M Tris-HCl, 0.1 M KCl, 5 mM MgCl₂, 1% TX100, 0.5% Na deoxycholate, 0.3% SDS, 1 mM PMSF, pH 8.0, and rabbit antisera to cathepsin D or pepsinogen were added as appropriate (CP52, 55, 56, and 69 were immunoprecipitated with anti-cathepsin D; all other chimeric proteins were immunoprecipitated with anti-pepsinogen). Samples were incubated for 6–16 h at 4°C with constant mixing. 25 μ l of protein A-agarose (Repligen) was added, and the incubation was continued for another 60 min at 4°C. Agarose beads were pelleted by 10-s centrifugation in a microfuge, washed four times with 500 μ l 0.1 M Tris-HCl, 0.1 M NaCl, 1% TX-100, pH 8.0, and then twice with 500 μ l 0.1 M Tris-HCl, pH 8.0. Immunoprecipitates were eluted by heating to 100°C for 5 min in nonreducing SDS sample buffer and loaded on 10% SDS-polyacrylamide gels (Laemmli, 1970). Gels were fixed and stained with Coomassie blue, impregnated with Amplify fluorographic enhancer (Amersham Corp., Arlington Heights, IL), dried, and then exposed to preflashed Kodak XAR film (Eastman Kodak, Rochester, NY).

Radioactive bands were quantitated by densitometric scanning or by excising the appropriate region of the dried gel and solubilizing in 30% H₂O₂ at 50°C. 10 ml ScintiVerse I (Fisher Scientific Co.) was added, and samples were counted in a liquid scintillation counter (LS 6800; Beckman Instruments).

Percoll Gradient Fractionation

All operations were carried out at 4°C. [³⁵S]methionine-labeled cells were collected by 1-min centrifugation in a microfuge, washed twice with ice-cold PBS, and once with ice-cold homogenization buffer (0.25 M sucrose, 10 mM triethanolamine-HCl, 1 mM EDTA, pH 7.4), and resuspended in 500 μ l homogenization buffer plus protease inhibitors (see above). Cells were homogenized by 15 passes through a 200 μ l pipet tip fitted to a 1-cc syringe and centrifuged for 10 min at 500 g. The supernatant was decanted, and the pellet was resuspended in 300 μ l homogenization buffer, rehomogenized, and spun as above. The pooled postnuclear supernatants were applied to 11 ml 36% Percoll (in homogenization buffer) layered on a 2 ml cushion of 1.25 M sucrose in a 16 \times 102 mm Ultra Clear centrifuge tube (Beckman Instruments, Inc., Palo Alto, CA). The gradient was centrifuged for 90 minutes at 35,000 g in a rotor (SS34; Sorvall Instruments, Newton, CT), and 1 ml fractions were collected through a needle hole in the bottom of the tube. After removal of Percoll by high-speed centrifugation, fractions were immunoprecipitated with antisera to cathepsin D or pepsinogen, and assayed for marker enzymes as described elsewhere in Materials and Methods. Density of fractions was determined by direct weighing and by refractometry.

Oligosaccharide Analysis

Endo H digestion and Man-6-P receptor affinity chromatography were performed as described previously (Baranski et al., 1990). For neuraminidase digests, samples were adjusted to 50 mM MES-NaOH, 5 mM CaCl₂, 0.1% TX-100, 1 mM PMSF, pH 6.0, and 6 mU *Vibrio cholerae* neuraminidase were added. After 4–6 h at 37°C, digestion was terminated by placing the sample on ice and adding immunoprecipitation buffer. Immunoprecipitation was performed as described above.

Pepstatin Binding Assays

50 μ l [³⁵S]methionine-labeled cell lysate or media samples were added to 500 μ l of a 1:10 slurry of pepstatin A-agarose (Sigma Immunochemicals) equilibrated in 50 mM Na acetate/150 mM NaCl/0.5% TX-100, 0.5 mM PMSF, 0.25 TIU/ml aprotinin, 10 μ M leupeptin, pH 3.6, rapidly mixed, and then incubated overnight at 4°C with constant mixing. After removal of the unbound fraction, the agarose beads were washed five times with this buffer and then incubated with 250 μ l 50 mM Tris-HCl, 150 mM NaCl, 0.5% TX-100, pH 8.0. The unbound and pH 8.0 eluted fractions were immunoprecipitated with anti-cathepsin D or pepsinogen antisera as appropriate and analyzed by SDS-PAGE and fluorography.

Assays and Miscellaneous Methods

β -hexosaminidase and α -glucosidase II were assayed using 1 mM of the appropriate 4-methylumbelliferyl substrate at pH 4.5 and 7.0, respectively, in the presence of 0.1% Triton X-100 (Little et al., 1987). Fluorescence was measured on an Aminco fluorimeter and compared to enzyme-free blanks. Protein was assayed with the Bio-Rad dye reagent using bovine serum albumin as a standard.

Results

Biosynthesis of Cathepsin D in ICD B Lymphoblasts

To assess the ability of ICD lymphoblasts to target newly synthesized lysosomal enzymes, we first examined the trafficking of the aspartyl protease cathepsin D. B lymphoblasts from an ICD patient and a normal individual were labeled with [³⁵S]methionine for 30 min at 37°C and chased for 4 h in an excess of unlabeled methionine. At the end of the chase, cells and media were collected separately, the cells were lysed, and equal portions of cell lysate and media samples were immunoprecipitated with antisera specific for human cathepsin D. Both ICD and normal lymphoblasts synthesized cathepsin D as a 47-kD precursor (Fig. 1 A, lanes C). ICD lymphoblasts retained ~56% of cathepsin D intracellularly, while the remainder was secreted as procathepsin D (Fig. 1 A, lanes M). Cell-associated cathepsin D underwent two proteolytic cleavages – removal of the ~5-kD prosegment to generate mature cathepsin D and further cleavage to the 31-kD heavy chain – which in other cells indicate delivery to lysosomes (Hasilik and Neufeld, 1980a; Gieselmann et al., 1983; Faust et al., 1987; Rijnboutt et al., 1992). In normal lymphoblasts, >95% of cathepsin D remained intracellular and was almost completely processed to the 31-kD heavy chain. By contrast, ICD fibroblasts secreted >90% of newly synthesized cathepsin D, while normal human fibroblasts sorted >90% of cathepsin D to lysosomes as judged by proteolytic processing (Fig. 1 B).

