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. 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-phosphotransferase α-N-acetylglucosaminidase to generate an exposed mannosyl 6-phosphat (Man-6-P) 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; 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,
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 pepstatin, 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 (Varik and Kornfeld, 1983). Rabbit antisera to human pepstatinogen were generated by injection of the purified protein (gift of D. L. Michael Samioff, University of California, Los Angeles, CA). EXPRES35S protein-labeling mix was from DuPont/NEN (Wilmington, DE). All restriction and other DNA modifying enzymes were from New England Biolabs (Beverley, 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. (Madison, WI). Endo Hase neuraminidase H 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 Immunologicals (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). CGL-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 x 10^6/ml in a humidified 5% CO_2 atmosphere.Skin fibroblasts from normal individuals and ICD patients, obtained from the Human Genetics Mutant Cell Repository (Bethesda, MD), were maintained in MEM-a, 10% FCS (Hazleton Biologics, Lexena, KS), 100 U/ml penicillin, 100 U/ml streptomycin.

ICD lymphoblasts were electroporated essentially as in Margolles et al. (1988). All procedures were carried out under aseptic conditions. 5 x 10^6 cells in logarithmic growth phase were pelleted, washed once with PBS, and resuspended in 500 μl HEPES buffered saline containing 100-150 μg plasmid DNA and 500 μg sheared salmon sperm DNA. 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, 1050 μF (pulse length 6-9 ms). Electroporated cells were maintained at 5 x 10^6/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 x 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 μM leupeptin and 200 μM pepstatin A.

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 similarly. The cDNA for human cathepsin D from the appropriate region of the dried gel and solubilized in 30% H_2O_2 at 65°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. 1,25[Methyl-3H]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 μl homogenization buffer plus protease inhibitors (see above). Cells were homogenized by 15 passes through a 200 μl 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 μl 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 10 ml ScintiVerse I (Fisher Scientific Co.) was added, and samples were counted in a liquid scintillation counter (LS 6800; Beckman Instruments).
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 PMSE, pH 6.0, and 6 μM Vibri 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 μl of [35S]methionine-labeled cell lysate or media samples were added to 500 μl 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 PMSE 0.25 TIU/ml aprotinin, 10 μM 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 μl 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 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 antisera and analyzed by SDS-PAGE and fluorography.

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 antisera. 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.

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 (1= 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 ± 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 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 ± 1% (n = 29 under all conditions)

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.
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 D 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 processing 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 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-
define the phosphotransferase recognition marker (Baranski et al., 1990).

% intracellular (n) % in dense lysosomes (n)

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<th>Protein Type</th>
<th>n (intracellular)</th>
<th>n (dense lysosomes)</th>
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<td>Cathepsin D</td>
<td>46 ± 1 (22)</td>
<td>32 (3)</td>
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<tr>
<td>mpep12</td>
<td>20 ± 1 (16)</td>
<td>5 (3)</td>
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<td>45 ± 3 (7)</td>
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<td>CP2</td>
<td>41 ± 2 (7)</td>
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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 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 (Braulke 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. 7A). 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...
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 proteins 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 ± 3% for CP4 and 33 ± 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 230 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 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 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 (i = bottom) were analyzed by immunoprecipitation, SDS-PAGE, and fluorography.](image)

![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 (≈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 ± SE): Cathepsin D (136 ± 8%), CP55 (123 ± 11%), CP56 (131 ± 7%), CP52 (72 ± 6%), CP51 (141 ± 14%), and CP65 (67 ± 2%).](image)
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 ± 3%, 55 ± 6%, and 43 ± 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 reversibly 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.

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, 1980a,b; and this study). DiCioccio 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 this 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 lysosomal targeting, consistent with the notion that the intercisternal 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
zyme binding protein which could play a role in targeting. Several investigators (Diment et al., 1988; McIntyre and Erickson, 1991; Rijnbout 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 proteasine 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; Vals et al., 1990). This contrasts with our findings which fail to show a role for the cathepsin D propiece in lysosomal targeting. However, in the case of proteasine 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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