Cathepsin D Is Membrane-associated in Macrophage Endosomes*

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Previously we identified an acid protease activity which was located in the endosomes of rabbit alveolar macrophages (Diment, S., and Stahl, P.D. (1985) J. Biol. Chem. 260, 15311-15317). In this study, the endosomal protease is identified as cathepsin D by immunoprecipitation with polyclonal antibodies raised against rabbit cathepsin D and by NH₂-terminal sequence. In order to elucidate the mechanism for targeting of cathepsin D to endosomes, we first examined the membrane association of cathepsin D with light (ρ = 1.05 g/ml) and heavy density (ρ = 1.1 g/ml) vesicles from Percoll density gradients. After sequential washes, 8.4 and 21.9% of cathepsin D activity remained associated with heavy and light density vesicles, respectively. This membrane-associated cathepsin D could not be solubilized in either buffer at pH 5.0 containing mannose 6-phosphate and EDTA or in buffer at pH 10.6. Solubilization required the detergent Triton X-100. To determine whether membrane-associated cathepsin D was found in endosomes, the enzyme was radiiodinated within endosomes and lysosomes with internalized lactoperoxidase. The membrane-associated form was detected in endosomes, but much less in lysosomes. Biosynthetic studies combined with the same extraction procedure revealed that macrophage cathepsin D is first synthesized as an inactive membrane-associated precursor. The precursor is processed to an active, membrane-associated form and then to the active soluble form found in lysosomes. Our studies provide evidence that 1) cathepsin D is in endosomes of macrophages; 2) cathepsin D is transported to endosomes as a membrane-associated form; and 3) the membrane-associated form is a biosynthetic precursor for the soluble form found in endosomes and lysosomes.

When rabbit macrophages were allowed to take up the neoglycoprotein mannose-BSA, rapid degradation of the ligand was observed (1). Kinetic experiments combined with subcellular fractionation indicated that degradation took place in the endosomes before delivery of the ligand to lysosomes had occurred and that peptatin A-sensitive endosomal protease activity was responsible. Recent evidence from other laboratories suggests that other enzymes (for example, cathepsin E in human and mouse fibroblasts (2, 3), carboxypeptidase in mouse 3T3 fibroblasts (4), leucyl β-naphthylamidase in HeLa cells (5)) may also be present in endosomes. Such data challenge the notion that acidic enzymes are found only in lysosomes and suggest that endosomal forms of these enzymes may possess structural information that allows them to accumulate in endosomes.

We first set out to determine the identity of the peptatin-sensitive protease isolated from rabbit macrophages. In our studies, the enzyme responsible for degradation of mannose-BSA was identified as an acidic protease, subunit size 46 kDa, which was sensitive to peptatin A. Approximately 35% of this activity was associated with non-lysosomal membranes in the macrophages (1). Peptatin A is a specific inhibitor of aspartyl proteases such as pepsin, renin (extracellular), and cathepsins D and E (intracellular) (6). Since cathepsin D is the more abundant intracellular aspartyl protease in alveolar macrophages (7), we attempted to determine if this was the identity of the proteolytic activity we had observed in endosomes.

In these studies, we have purified the peptatin-sensitive protease from whole rabbit macrophages and have confirmed by amino-terminal sequence analysis that this protein is cathepsin D. In addition, we have examined the differences in membrane association between the lysosomal form of cathepsin D, the lysosomal marker β-hexosaminidase, and the non-lysosomal form of cathepsin D. Finally, using polyclonal antibodies, we have investigated the biosynthesis of a membrane-associated form of cathepsin D in an attempt to clarify the relationship between the endosomal and lysosomal forms.

EXPERIMENTAL PROCEDURES

Materials

Peptatin A-agarose was purchased from Pierce. Protease inhibitors, protein A-Sepharose, lactoperoxidase, Percoll, and ninhydrin were supplied by Sigma. Dextroxyribonuclease I, swine anti-goat IgG, and glutaraldehyde affinity matrix were from Boehringer Mannheim. Radiochemicals were from Amersham.

Methods

Purification of Cathepsin D from Rabbit Cells—Rabbit macrophages were harvested by lung lavage (8). The cells were solubilized in 1% Triton/PBS containing 1 μg/ml of leupeptin and chymostatin and centrifuged at 100,000 × g for 90 min at 4 °C to remove undissolved material. The supernatant was taken to pH 4.5 by adding 1 M sodium acetate buffer at pH 4.0 (final concentration, 100 mM sodium acetate). Cathepsin D was then purified by affinity chromatography on peptatin A-agarose as previously described (1). An average yield was 2.5 μg of purified cathepsin D/10⁶ macrophages, and the specific activity was 0.66 units/μg. The purified protein and all immunoprecipitates obtained in these studies were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) according to the method of Laemmli (9).