To confirm that cathepsin D was sorted to lysosomes in ICD lymphoblasts, lymphoblasts labeled with [³⁵S]methionine were homogenized and fractionated on 36% Percoll density gradients. Gradient fractions were then immunoprecipitated with antisera to cathepsin D and assayed for a number of organelle marker enzymes. Virtually all of the mature, proteolytically processed cathepsin D was present in a dense

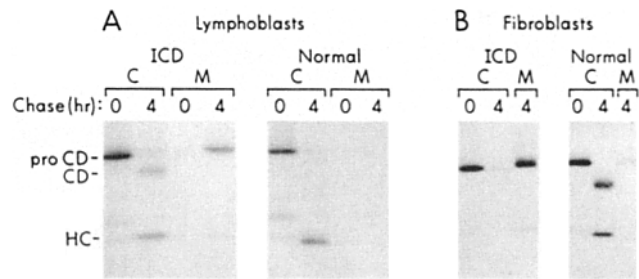


Figure 1. Sorting of cathepsin D in I-cell disease and normal cells. Cultures of (A) ICD and normal lymphoblasts or (B) ICD and normal fibroblasts were labeled with [³⁵S]methionine for 30 min at 37°C and chased in excess unlabeled methionine for 0 or 4 h, as indicated. Equivalent samples of a detergent lysate of cells (C) and media (M) were immunoprecipitated with anti-cathepsin D antiserum. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. Positions of procathepsin D (*proCD*), mature cathepsin D (*CD*), and cathepsin D heavy chain (*HC*) are indicated to the left of each panel.

(~1.08–1.12 g/ml) compartment enriched in the lysosomal marker β -hexosaminidase (Fig. 2, A and B, fractions 3–5). The small amount of procathepsin D remaining in the cells was present in lighter compartments (Fig. 2 A, fractions 11 and 12) enriched in the ER marker α -glucosidase but also containing Golgi and endosomal membranes. Quantitation

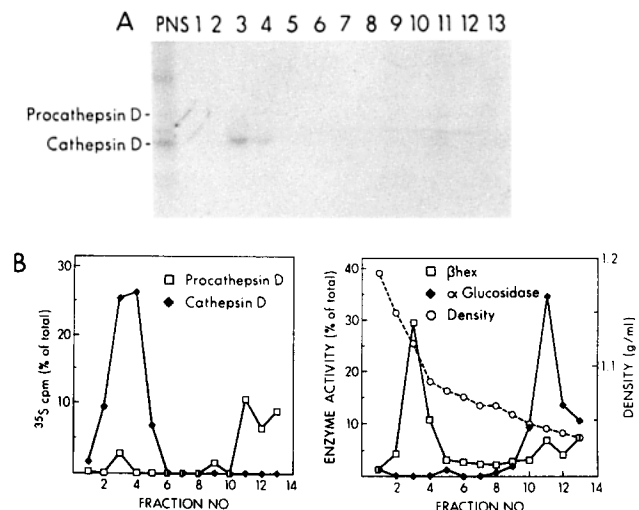


Figure 2. Cathepsin D is transported to dense lysosomes. ICD lymphoblasts were labeled as described in the legend to Fig. 1. Cells were homogenized, and the postnuclear supernatant (PNS) was fractionated on a 36% (vol/vol) Percoll density gradient. 1-ml fractions (*l* = bottom) were collected and analyzed. (A) cathepsin D immunoprecipitation. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Positions of procathepsin D and cathepsin D are indicated. In this experiment, cells were preincubated with the protease inhibitors pepstatin A and leupeptin before labeling. Therefore, cathepsin D heavy chain is not seen. (B) Distribution of cathepsin D forms in A. Appropriate regions of the dried gel were excised, and radioactivity determined by liquid scintillation counting. Values are expressed as a percentage of the total radioactivity recovered from the gradient for that species. (C) Distribution of the lysosomal marker β -hexosaminidase, the ER marker α -glucosidase II, and density profile across the gradient. Recovery of enzyme activity from the gradient was >80% of that loaded.

of multiple experiments revealed that $56 \pm 2\%$ ($n = 14$) of newly synthesized cathepsin D was retained, and that 80% of this was present in dense lysosomes. Transfer of cathepsin D to the lysosomal compartment was first detected after 60-min chase at 37°C (not shown), indicating rapid delivery to this organelle. Similar experiments performed with normal lymphoblasts showed that virtually all cell-associated cathepsin D was present in dense lysosomes (not shown).

Analysis of Cathepsin D Oligosaccharides

Cathepsin D has two N-linked glycosylation sites, both of which are used. In normal mammalian cells (Hasilik and Neufeld, 1980b; Schulze-Lohoff et al., 1985) and *Xenopus* oocytes (Faust et al., 1987), cathepsin D is an excellent substrate for phosphotransferase and acquires Man-6-P residues. Despite the nearly total absence of phosphotransferase activity in ICD lymphoblast lysates (Little et al., 1987), we wished to directly exclude the possibility that lysosomal sorting could be due to a low but detectable level of phosphorylation. The processing of cathepsin D oligosaccharides was first examined by treating cathepsin D immunoprecipitates with endo H, which releases high mannose-type but not complex-type oligosaccharides from the polypeptide backbone. Cathepsin D synthesized during a 30-min pulse with [³⁵S]methionine contained two Endo H-sensitive oligosaccharides (Fig. 3 A, *Pulse*). After a four hour chase, both secreted and cellular cathepsin D contained a mixture of molecules, some of which were partially sensitive to endo H while the others were completely resistant (Fig. 3 A, *Cell and Media*). This indicates that a portion of the molecules contain one high mannose and one complex-type oligosaccharide while the rest contain two complex-type units. Since the 31-kD heavy chain, which contains the carboxyl lobe oligosaccharide, was completely resistant to endo H digestion (Fig. 3 A, *Cell*) and shifted slightly on neuraminidase digestion (Fig. 3 B), the carboxyl lobe oligosaccharide must be completely processed to a sialylated complex-type species. The amino lobe oligosaccharide, therefore, must contain a mixture of high mannose and complex-type units. The molecular weight decrease observed for the cell-associated and secreted cathepsin D upon neuraminidase digestion (Fig. 3 B) can be accounted for by the release of sialic acid from the complex-type oligosaccharides. In normal lymphoblasts, both of the cathepsin D oligosaccharides remained Endo H-sensitive during a four hour chase (not shown).

