Intermolecular Association of Lysosomal Protein Precursors during Biosynthesis*

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To study the mechanism involved in mannose-6-phosphate (Man-6-P) independent lysosomal proenzyme membrane association, we used a reversible cross-linker to probe radiolabeled human HepG2 cells permeabilized with saponin in the presence of Man-6-P. After immunoprecipitation of the extracted and cross-linked cells with anti-cathepsin D antibody, followed by complete reduction of the immunoprecipitates and SDS-polyacrylamide gel electrophoresis analysis, we found that procathepsin D was specifically and transiently associated, independent of Man-6-P, with two co-synthesized glycoproteins having molecular masses of 68 and 72 kDa. Pulse-chase and cell fractionation experiments showed that the Man-6-P independent association of procathepsin D with the 68-kDa protein started in the rough endoplasmic reticulum, continued in the Golgi, but had no association with either membrane. The Man-6-P independent association of procathepsin D with the 72-kDa protein and the membrane was found in compartments all the way from the Golgi to the dense lysosome, where processing of procathepsin D is believed to occur and where procathepsin D dissociated from the 72-kDa protein and the membrane. Endo H digestion of the 72-kDa protein showed that this protein was partially resistant to Endo H, suggesting that membrane association of the procathepsin D-72-kDa protein complex probably began in a late Golgi compartment. Endo F digestion of the proteins showed both have the same molecular mass around 58 kDa. Using antisera against human saposin C, we identified the two glycoproteins as forms of prosaposin with different glycosylation. The transient, Man-6-P independent, membrane association of the procathepsin D-prosaposin complex and the presence of this complex in heavy lysosomes indicated that the proteins were transported to the lysosome as a complex. The association of two lysosomal proteins in the endoplasmic reticulum early after synthesis suggested that preassembly of some lysosomal components occurs before the earliest previously identified steps in the sorting pathway.

Maintenance of the complex structural organization of eucaryotic cells requires that each newly synthesized protein be delivered to its correct location. The best characterized targeting pathway for lysosomal enzymes is the Man-6-P dependent pathway (for review, see Kornfeld and Mellman (1989) and Von Figura and Hasilik (1986)), in which the lysosomal enzymes acquire a common recognition signal, the Man-6-P recognition marker. Lysosomal enzymes bearing this recognition marker are recognized in the trans-Golgi by different receptors (MPR) and segregated from the secretory proteins via clathrin-coated vesicles to late endosomes where the low pH dissociates lysosomal enzymes from the MPRs. The MPRs either recycle back to the Golgi or to the plasma membrane for further rounds of transportation. The discharged lysosomal enzymes are somehow transported to heavy lysosomes.

Although the importance of the Man-6-P dependent targeting pathway for lysosomal enzymes is clear, several lines of evidence suggest that Man-6-P independent alternative pathways must also exist. In I-cell disease (mucolipidosis II) and pseudo-Hurler polydystrophy (mucolipidosis III), a critical enzyme for the Man-6-P dependent pathway (UDP-N-acetylgalactosami ne:lysosomal enzyme, N-acetylgalactosamine phosphotransferase) is defective (Rettman and Kornfeld, 1981; Waheed et al., 1982). Thus, cells with these diseases are unable to synthesize the Man-6-P recognition marker and as a consequence, fibroblasts with I-cell disease or pseudo-Hurler disease are abnormal in lysosomal enzyme targeting. Most of the newly synthesized enzymes are secreted instead of being targeted to the lysosome due to failure of Man-6-P dependent pathway, resulting in a low level of intracellular lysosomal enzyme activity compared to normal control cells. Lack of enzymes in the lysosome leads to the accumulation of metabolites in the lysosome which form the inclusion bodies and gave the name of I-cell disease (Tondeur et al., 1971). However, this is not true for all lysosomal enzymes. α-Glucosidase (Tsuji and Suzuki, 1987), β-glucosidase (Owada and Neufeld, 1982; Van Weely et al., 1990), lysosomal acid phosphatase (Leroy et al., 1972), and cathepsin D (Hasilik and Neufeld, 1980) activities are nearly normal. Furthermore, several other cell types such as Kupffer cells, leukocytes, hepatocytes, and several organs such as brain, liver, spleen, and kidney, also have normal or nearly normal levels of lysosomal enzyme activity (Leroy et al., 1972; Owada and Neufeld 1982; Little et al., 1987). Therefore, a number of cells in different tissues must use a Man-6-P independent mechanism for lysosomal enzyme sorting. Also fibroblasts in I-cell disease and pseudo-Hurler disease have to use Man-6-P independent sorting pathway for the enzymes with normal levels (Owada and Neufeld, 1982; Kornfeld, 1986; Dinten and Fransen, 1991).