Anti-rabbit cathepsin D antiserum was raised in a goat and specific
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IgG isolated by affinity chromatography on cathepsin D coupled to cyanogen bromide-activated Sepharose (200 μg of cathepsin D/ml of Sepharose). The antiserum yielded 324 μg of purified IgG/ml.

Fractionation of Rabbit Macrophages and Extraction of Cathepsin D—Rabbit macrophages were disrupted by nitrogen cavitation. Nuclei and mitochondria were removed by centrifugation at 200 × g. Membrane vesicles were collected from the supernatant, and these were fractionated by centrifuging through 1.07 g/ml Percoll density gradients to separate dense vesicles (lysosomes) (ρ = 1.1 g/ml) from light membranes (endosomes) (ρ = 1.05 g/ml). Lysosomes were identified by assaying for the marker enzyme β-hexosaminidase (1). In order to determine subcellular localization of light membranes (endosomes) and dense membranes (lysosomes) in the lumen of the vesicles and membrane-associated proteins, the vesicle membranes were subjected to the following washing procedure. First the vesicles were disrupted by one cycle of freeze-thawing and then homogenized in 5 mM Tris buffer, pH 7.4, containing 5 mM mannose 6-phosphate (buffer A); the membranes were harvested by centrifugation at 100,000 g for 15 min. Contaminating cytoplasmic and soluble proteins were removed by washing with 100 mM KCl and 0.1% Triton X-100. Undegraded substrate was precipitated by adding ice-cold trichloroacetic acid (final concentration 3.5%), and acid-soluble fragments were determined by reaction with ninhydrin after incubation for 1 h with enzyme. Protein concentrations were assayed using Bio-Rad reagent and BSA as standard.

Identification of the Pepstatin-sensitive Endosomal Protease—Pepstatin-sensitive protease was purified from alveolar macrophages by affinity chromatography on pepstatin A-agarose (see "Methods"). When analyzed by SDS-PAGE, the purified enzyme migrated as a single protein of 46 kDa (Fig. 1). The amino-terminal sequence of this protein (Table I) was very similar to the amino-terminal sequence of the light chain of porcine cathepsin D (13). Compositional analysis also revealed a marked similarity between the rabbit protease and porcine cathepsin D (data not shown). Affinity-purified goat IgG raised against this protein was used to confirm that the endosomal pepstatin-sensitive protease is cathepsin D. First, endosomal proteins were iodinated using internalized lactoperoxidase as described previously (1, also "Methods"). The radiolabeled cells were then washed and lysed with 0.5% Triton X-100/PBS prior to immunoprecipitation. Fig. 2 shows that anti-cathepsin D IgG specifically immunoprecipitated this endosomal protein from the solubilized cells.

Extraction of Cathepsin D from Non-lysosomal and Lysosomal Vesicles—Rabbit macrophages were disrupted by nitrogen cavitation, and the subcellular compartments were fractionated according to density on Percoll gradients. Gradient fractions were collected and assayed for β-hexosaminidase, a lysosomal marker, or for cathepsin D activity. Greater than 90% of the total β-hexosaminidase activity was detected in the densest fractions on these gradients (ρ = 1.10 g/ml), whereas cathepsin D activity was distributed 65 and 35% in heavy and light (ρ = 1.05 g/ml) fractions, respectively (Fig. 3). When these vesicles were washed sequentially with buffers

TABLE I

<table>
<thead>
<tr>
<th>Amino-terminal sequence of rabbit cathepsin D</th>
</tr>
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<tbody>
<tr>
<td>Purified cathepsin D from rabbit macrophages was sequenced from the amino-terminal end. The sequence is compared with that obtained for the light chain of cathepsin D from porcine spleen (13).</td>
</tr>
</tbody>
</table>

Rabbit

Porcine light chain

GVPEILRNMDAQQYVGEIGITPP-

GPIEVLKNMDAQQYVGEIGITPP-
cathepsin D was immunoprecipitated with goat anti-cathepsin D IgG. Dosomal proteins were iodinated using internalized lactoperoxidase control IgG.

To density by centrifuging through a 1.07 g/ml self-forming Percoll gradient (1). Each fraction was assayed for both cathepsin D and p-hexosaminidase activities as described under "Methods." O, cathepsin D activity; S, specific anti-cathepsin D IgG; NS, nonspecific control IgG.

Immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography. S, specific anti-cathepsin D IgG; NS, nonspecific control IgG.

FIG. 2. Immunoprecipitation of endosomal cathepsin D. Endosomal proteins were iodinated using internalized lactoperoxidase as a catalyst (see "Methods"). The labeled cells were lysed and cathepsin D was immunoprecipitated with goat anti-cathepsin D IgG. Immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography. S, specific anti-cathepsin D IgG; NS, nonspecific control IgG.

FIG. 3. Fractionation of rabbit alveolar macrophages on Percoll gradients. Rabbit macrophages were fractionated by nitrogen cavitition, and then vesicle membranes were separated according to density by centrifuging through a 1.07 g/ml self-forming Percoll density gradient (1). Lysosomes sediment at approximately 1.10 g/ml in these gradients (Fraction 1); endosomes sediment at 1.05 g/ml (top). Each fraction was assayed for both cathepsin D and p-hexosaminidase activities as described under "Methods." ○, cathepsin D activity; ●, p-hexosaminidase activity.

A–D, more than 90% of the total p-hexosaminidase and cathepsin D activities were solubilized when the dense lysosomal fractions were lysed in buffer A (Table II). Buffer A contained 5 mM Tris at pH 7.4, which was used to lyse the vesicles by hypotonic shock. Mannose 6-phosphate was included in this buffer to inhibit binding of either enzyme to phosphomannosyl receptors exposed during lysis. In contrast, 68% of cathepsin D and 78% of p-hexosaminidase were recovered in the buffer A wash of non-lysosomal vesicles. Washing with buffer B resulted in the recovery of small amounts of each enzyme from lysosomal fractions, and the remaining p-hexosaminidase in non-lysosomal vesicles was recovered in this wash. Buffer B consisted of 100 mM sodium acetate at pH 5.0, containing 5 mM mannose 6-phosphate and 5 mM bicarbonate buffer, pH 10.6 (buffer C), was chosen since this was found to be sufficient to solubilize all of the remaining cathepsin D activity (Fig. 4). 8.4% of the total lysosomal cathepsin D and 21.9% of the total p-hexosaminidase activity required detergent extraction from lysosomes, and no p-hexosaminidase activity was detected in the detergent extracts from non-lysosomal membranes. Finally, proteins remaining associated with the membranes after washes A–C were solubilized with Triton X-100. Triton X-100 (0.5%) was chosen to dissociate any remaining proteins which may adhere to membranes through a charge interaction (14). Less than 1% of each enzyme was detected in this buffer wash of lysosomal membranes, but a small fraction (3.7%) of cathepsin D activity was dissociated from non-lysosomal membranes. Finally, proteins remaining associated with the membranes after washes A–C were solubilized with Triton X-100 in PBS. 100,000 × g supernatants from each extraction were assayed for cathepsin D activity and compared with total membrane-associated activity solubilized in 1% Triton X-100/ PBS.

TABLE II

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Lysosomal C-D</th>
<th>Non-lysosomal C-D</th>
<th>Lysosomal p-Hex</th>
<th>Non-lysosomal p-Hex</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>90.1</td>
<td>68.2</td>
<td>93.1</td>
<td>78.1</td>
</tr>
<tr>
<td>B</td>
<td>1.8</td>
<td>2.2</td>
<td>4.2</td>
<td>21.9</td>
</tr>
<tr>
<td>C</td>
<td>0.9</td>
<td>3.7</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>D</td>
<td>8.4</td>
<td>21.9</td>
<td>1.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>

pH 5.0 containing 5 mM mannose 6-phosphate and 5 mM EDTA, conditions which are sufficient to dissociate enzymes attached to membrane receptors. The third wash, sodium bicarbonate buffer, pH 10.6 (buffer C), was chosen to dissociate any remaining proteins which may adhere to membranes through a charge interaction (14). Less than 1% of each enzyme was detected in this buffer wash of lysosomal membranes, but a small fraction (3.7%) of cathepsin D activity was dissociated from non-lysosomal membranes. Finally, proteins remaining associated with the membranes after washes A–C were solubilized with Triton X-100. Triton X-100 (0.5%) was chosen since this was found to be sufficient to solubilize all of the remaining cathepsin D activity (Fig. 4). 8.4% of the total lysosomal cathepsin D and 21.9% of the non-lysosomal activity required extraction in detergent, whereas less than 2% of the total p-hexosaminidase activity required detergent extraction from lysosomes, and no p-hexosaminidase activity was detected in the detergent extracts from non-lysosomal fractions.