Since previous studies have established that only high mannose-type oligosaccharides are phosphorylated (Varki and Kornfeld, 1980), the predominance of complex-type oligosaccharides on ICD lymphoblast cathepsin D indicated that they contained little if any Man-6-P. To further assess phosphorylation, we applied [³⁵S]methionine-labeled lymphoblast lysates to Man-6-P/IGF-II receptor affinity columns and immunoprecipitated the unbound and Man-6-P-eluted column fractions with cathepsin D antiserum. As shown in Fig. 3 C, virtually all of the cathepsin D synthesized by normal B lymphoblasts bound to the column and was eluted by 10 mM Man-6-P, demonstrating efficient phosphorylation. By contrast, all cellular cathepsin D synthesized by ICD lymphoblasts passed through the column. Identical results were obtained with cathepsin D secreted into the medium (not shown). Since binding to the Man-6-P/IGF-II

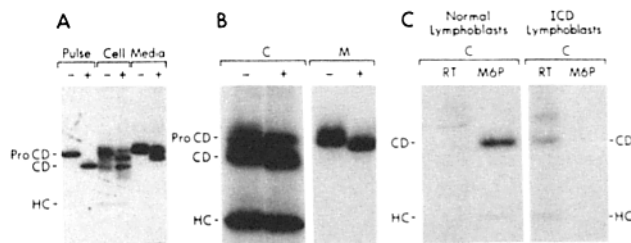


Figure 3. Analysis of cathepsin D oligosaccharides. ICD lymphoblasts transfected with human cathepsin D (A and B) or untransfected normal and ICD lymphoblasts (C) were labeled with [³⁵S]methionine as described in the legend to Fig. 1 unless otherwise noted. (A) Endo H digestion. Cell lysates and media samples were immunoprecipitated with anti-cathepsin D antisera. Immunoprecipitates were incubated in the presence (+) or absence (-) of endo H and analyzed by SDS-PAGE and fluorography. (B) Neuraminidase digestion. Cell lysate (C) and media samples (M) were incubated in the presence (+) or absence (-) of *Vibrio cholerae* neuraminidase. Digested samples were immunoprecipitated with anti-cathepsin D antisera and analyzed as in A. (C) Man-6-P/IGF-II receptor affinity chromatography. Normal and ICD lymphoblasts were labeled for 30 min with [³⁵S]methionine and chased for 2 h at 37°C. Cell lysates were applied to receptor columns and unbound (RT) and Man-6-P eluted (M6P) fractions were analyzed by immunoprecipitation as in B.

receptor is a sensitive indicator of acquisition of Man-6-P residues (Baranski et al., 1990), we conclude that cathepsin D is not detectably phosphorylated in ICD lymphoblasts, consistent with the diagnosis of ICD.

Glycopepsinogen Is Not Sorted to Lysosomes of ICD Lymphoblasts

Cathepsin D and pepsinogen are both members of the aspartyl protease family, and the human enzymes share about 45% amino acid sequence identity. The crystallographic structures of seven members of this family have been solved, and all family members show a remarkable conservation of secondary and tertiary structure (Davies, 1990). While cathepsin D is a lysosomal enzyme, pepsinogen is a non-glycosylated secretory protein. We reasoned that if the lysosomal targeting pathway in ICD lymphoblasts is selective for lysosomal enzymes, then pepsinogen should not be targeted to lysosomes in these cells. Previous work (Baranski et al., 1990) has demonstrated that a glycosylated form of pepsinogen (glycopepsinogen) is a poor substrate for phosphotransferase and so is excluded from the Man-6-P-dependent lysosomal targeting pathway.

ICD lymphoblasts were electroporated with vectors containing a gene for hygromycin B resistance and cDNAs coding for human cathepsin D, human glycopepsinogen, or no insert, and stably transfected cell lines expressing the transfected genes were then selected for resistance to hygromycin B. These cell lines were used in metabolic labeling experiments similar to those described in Figs. 1 and 2. The results of typical experiments are shown in Fig. 4. While cathepsin D-transfected cells express the enzyme at approximately 10 times the endogenous level, the transfected protein was transported to lysosomes almost as efficiently as the endogenous cathepsin D (Fig. 4 A). Quantitation of multiple experiments showed that $47 \pm 1\%$ ($n = 29$ under all conditions)

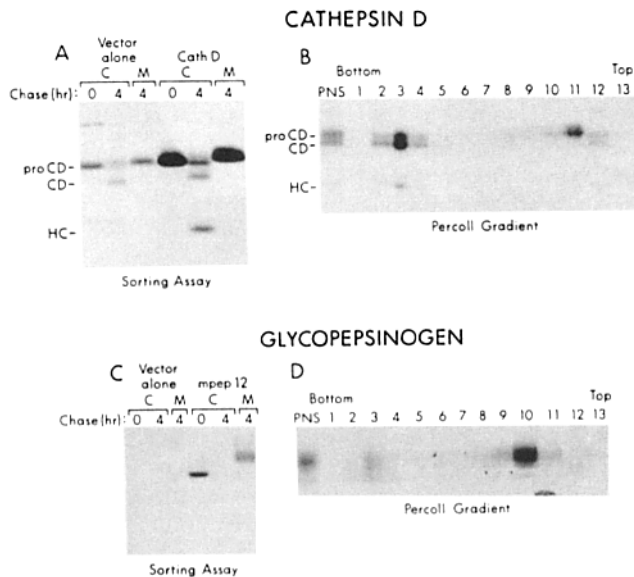


Figure 4. Sorting of transfected human cathepsin D and glycopepsinogen. Stably transfected ICD lymphoblasts were labeled with [³⁵S]methionine as described in the legend to Fig. 1. Percoll gradient analysis was performed as described in the legend to Fig. 2. Samples were immunoprecipitated with antisera to cathepsin D (A and B) or pepsinogen (C and D). (A) Sorting assays of vector transfected and cathepsin D (*cath D*) transfected cells. (B) Percoll gradient analysis of cathepsin D transfected cells. (C) Sorting assays of vector transfected and glycopepsinogen (*mpep12*)-transfected cells. (D) Percoll gradient analysis of glycopepsinogen transfected cells. In these experiments, cells were preincubated with protease inhibitors before labeling as described in Materials and Methods.

of transfected cathepsin D remained intracellular vs 56% of endogenous cathepsin D. Percoll gradient fractionation of [³⁵S]methionine labeled lymphoblast lysates revealed that 70% of intracellular transfected cathepsin had been transported to dense lysosomes versus 80% for endogenous cathepsin D (Fig. 4 B). Thus, overexpression of cathepsin D resulted in a 29% decrease in the efficiency of its sorting, although the absolute number of molecules targeted to dense lysosomes was increased.