Recently, several groups have reported that precursor forms of soluble lysosomal enzymes are transiently associated with cell membranes independent of Man-6-P (Diment et al., 1988; Rijnboutt et al., 1991a; McIntyre and Erickson, 1991; Burge et al., 1991). Although the mechanism of this membrane association remains unsolved, the results of Rijnboutt et al. (1991b)
and McIntyre and Erickson (1991) suggest that the membrane association of lysosomal proenzymes may occur through protein-protein interaction with membrane proteins.

In this study of the Man-6-P independent membrane association of procathepsin D, we found that a portion of procathepsin D was complexed with prosaposin immediately after synthesis. This complex, however, did not associate with the membrane until the complex reached the Golgi apparatus and received complex carbohydrate modification. Upon reaching the heavy lysosome and undergoing proteolytic processing, procathepsin D concomitantly lost both prosaposin interaction and that the procathepsin D-prosaposin complex was targeted to heavy lysosomes as a unit.

**MATERIALS AND METHODS**

**Culture of Cells and Metabolic Labeling**—Human HepG2 cells were cultured in minimum essential medium with Earle's salts (Life Technologies Inc.) with 10% fetal calf serum (Life Technologies Inc.). 1 × nonessential amino acids, 1 × glutamine, plus 0.05% fungizone and gentamicin under 5% CO₂ at 37 °C. For pulse-chase metabolic labeling, cells were washed three times with Ca²⁺ and Mg²⁺-free PBS and pre-incubated with Dulbecco's modified Eagle's medium without methionine (Life Technologies Inc.) and with dialyzed fetal calf serum for 20 min at 37 °C, then labeled for 15 min with 100 μCi/ml [³⁵S]methionine (DuPont-New England Nuclear) and chased for various times in regular Dulbecco's modified Eagle's medium with IgG-free fetal calf serum.

**Cell Permeabilization and Cross-linking**—After radiolabeling, cells were permeabilized according to Rijnboutt et al. (1991a) with slight modification. In brief, cells in 60-mm dishes were permeabilized in 1.5 ml of ice-cold PBS with 2 mg/ml saponin, 5 mM Man-6-P, 100 μM phenanthroline, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride by shaking on ice for 30 min. Permeabilization was repeated once more to ensure soluble proteins were totally washed out. The first wash supernatants were collected and spun at 50,000 rpm for 30 min for either cross-linking of permeabilization supernatants or immunoprecipitation.