Membrane Association of Cathepsin D in Endosomes—The results described in Table II indicated that a subpopulation of cathepsin D may be closely associated with the membranes sedimenting in the light density fractions from Percoll gradients. While these gradients are designed to separate dense lysosomes from other cell membranes, the light fractions consist of a mixture of Golgi, plasma membrane, and endo-
proteins were iodinated using lactoperoxidase internalized for 5 min (see “Methods”). Under these conditions, lysosomal vesicles were not labeled (1); however, iodinated lysosomes were prepared for comparison by allowing internalization of lactoperoxidase to proceed for 60 min and then harvesting the dense lysosomal fractions from Percoll gradients. After lysing the labeled membranes and extracting in buffers A–D, the cathepsin D in each buffer was isolated by affinity chromatography on pepstatin-agarose. Fig. 5 shows the results of SDS-PAGE analysis and autoradiography of the cathepsin D extracted in each buffer. In these experiments, only cathepsin D which is in contact with the endocytosed lactoperoxidase can be labeled (10). However, a comparison of the cathepsin D labeled in lysosomes with the same enzyme labeled in endosomes revealed that the distribution of enzyme in each vesicle was different. All of the detectable cathepsin D labeled in lysosomes was released by lysis in buffer A, suggesting that it was free in the lumen of these vesicles. In contrast, approximately half of the detectable cathepsin D labeled in endosomes remained membrane-associated through washes with buffers A and B. A small portion of the enzyme was released in buffer C from the endosomal membranes, but the remaining cathepsin D required extraction in detergent buffer D.

**Biosynthesis of Cathepsin D in Rabbit Macrophages**—The cathepsin D distribution in endosomes illustrated in Fig. 5, indicated that at least a portion of this enzyme was closely associated with the membranes. To determine whether such membrane association was a result of post-translational modifications of the enzyme (in which case, the membrane-associated form might be expected to represent a separate subpopulation of the cathepsin D pool), or whether the membrane-associated form of the enzyme was a precursor of the soluble form, we studied the biosynthesis of cathepsin D in rabbit macrophages. Fig. 6 shows that during 60-min pulse-labeling with 35S-methionine, rabbit cathepsin D was synthesized initially as a 53-kDa precursor. After a 1-h chase, most of the precursor was cleaved to an intermediate form of 47 kDa which persisted through a 2-h chase. Minor modifications were then made as the enzyme was chased to its mature form of 46 kDa between 2 and 8 h. Less than 10% of the total immunoprecipitable radioactivity was released into the medium during these incubations. There was no significant loss of label for up to 11 h suggesting that negligible degradation of cathepsin D had occurred. To determine which of the cathepsin D precursors were catalytically active, we isolated the enzyme from labeled cell lysates by affinity chromatography on pepstatin A-agarose, followed by elution of labeled cathepsin D and SDS-PAGE analysis (Fig. 7). No 53-kDa species from the 1-h pulse was found, indicating that this form was inactive. The 47-kDa form which was found following the 2-h chase (Fig. 6) bound to pepstatin A-agarose and after an 8-h chase, the cathepsin D bound to the pepstatin A-agarose was eluted and detected after SDS-PAGE and autoradiography.

**Membrane Association of Cathepsin D**—To determine whether the newly synthesized 53-kDa cathepsin D precursor was membrane-associated, macrophages were pulse-labeled with 35S-methionine for 1 h as described in Fig. 6. The pulsed cells were not chased, but lysed in buffer A, sequentially
washed with buffers B and C, and finally solubilized in buffer D. The washes were all adjusted to neutral pH and were subjected to immunoprecipitation with anti-cathepsin IgG as described for Fig. 6. When analyzed by SDS-PAGE and autoradiography, more than 90% of the precipitated 53-kDa precursor was detected in extraction buffer D, while the remaining portion of cathepsin D precursor was observed in wash buffer A (Fig. 8). To discover if the 47- and 46-kDa forms were also membrane-associated and to investigate whether membrane-associated cathepsin D was a precursor for the soluble form found in lysosomes, the cells were labeled for 1 h as for Fig. 6, but the labeled macrophages were chased in unlabeled medium for 2 and 8 h. After immunoprecipitation of labeled cathepsin D, the distribution of the protease between soluble buffers A–C and the detergent-containing buffer D was determined (Fig. 9). As in Fig. 8, the 53-kDa precursor was detected as the membrane-associated form after a 1-h pulse-label. After chasing for 2 h, cathepsin D was partially processed to the 47-kDa form, which also required detergent extraction from the membranes. The soluble form of cathepsin D was first detected in 8-h chase cell lysates, when both soluble and membrane-associated forms had been processed to the mature 46-kDa form.