In contrast to these findings with cathepsin D, the transfected glycopepsinogen was largely secreted by the ICD lymphoblasts (Fig. 4 C). Endo H digestion demonstrated that both oligosaccharides on glycopepsinogen were processed to complex-type chains (data not shown). Over multiple sorting assays, 12 ± 1% (*n* = 8) of newly synthesized glycopepsinogen remained intracellular. To protect intracellular protein from degradation, cells were routinely preincubated in media containing the protease inhibitors leupeptin and pepstatin A before labeling (see Materials and Methods). Although this treatment had no effect on the intracellular level of cathepsin D, the corresponding value for glycopepsinogen rose to 20 ± 1% (*n* = 16). Under these conditions, the majority of cell-associated glycopepsinogen contained processed oligosaccharides and was found in light-density compartments (Fig. 4 D), probably Golgi complex or endosomes. This relative distribution did not change with longer chase times (data not shown), indicating that the light-density pool of glycopepsinogen was not simply being transported to lysosomes very slowly. Overall, 5 ± 1% of newly

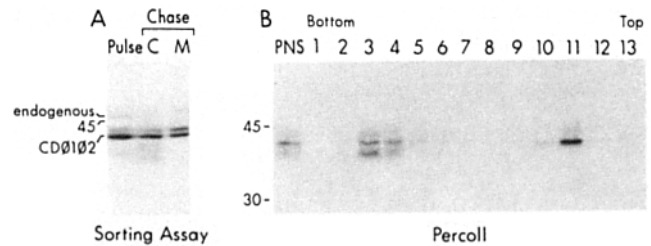


Figure 5. Sorting of nonglycosylated cathepsin D. ICD lymphoblasts transfected with a mutant cathepsin D lacking glycosylation sites (*CDφ1φ2*) were labeled for 1 h with [³⁵S]methionine at 28°C and chased for 16 h at this temperature. Cells were preincubated with protease inhibitors before labeling as described in Materials and Methods. Samples were immunoprecipitated with antisera to cathepsin D and analyzed by SDS-PAGE and fluorography. (A) Sorting assay. Positions of endogenous cathepsin D, *CDφ1φ2*, and a 45-kD molecular weight standard are indicated. (B) Percoll gradient fractionation, performed as described in the legend to Fig. 2. Positions of 45- and 30-kD molecular weight standards are indicated.

synthesized glycopepsinogen was transported to dense lysosomes in our experiments. In addition, similar studies with immunoglobulin M, a major secretory protein of B lymphocytes, failed to show any significant targeting of this protein to lysosomes, even when cells were preincubated with protease inhibitors (results not shown). We conclude that these two secretory proteins are poor substrates for the ICD lymphoblast lysosomal transport pathway.

Nonglycosylated Cathepsin D Is Sorted to Lysosomes

The ICD lymphoblasts' ability to target cathepsin D but not glycopepsinogen suggests that a selective recognition event underlies lysosomal transport of cathepsin D. This could occur through recognition of a cathepsin D protein determinant absent from pepsinogen, or, by analogy to the Man-6-P-dependent pathway, through a previously unrecognized difference in oligosaccharide structure. To test more directly whether a carbohydrate determinant was necessary for sorting, ICD lymphoblasts were transfected with a cDNA encoding a mutant cathepsin D lacking both its N-linked glycosylation sites. This protein was sorted to dense lysosomes and underwent the same proteolytic processing as glycosylated cathepsin D (Fig. 5), although the efficiency of sorting was slightly lower than that of the wild-type transfected cathepsin D (44% vs. 47% intracellular, *n* = 3, with 60% vs. 70% of this amount in dense lysosomes). These results establish that N-linked carbohydrate residues are not essential for Man-6-P-independent sorting in ICD lymphoblasts.

Identification of Cathepsin D Residues Involved in Lysosomal Sorting

Having excluded N-linked carbohydrates as sorting determinants for the Man-6-P-independent pathway, we hypothesized that amino acid sequence differences between cathepsin D and pepsinogen most likely account for the selective lysosomal targeting of cathepsin D. To identify the sequences required for sorting, we analyzed the behavior of transfected cathepsin D/glycopepsinogen chimeras, reasoning that the substitution of appropriate cathepsin D residues into the homologous position in the pepsinogen sequence should con-