Cross-linking of cells was done by adding to each dish 1.5 ml of ice-cold PBS/saponin (2 mg/ml) with 1 mM dithiobisuccinimidyl propionate (Pierce Chemical Co.) from 100 mM stocks freshly made in Me₂SO. After shaking on ice for 30 min, the reaction was quenched with 40 μM glycine (pH 7.5) at room temperature for 30 min. Cross-linking of supernatants was done by adding dithiobisuccinimidyl propionate to 1 ml and preclearing the cell extracts (Pierce Chemical Co., 100 mg/ml) with protein A-Sepharose 4B-CL (Sigma) and washed, was eluted and completely reduced at 100 °C in 1 × sample buffer with 100 mM dithiothreitol for 5 min and at 65 °C for 20 min before loading on 10% SDS-PAGE (Fisher et al., 1982). Gels were fluorographed according to Laskey and Mills (1975) and autoradiograms were quantitated on a Molecular Dynamics scanning laser densitometer or Phosphor-Imager. For Endo F and Endo H digestion, immunoprecipitates were eluted into Endo F buffer or Endo H buffer and treated with 0.05 unit of Endo F or 10 μg of Endo H in the presence of protease inhibitors according to Conner (1992). Digests were precipitated with 15% trichloroacetic acid, dissolved in 1 × sample buffer, and reduced before loading on SDS-PAGE. For sequential immunoprecipitation with anti-human saposin C antiserum, immunoprecipitates were precipitated with anti-cathepsin D antibodies, from both membrane bound and saponin extracts, were completely reduced with dithiothreitol in SDS lysis buffer and re-immunoprecipitated with either anti-cathepsin D antibody or anti-human saposin C antiserum (generously provided by Dr. John O'Brien, University of California, San Diego).

**RESULTS**

**Transient Man-6-P Independent Association of Procathepsin D with Membranes and with a 72-kDa Glycoprotein**—Procathepsin D has been shown to have transient Man-6-P independent membrane association in mouse macrophages (Diment et al., 1988), human HepG2 cells (Rijnboutt et al., 1991a), and 3T3 fibroblasts (McIntyre and Erickson, 1991) and the interaction seems to be through protein-protein interaction. Theoretically, this kind of protein-protein interaction can be studied by chemical cross-linking. We used a reversible homobifunctional cross-linker (dithiobisuccinimidyl propionate) to cross-link radiolabeled HepG2 cells which had been permeabilized with PBS/saponin in the presence of 5 mM Man-6-P, to see if the Man-6-P independent membrane bound procathepsin D was associated with any membrane protein. We found that after a 15-min pulse, procathepsin D was not bound to the membrane (Fig. 1, lanes 1 and 2). However, after 1 h of chase (Fig. 1, lanes 3 and 4), procathepsin D not only was associated with procathepsin D and membrane.
with the membrane but also interacted with another protein of 72 kDa which we called MIB-1 as Man-6-P independent binding protein-1. After 5 h of chase, which is sufficient time for procathepsin D to be targeted to heavy lysosomes and to be proteolytically processed, there was a concomitant loss of both membrane association of procathepsin D and the intermolecular interaction of procathepsin D and MIB-1 (Fig. 1, lanes 5 and 6). The interaction of procathepsin D and MIB-1 was shown to be specific by control immunoprecipitations without cross-linking or after complete reduction of the cross-linker which showed only procathepsin D. When anti-β-hexosaminidase and anti-α-glucosidase antisera were used instead of anti-cathepsin D antibody, no interaction of procathepsin D or MIB-1 with these two lysosomal enzymes could be detected (data not shown).

Although the membrane association of the procathepsin D-MIB-1 complex was resistant to 5 mM Man-6-P, saponin permeabilization in the presence of Man-6-P did reduce both procathepsin D and MIB-1 membrane association (compare lanes 3 and 4 in Fig. 1). Quantitation of membrane and soluble forms demonstrated that after the 15-min pulse, 2% of the procathepsin D was membrane associated (lanes 1 and 2) while after 1 h of chase, 6 and 15% of the procathepsin D was membrane associated in the presence or absence of Man-6-P, respectively (lanes 3 and 4). After 5 h of chase 12% of the procathepsin D remained in the cell and 1–2% of the procathepsin D made during the 15-min pulse remained associated with the membrane. No processed forms of cathepsin D were detectable in the membrane-associated fractions. Man-6-P reduced membrane-associated procathepsin D by about 62 and 57% at the 1- and 5-h points, respectively. Similarly, membrane-associated MIB-1 was reduced by 55% when permeabilization was performed in the presence of Man-6-P. These results were consistent with those of Rijnboutt et al. (1991a). They attribute this Man-6-P sensitivity to the MPR dependent binding. When cells were continuously labeled for 24 h and then permeabilized, cross-linked, and immunoprecipitated with anti-cathepsin D antibodies, one of the cross-linked proteins was seen only in the absence of Man-6-P (data not shown). Based on its apparent molecular mass it may represent the cation-independent MPR.