**DISCUSSION**

The first aim of these studies was to identify the endosomal pepstatin-sensitive protease found in rabbit alveolar macrophages. After purification of the protease from macrophage lysates, this protein was sequenced from the amino-terminal end, and the sequence was compared with those reported for cathepsin D from other sources. The amino terminus of the rabbit enzyme (Table I) was found to be closely related to the sequence of the light chain of porcine (13) and human (15) cathepsin D. Unlike bovine, porcine (16), rat (17), and human (18) cathepsin D, mature rabbit cathepsin D is not composed of two subunits of approximately 14 and 31 kDa, but migrates as a single protein of 46 kDa under reducing conditions (Fig. 1). In nonreducing SDS-polyacrylamide gels, rabbit cathepsin D migrated as a 43-kDa protein (data not presented), suggesting that internal disulfide bonds exist similar to those which connect the light and heavy subunits of cathepsin D from other species. It remains to be determined why cathepsin D should be cleaved into light and heavy chains in other species but not in rabbit cells. It is possible that the internal sequence of rabbit cathepsin D differs sufficiently from other species that the enzyme is rendered resistant to processing during biosynthesis. Alternatively, the rabbit may lack processing enzymes associated with maturation of cathepsin D to the light and heavy subunits. In either case, the lack of cleavage is not confined to macrophages, but has been observed for cathepsin D from rabbit heart cells (19).

Using antibodies raised against purified rabbit cathepsin D, we were able to immunoprecipitate $^{131}$I-cathepsin D which had been radiolabeled in macrophage endosomes (Fig. 2), confirming the identity of the pepstatin-sensitive protease (1). Recently, these antibodies have been used in morphological studies to immunolocalize cathepsin D in multivesicular endosomes of macrophages (20), while other researchers have identified the enzyme in multivesicular endosomes of hepatoma cells (21). We next set out to determine how cathepsin D might be targeted to endosomes. There are at least two possible mechanisms by which cathepsin D may come to be localized in endosomes. The endosomal form may be a precursor of the lysosomal form which passes through endosomes en route to lysosomes. Alternatively, cathepsin D may be targeted specifically to endosomes where it is retained in an intracellular pool separate from lysosomal cathepsin D. Previous morphological data might favor the former, since mannose 6-phosphate receptors have been identified in endosomes (22–24), and dissociation of lysosomal enzymes from mannose 6-phosphate receptors is now thought to occur in endosomes (22). However, there have been only a few reports of endosomal enzyme activities (2–5) beside our own. In our studies, 9% of the total intracellular β-hexosaminidase activity sedimented with non-lysosomal membranes in Percoll density gradients of fractionated macrophages (Fig. 2). In contrast, 35% of the total cathepsin D activity was detected in non-lysosomal fractions, and similar heterogeneity of distribution has been observed for other acidic hydrolases (25, 26). If entry of these enzymes to endosomes is coordinated by mannose 6-phosphate receptors, a regulated or differential exit from the endosomes must be assumed to take place to explain the observed heterogeneity of enzyme distribution among subcellular fractions.

Data that may explain why cathepsin D accumulates in endosomes are described in Table II and Figs. 4 and 5. Under conditions sufficient to dissociate ligands from mannose 6-
phosphate receptors (27), β-hexosaminidase was extracted in full from lysosomes, whereas a portion of cathepsin D activity remained membrane-associated. In non-lysosomal membranes, a significant amount of cathepsin D was resistant to washes A and B. Little of the remaining activity was extracted by careful washing of the membranes at pH 10.6. The cathepsin D resistant to the dissociating buffers A-C was only solubilized by nonionic detergent Triton X-100 (Fig. 4). To determine whether the detergent-soluble form of cathepsin D found in non-lysosomal membranes could be detected in endosomes, endosomal and lysosomal forms of cathepsin D were radioiodinated. The distribution of [125I]-cathepsin D isolated from each of buffers A–D revealed that the membrane-associated form was present in endosomes, but absent from lysosomes (Fig. 5). These data differ slightly from the data obtained in Table II, in which 8% of the total lysosomal cathepsin D required detergent for solubilization. The apparent difference may result from a greater sensitivity obtained in enzyme assays compared with the sensitivity of detection by autoradiography, or it may result from differences in the activity of lactoperoxidase to catalyze iodination of membrane-associated cathepsin D compared with unbound form. In either case, both types of experiment provide evidence that membrane-associated cathepsin D is enriched in non-lysosomal membranes. In addition, the requirement for detergent to solubilize the membrane-associated enzyme (Fig. 4) suggested that the interaction between cathepsin D and the membranes may require the insertion of a hydrophobic moiety on the enzyme into the lipid bilayer.