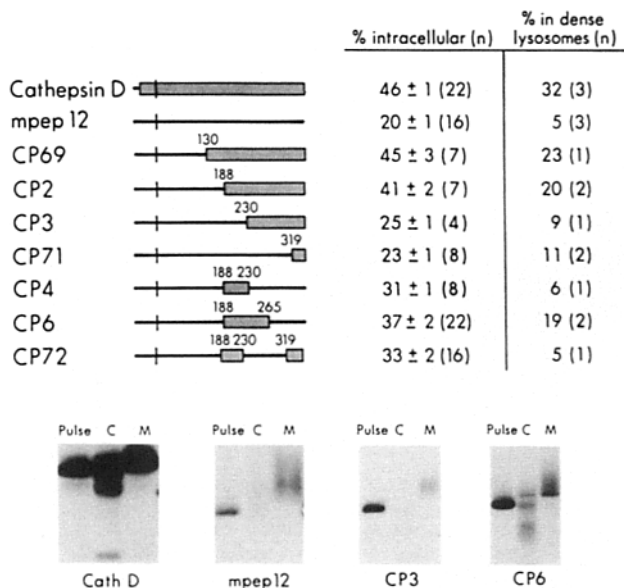


Figure 6. Cathepsin D residues 188 to 265 are sufficient for lysosomal sorting. The various chimeric proteins tested are shown schematically with the open bars representing cathepsin D sequence and the solid lines representing pepsinogen sequence. Transfected lymphoblasts were preincubated with protease inhibitors as described in Experimental Procedures, labeled with [³⁵S]methionine for 1 h at 28°C and chased for 16 h at 28°C followed by 4 h at 37°C. Control experiments (not shown) indicated that the additional chase at 37°C improved the secretion of chimeric proteins without affecting their stability. Immunoprecipitation, SDS-PAGE and fluorography of pulse (P), cell lysate (C), and media (M) samples were performed as described in Materials and Methods. Appropriate regions of dried gels were excised and counted, and percent sorting was calculated as cellular radioactivity divided by total recovered radioactivity (cells plus media). Percent sorting values represent the mean ± standard error of multiple determinations. The fraction of intracellular chimeric protein in dense lysosomes (enriched in β-hexosaminidase activity; typically Percoll gradient fractions 2–4) was determined by Percoll gradient fractionation of [³⁵S]methionine-labeled cell homogenates followed by immunoprecipitation, SDS-PAGE, and fluorography. This fraction (single value or mean of multiple determinations, as indicated) was multiplied by the percent sorting value to obtain percent in dense lysosomes. The recovery of immunoprecipitable protein at the end of the chase compared to the initial pulse value was as follows (mean ± S.E.): Cathepsin D (136 ± 8%), mpep12 (92 ± 6%), CP69 (106 ± 8%), CP2 (106 ± 6%), CP3 (93 ± 6%), CP71 (129 ± 18%), CP4 (45 ± 3%), CP6 (98 ± 10%), and CP72 (33 ± 4%).

vert this secreted protein into a lysosomally targeted one. Similarly, the substitution of the homologous pepsinogen sequence for the critical cathepsin D elements should give rise to a chimeric protein that is secreted rather than targeted to lysosomes. This approach has been successfully used to define the phosphotransferase recognition marker (Baranski et al., 1990).

In our previous study, a large number of cathepsin D/pepsinogen chimeric proteins were expressed in *Xenopus* oocytes and, with a few exceptions, were noted to fold properly and exit the ER similar to the native proteins (Baranski et al., 1990, 1991). However, when the biosynthesis of several of these same chimeric proteins was examined in transfected

B lymphoblasts, we found that most of these proteins were degraded soon after synthesis at 37°C (data not shown). This degradation presumably took place in the ER as a result of improper folding of the chimeric protein (Hurtley and Helenius, 1989). Since these chimeric proteins had folded stably in *Xenopus* oocytes maintained at 19°C, we tested whether lowering the incubation temperature during the metabolic labeling period would allow proper protein folding and exit from the ER in B lymphoblasts. We found that when the metabolic labeling experiments were performed at 28°C rather than 37°C, most of the chimerics were no longer degraded and were transported out of the ER with good efficiency (see below). Lowering the temperature to 28°C did not significantly alter the behavior of cathepsin D or glycopepsinogen in the sorting assays, although longer chase times (12–18 h) were required for complete lysosomal delivery or secretion of the labeled proteins, consistent with earlier studies (Brault et al., 1988). Accordingly, the sorting of the various chimeric proteins was analyzed at 28°C. In addition, the cells were preincubated with protease inhibitors to minimize intracellular degradation, as described above. Under these conditions, the recovery of eight of the chimeric proteins after a 20-h chase period ranged from 93 to 141% of the value obtained at the end of the pulse period. These values were the same as those determined for cathepsin D (136 ± 8% recovery) and glycopepsinogen (92 ± 6% recovery). In seven other chimeric proteins partial degradation was observed with recoveries ranging from 72% to 33%. The recovery of each construct is given in the legends to Figs. 6, 8, and 9.

Carboxyl Lobe Sequences Contain Man-6-P-Independent Lysosomal Targeting Information

We first analyzed a series of chimeric proteins in which amino-terminal cathepsin D residues were progressively replaced by pepsinogen residues. The results are summarized in Fig. 6. Cathepsin D and pepsinogen are bilobed proteins, and in construct CP69 most of the cathepsin D amino lobe is replaced by pepsinogen residues whereas in construct CP2 all of the amino lobe plus some of the carboxyl lobe elements are replaced by pepsinogen sequences. When these constructs were expressed, the resultant chimeric proteins were retained intracellularly and sorted to lysosomes nearly as well as cathepsin D (23 and 20% in dense lysosomes versus 32% for cathepsin D and 5% for glycopepsinogen). Lysosomal targeting of these chimeric proteins was accompanied by efficient cleavage of the pro-segment, indicating that these proteins had folded well enough to undergo acid-induced autoactivation (Fig. 7 A). These results show that the carboxyl lobe elements of cathepsin D are sufficient to direct efficient lysosomal sorting.

The next constructs were designed to localize the critical elements in the carboxyl lobe. In CP3, an additional 42 amino acids in the carboxyl lobe of cathepsin D were replaced by analogous pepsinogen residues. Expression of this construct resulted in a chimeric protein that was poorly sorted relative to CP2 (9% in dense lysosomes versus 20%). This result indicates that cathepsin D amino acids 188 to 230 (cathepsin D numbering [Faust et al., 1985] is used throughout) are necessary for efficient sorting. The poor sorting of CP71, which contains only the 26 COOH-terminal residues

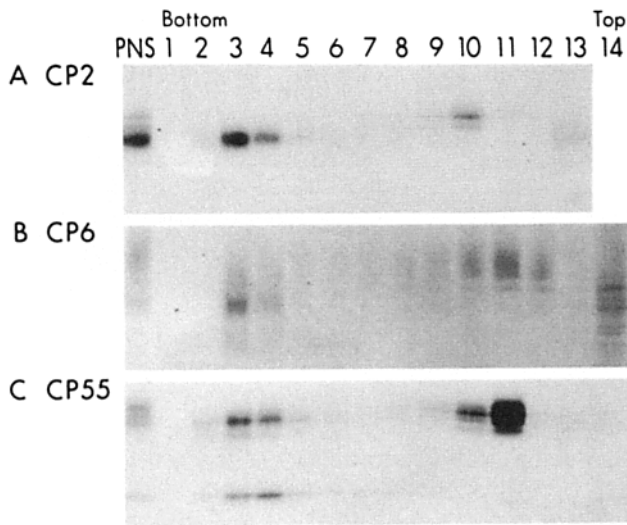


Figure 7. Percoll gradient analysis of representative chimeric proteins. Transfected ICD lymphoblasts were labeled with [³⁵S]methionine as described in the legend to Fig. 6, cells were homogenized, and postnuclear supernatants were fractionated on 36% Percoll gradients. Equal portions of each fraction (*l* = bottom) were analyzed by immunoprecipitation, SDS-PAGE, and fluorography.

of cathepsin D, is consistent with this conclusion. Both CP3 and CP71 were highly secreted, indicating that they had folded adequately to exit the ER.

Constructs CP4, CP6, and CP72 were prepared to determine which portions of the cathepsin D carboxyl lobe were sufficient to direct the chimeric protein to the lysosome. CP4, which contains cathepsin D residues 188 to 230 in the pepsinogen background, was sorted relatively poorly (6% in dense lysosomes). The inclusion of cathepsin D residues 320 to 346 with residues 188 to 230 (construct CP72) did not augment the lysosomal sorting (5% in dense lysosomes). However, since the recovery of both of these chimeric proteins was relatively low ($45 \pm 3\%$ for CP4 and $33 \pm 4\%$ for CP72), the targeting of these proteins to lysosomes may have been underestimated. CP6, which contains residues 188 to 265 of cathepsin D sequence, was targeted to lysosomes as well as CP2, which contains most of the cathepsin D carboxyl lobe (19% in dense lysosomes versus 20%, respectively; Figs. 6 and 7 *B*). These results show that cathepsin D residues 188 to 265 can act to direct lysosomal sorting of glycopepsinogen.

Cathepsin D Residues 188 to 230 Are Required for Efficient Lysosomal Sorting

While the preceding experiments show that the carboxyl lobe segment 188 to 265 of cathepsin D is sufficient to form a lysosomal sorting signal, the significant difference in sorting efficiency between CP6 and cathepsin D (19 vs 32%) clearly indicates that other cathepsin D regions contribute to sorting. In addition, these cathepsin D regions could potentially direct lysosomal sorting in the absence of the carboxyl lobe region. To assess this possibility, we assayed the sorting of chimeric proteins in which various carboxyl lobe elements of cathepsin D were replaced by analogous pepsinogen sequences. Constructs CP55 and CP56, in which cathepsin D residues 188 to 230 and 188 to 265, respectively, were

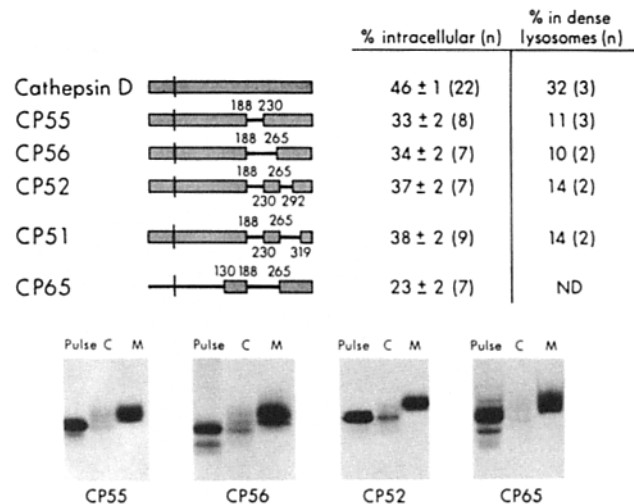


Figure 8. Cathepsin D carboxyl lobe residues 188 to 230 are necessary for efficient lysosomal sorting. Experimental details are described in the legend to Fig. 6. CP52, CP55, and CP56 were immunoprecipitated with antisera to cathepsin D; therefore, some ($\approx 10\%$) of the radioactive signal detected corresponds to the endogenously expressed cathepsin D. The recovery of immunoprecipitable protein at the end of the chase compared to the initial pulse value was as follows (mean \pm SE): Cathepsin D ($136 \pm 8\%$), CP55 ($123 \pm 11\%$), CP56 ($131 \pm 7\%$), CP52 ($72 \pm 6\%$), CP51 ($141 \pm 14\%$), and CP65 ($67 \pm 2\%$).

replaced, were significantly impaired in sorting (11 and 10% in dense lysosomes, respectively; Fig. 8). Both of these chimeric proteins were highly secreted, and the majority of the retained material was present as the proenzyme in light density compartments (Fig. 7 *C*). The chimeric protein in this compartment contained complex-type oligosaccharides, indicative of passage through the Golgi cisternae, although its exact intracellular location is unclear. This pool appeared to be stable since the extent of secretion and the distribution of the chimeric proteins on Percoll gradients did not change even after extended chase times (not shown). These results are consistent with cathepsin D residues 188 to 265 containing the most potent lysosomal sorting determinants. Further evidence to support this conclusion comes from a comparison of the results obtained with constructs CP69 (Fig. 6) and CP65 (Fig. 8). CP69, which contains cathepsin D residues 130 to 348, is efficiently sorted to dense lysosomes (23%) whereas CP65, which differs from CP69 by lacking the cathepsin D residues 188 to 265, is mostly secreted.

Constructs CP52 and CP51 differ from CP55 in also having cathepsin D residues 265 to 292 and 265 to 319, respectively, substituted with pepsinogen sequence (Fig. 8). Both of these chimeric proteins were impaired in sorting although they were targeted to dense lysosomes slightly more efficiently than CP55 (14% for each versus 11% for CP55). One possibility is that these chimeric proteins fold in such a way as to express a lysosomal sorting signal located within cathepsin D residues 230 to 265 that remains cryptic in CP55.

Involvement of Phosphotransferase Recognition Marker Elements in Man-6-P-independent Lysosomal Sorting

In previous studies, we demonstrated that two noncontiguous primary sequences from the carboxyl lobe of cathepsin D

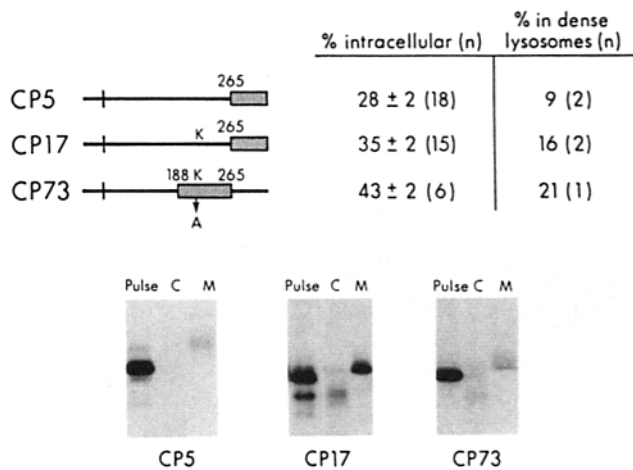


Figure 9. Involvement of lysine 203 in lysosomal sorting. Experimental details are described in the legend to Fig. 6. The recovery of immunoprecipitable protein at the end of the chase compared to the initial pulse value was $48 \pm 3\%$, $55 \pm 6\%$, and $43 \pm 13\%$ of CP5, CP17, and CP73, respectively.