To determine when newly synthesized cathepsin D became membrane-associated, the biosynthesis and processing of the enzyme was studied. Cathepsin D in rabbit macrophages, like the human enzyme (18), was synthesized initially as an inactive 53-kDa precursor, which was processed to a catalytically active form after a 2-h chase period. The time course for activation and processing of cathepsin D agrees with that reported for cathepsin D in perfused cardiac tissue from rabbits (19), although the mature form of the enzyme immunoprecipitated by these researchers was slightly larger. Immunoprecipitation of newly synthesized cathepsin D from soluble buffer washes and detergent extracts shows that more than 90% of the cathepsin D was membrane-associated after a 1-h labeling (Fig. 9). (Preliminary data indicate that this form also partitions in the detergent phase when extracted with Triton X-114 (28).) In subsequent chase periods, the immunoprecipitable radioactivity disappeared from membrane fractions and appeared in the soluble washes (Fig. 9), indicating that the membrane-associated form was the precursor for the soluble form.

In summary, our data provide evidence that cathepsin D is synthesized as a membrane-associated form in rabbit macrophages. This form is processed from an inactive precursor to an active form while attached to the membranes. The membrane-associated form is found in endosomes and is converted to a soluble form over several hours. Many questions arise from these observations. First, is cathepsin D delivered to endosomes aboard mannose 6-phosphate receptors? Our data have not excluded this possibility, but it is clear that there is an additional membrane association of the enzyme both in endosomes and, presumably, in the Golgi. Second, given the dynamic nature of endosomal compartments, how is cathepsin D conserved in these vesicles? Several models may be considered for intracellular transport of cathepsin D. It is possible that a membrane-associated form could recycle between endosomes and the cell surface; such recycling has been described for a number of plasma membrane receptors (29).

Alternatively, cathepsin D may be transported from endosomes to lysosomes together with incoming ligands, but the membrane-associated form may be recovered with membrane recycling to endosomes. Another possible route for cathepsin D transport may include sequestration in small vesicles or tubules which sediment with light density vesicles and are able to fuse with endosomes under given conditions. Third, what is the mechanism for membrane association of cathepsin D in macrophages? Our data indicate that binding to the membrane through known glycoprotein receptors is unlikely, since such interactions may be disrupted at low or high pH. Alternatively, the interaction may be mediated by a hydrophobic moiety attached covalently to the enzyme. Two classes of membrane association have been described which allow accumulation of proteins in endoplasmic reticulum. First, the acid hydrolase β-glucuronidase is synthesized as two forms. One form of the enzyme is found in the lumen of lysosomes; the other form remains in the endoplasmic reticulum where it is anchored to an auxiliary protein, egasyn (30). Under the experimental conditions described in this paper, an egasyn-like anchoring protein has not been identified in association with cathepsin D. A second class of membrane-associated proteins (grp78, grp94, and protein disulfide isomerase), which reside in the endoplasmic reticulum, were found to contain a common amino acid sequence (KDEL) at the carboxyl terminus (31). This sequence was shown to be essential for retention of proteins in the endoplasmic reticulum, but the membrane component which recognizes KDEL has not yet been identified. While the carboxyl sequence of membrane-associated cathepsin D remains to be determined, biosynthetic studies carried out by Erikson and Blobel (32), revealed a reduction in molecular mass of 1 kDa (by SDS-PAGE) as the final processing step for porcine cathepsin D. The 1-kDa peptide was cleaved from the carboxyl terminus of the heavy subunit of the enzyme. It is therefore possible that an anchoring signal analogous to KDEL is present in cathepsin D precursors but is lost on maturation of the enzyme and transport to lysosomes. Alternatively, macrophage cathepsin D may contain a lipid or phospholipid responsible for attachment to membranes. Such hydrophobic moieties have been described for a number of membrane-bound proteins (for a review, see Ref. 33). It remains to be seen if membrane-associated cathepsin D contains either lipid or a peptide moiety covalently attached to the protein that accounts for the tethering to the vesicle membranes.

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