(amino acids 188 to 230, particularly lysine 203, and 265 to 292) were sufficient to generate an efficient phosphotransferase recognition domain in glycopepsinogen. (Baranski et al., 1990). The involvement of the first region as a component of the Man-6-P-independent lysosomal sorting signal suggested the possibility that there may be a relationship between the two signalling motifs. Since lysine 203 is a key component of the phosphotransferase recognition motif, we tested whether this residue is important for the Man-6-P-independent lysosomal sorting pathway. When lysine 203 was changed to an alanine in the context of cathepsin D residues 188 to 265 (construct CP73), the resultant chimeric protein was sorted as well as CP6, the equivalent chimeric protein containing lysine 203 (21% of CP73 in dense lysosomes, Fig. 9, versus 19% of CP6, Fig. 6). CP5 and CP17 were tested to determine whether lysine 203 might enhance lysosomal sorting when expressed alone in the pepsinogen background. CP5 contains only cathepsin D residues 265 to 348 whereas CP17 is identical except for a glutamic acid-to-lysine mutation at position 203 (Fig. 9). When expressed, CP5 was sorted at the baseline level (9% in dense lysosomes) while CP17 was sorted at an intermediate level (16% in dense lysosomes). These results indicate that lysine 203 contributes to the Man-6-P-independent lysosomal sorting, but that other residues in the 188 to 265 region of cathepsin D can compensate for its absence. The analysis of further constructs involving residue 203 will be necessary to better define the relationship between this residue and other elements of the sorting signal.

The Chimeric Proteins Are Catalytically Active

A useful parameter to evaluate the proper folding of an enzyme is whether it is catalytically active. This is particularly helpful in the case of the aspartyl proteases since the active site contains one aspartic acid residue (Asp 33 and Asp 231) contributed by each lobe of the enzyme. To examine whether the expressed chimeric proteins had folded correctly at 28°C, we took advantage of the fact that the aspartyl proteases are specifically inhibited by pepstatin A. This compound rever-

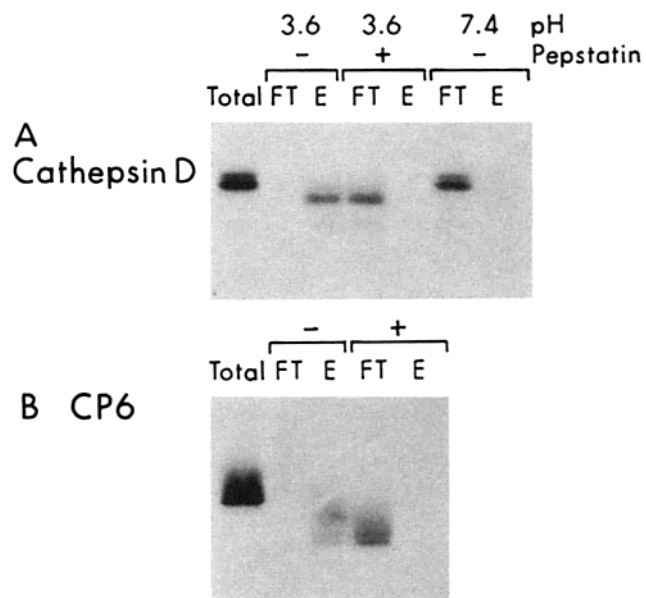


Figure 10. Binding of cathepsin D and CP6 to Pepstatin A-agarose. Aliquots of media from transfected B lymphoblasts labeled with [³⁵S]methionine were incubated with Pepstatin A agarose as described in Materials and Methods, in either the presence (+) or absence (-) of free pepstatin A. Unbound (FT) and pH 8.0-eluted (E) fractions were analyzed by immunoprecipitation, SDS-PAGE, and fluorography. (A) Cathepsin D-transfected cells. Samples were immunoprecipitated with antisera to cathepsin D. (B) CP6-transfected cells. Samples were immunoprecipitated with antisera to pepsinogen.

sibly binds to the active site in a strictly pH-dependent manner (Davies, 1990). As shown in Fig. 10 A, the expressed cathepsin D bound to pepstatin A-agarose at pH 3.6 and was eluted at pH 8.0. This binding was completely inhibited by the inclusion of free pepstatin A and did not occur at pH 7.4. Identical results were obtained with CP6 (Fig. 10 B) and with all the other chimeric proteins used in this study.

Interestingly, all of the proteins tested, including cathepsin D, exhibited acid-induced proteolysis, presumably due to autocatalytic cleavage of the prosegment, even in the presence of pepstatin A. The reason for this pepstatin A-resistant autoactivation is unclear, but it may reflect the specific pH conditions of the experiments. At pH 3.6, as opposed to more strongly acidic conditions, the prosegment may not be adequately displaced from the active site of admit the inhibitor before autoactivation occurs (Marciniszyn et al., 1976). Nevertheless, the ability of these proteins to autoactivate and to bind to pepstatin A argues strongly for the proper folding of their active sites.

Discussion

The Man-6-P-independent lysosomal targeting pathway in mammalian cells has been known to exist for over a decade, but the nature of the molecular signals that mediate this targeting have not been previously identified. The availability of a B lymphoblastoid cell line from a patient with ICD has allowed us to study the targeting of newly synthesized cathepsin D to lysosomes in the absence of a Man-6-P-sorting signal. We find that this acid protease traverses the secretory

pathway as far as the *trans*-most cisternae of the Golgi complex and that about 45% of the molecules are sorted to dense lysosomes while the rest of the enzyme is secreted. Since the cathepsin D synthesized by the ICD lymphoblasts contains no detectable Man-6-P, the lysosomal targeting must be occurring by a Man-6-P-independent mechanism. This Man-6-P-independent pathway is less efficient than the Man-6-P-dependent one expressed in normal lymphoblasts. ICD fibroblasts appear to lack the Man-6-P-independent pathway since they secrete almost all their newly synthesized cathepsin D (Hasilik and Neufeld, 1980*a,b*; and this study). Di-Cioccio and Miller (1991) have reported that the lysosomal hydrolase α -L-fucosidase is retained intracellularly in ICD lymphoblasts even though it is not phosphorylated. Although cell fractionation was not performed in that study to verify lysosomal targeting, comparison with the results presented here suggests that cathepsin D is sorted more efficiently than α -L-fucosidase.

The sorting of proteins to lysosomes via the Man-6-P-independent pathway is selective for acid hydrolases. In our experiments, 32% of transfected cathepsin D was targeted to dense lysosomes versus 5% of the homologous secretory protein, glycopepsinogen. Similar pulse-chase experiments following the fate of immunoglobulin M, a major secretory protein of B lymphocytes, failed to show any significant targeting of this protein to lysosomes, even when cells were preincubated with protease inhibitors to prevent lysosomal degradation. These results indicate that the lysosomal targeting of cathepsin D is not the result of nonspecific "spillover" from the constitutive secretory pathway.

The major goal of this study was to identify the structural determinants required for protein targeting to lysosomes via the Man-6-P-independent pathway. The finding that nonglycosylated cathepsin D was targeted to lysosomes almost as efficiently as normal cathepsin D indicated that the cathepsin D amino acid sequence must contain the specific sorting signal(s). By making chimeric proteins between cathepsin D and glycopepsinogen, which lacks the sorting signal even though it shares 45% amino acid sequence identity with cathepsin D, we have been able to identify elements of the signal. The analysis of a number of cathepsin D/glycopepsinogen chimeric proteins reveals that amino acids 188 to 265 of the cathepsin D carboxyl lobe are capable of directing efficient sorting to lysosomes. Within this region amino acids 188 to 230 are essential for this sorting, but appear to require residues 230 to 265 to form an active signal. In addition, since CP6, which contains cathepsin D residues 188 to 265, is not sorted as well as cathepsin D, it is likely that other regions of the cathepsin D molecule contribute to the sorting signal. Examination of the location of amino acids 188 to 265 in the crystal structure of porcine pepsinogen (Baranski et al., 1990) reveals that they form an extended surface domain on the carboxyl lobe. One residue within this domain, lysine 203, appears to contribute to the sorting signal although other residues in this region can compensate for its absence.

The finding that amino acids 188 to 265 function as a sorting signal for the Man-6-P-independent targeting pathway is of particular interest in view of our current understanding of the phosphotransferase recognition domain on cathepsin D. We have found that the combined substitution of two regions of cathepsin D (amino acids 188 to 230, particularly lysine

203, and amino acids 265 to 292) into the analogous positions of glycopepsinogen generates a strong recognition domain for phosphotransferase. These two sequences are in direct apposition on the surface of the molecule and appear to form a surface patch that contains multiple interacting sites (Baranski et al., 1990, 1991). It is apparent that the determinants required to form the signal for the Man-6-P-independent targeting pathway overlap with the first region of the phosphotransferase recognition motif. Furthermore, lysine 203 seems to form part of both signals. These results, taken together, lead us to conclude that the two determinants overlap but are not identical.

While the actual function of the cathepsin D carboxyl lobe elements in the Man-6-P-independent sorting pathway remains to be elucidated, the simplest explanation is that these determinants allow binding to a receptor in the Golgi complex which then delivers the bound ligand to the lysosome or to a prelysosomal compartment. The fact that the efficiency of endogenous cathepsin D sorting is 45% and the finding that the sorting is decreased to 32% when cathepsin D is tenfold overexpressed suggests that the putative receptor system may be of limited capacity. This model would also explain the observation that the Man-6-P-independent sorting pathway only functions in selected cell types if the receptor is expressed in a cell type-specific manner. However, other mechanisms could account for the Man-6-P-independent sorting, such as a model in which the various acid hydrolases would selectively aggregate with each other and interact with a membrane component in the Golgi complex. This type of mechanism is currently believed to mediate the sorting of proteins to secretory granules (Miller and Moore, 1990). We are currently trying to identify cellular component(s) that recognize non-phosphorylated cathepsin D in order to determine which of these models is operative.

In studying the delivery of transfected chimeric proteins to the lysosome, it is necessary to distinguish an active sorting process from nonspecific targeting of misfolded proteins. There are several reasons why we believe we are observing the former process rather than the latter. First, all the constructs used in this study acquired endo H-resistant oligosaccharides and were secreted to variable extents, indicating that they had folded sufficiently to exit the ER. Chimeric constructs which did not meet these criteria were considered noninformative. Second, the sorting values for the various chimeric proteins fell between those of cathepsin D and glycopepsinogen, consistent with the notion that the interchange of cathepsin D and pepsinogen residues destroys or generates a sorting signal present in cathepsin D but absent in pepsinogen. Finally, all the chimeric proteins bound specifically and quantitatively to pepstatin A-agarose. Therefore, the aspartyl protease active site of each of these proteins must have folded sufficiently well to bind a small molecule competitive inhibitor. Since each lobe contributes an active site aspartic acid to the binding cleft, the overall bilobed structure must also be intact. Nevertheless, five of the fifteen chimeric proteins (CP4, CP5, CP17, CP72, and CP73) did exhibit a significant turnover during the chase period, with between 45% and 67% of the molecules being degraded. Although most of this degradation probably occurred in the ER, it is possible that the actual delivery of these chimeric proteins to lysosomes was underestimated. Even if this were the case, the conclusions regarding the importance of

cathepsin D amino acids 188 to 265 in lysosomal targeting would not be significantly altered since all the critical constructs (CP2, CP3, CP6, CP55, and CP56) survived as well as cathepsin D and glycopepsinogen.

Several investigators (Diment et al., 1988; McIntyre and Erickson, 1991; Rijnbouts et al., 1991) have found that the precursor forms of cathepsin D and other lysosomal proteases become membrane associated during their biosynthesis in a variety of cell types, and that this association appears to occur independently of Man-6-P residues. These findings provide some evidence for an unrecognized lysosomal enzyme binding protein which could play a role in targeting. However, since membrane association in these studies was observed primarily in endosomes and only at relatively acidic pH, the relationship of this phenomenon to lysosomal enzyme sorting in the Golgi is uncertain at present. Studies in the yeast *Saccharomyces cerevisiae* have demonstrated that amino acids in the propeptides of proteinase A and carboxypeptidase Y are sufficient to direct the sorting of these soluble proteins to the lysosome-like vacuole (Johnson et al., 1987; Klionsky et al., 1988; Valls et al., 1990). This contrasts with our findings which fail to show a role for the cathepsin D propeptide in lysosomal targeting. However, in the case of proteinase A, an aspartyl protease which shows 46% identity in amino acid sequence with cathepsin D, the possibility of a second sorting signal in the mature protein could not be excluded (Klionsky et al., 1988). The possible relationship between the yeast vacuolar sorting pathway and the system we have characterized in human B lymphoblasts should become clearer as the components of the two systems are further characterized.

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