To further test the specificity of the partial Man-6-P sensitivity, we used several other monosaccharides in the saponin permeabilization. As can be seen in Fig. 2, only fructose-1-phosphate, which is known to interact with MPR, reduced the procathepsin D-MIB-1 membrane binding to the same extent as did Man-6-P (Tong and Kornfeld, 1989). Other monosaccharides did not have any effect on membrane binding, indicating it is Man-6-P specific.

Endoglycosidase F (Endo F) and H (Endo H) digestion (Fig. 3, A and B) showed that MIB-1 was a glycoprotein with probably 4–5 glycosylation sites (at least 5 bands could be seen on the Endo H digestion). After Endo F treatment, the molecular mass of this protein shifted from 72 kDa to about 58 kDa. The glycosylation of this protein was quite heterogenous, with some complex-type that were resistant to Endo H and some high mannose-type that were sensitive to Endo H, suggesting the actual site of membrane association could be in the late Golgi or distal to that compartment.

**Procathepsin D Associates with Another 68-kDa Glycoprotein as a Soluble Complex**—As procathepsin D associated with MIB-1 and the membrane, it was possible that MIB-1 is the membrane receptor which procathepsin D binds. To test this possibility, intact salt-washed microsomes were digested with either trypsin or proteinase K, spun through 100,000 x g for 60 min and further digested with a solution containing either trypsin or proteinase K, spun through 100,000 x g for 60 min and then reprecipitated with anti-cathepsin D antibodies. We found that MIB-1 was totally protected by the membrane with no shift of molecular mass (data not shown), suggesting that it was a luminal protein instead of a transmembrane protein. To see if the Man-6-P sensitive procathepsin D-MIB-1 complex was also present in the soluble phase, we cross-linked the saponin-permeabilized supernatants of the intact cells. As can be seen in Fig. 4, there was indeed a small amount of this complex in the saponin permeabilization supernatants (Fig. 4, lanes 3 and 4, arrow), further proving that MIB-1 was a luminal protein. Surprisingly, we found that procathepsin D was also associated with another protein, MIB-2 (68 kDa), independent of Man-6-P (Fig. 4, lanes 1–6). This complex did not associate with the membrane and therefore, it was only in the permeabilization supernatants and was not detected in Fig. 1. When MIB-1 and MIB-2 were isolated from the cross-linked permeabilized supernatants by immunoprecipitation with anti-cathepsin D antibody and digested with Endo F, both MIB-1 and MIB-2 bands shifted to the same molecular mass around 58 kDa (Fig. 5) and only a single band was detected, suggesting that both MIB-1 and MIB-2 were possibly derived from the same protein precursor. Since a vastly larger portion of procathepsin D was present in the immunoprecipitates of soluble fractions, the linear range of the

**FIG. 2. Sensitivity of membrane-associated procathepsin D and MIB-1 to monosaccharides.** HepG2 cells were pulse labeled for 15 min and chased 1 h. Cultures were then permeabilized in the presence of different monosaccharides and processed as described in the legend to Fig. 1. A, fluorograph of a gel showing the effects of the different monosaccharides on membrane association of procathepsin D and MIB-1. Control (−); mannose 6-phosphate (m6p); glucose 6-phosphate (g6p); fructose 1-phosphate (f1p); α-methylmannoside (mman); N-acetylglucosamine (GlcNAc). B, densitometric analysis of the fluorograph in A. Open bars correspond to the MIB-1 signal in A. Hatched bars correspond to the procathepsin D signal in A. Man-6-P and fructose 1-phosphate reduced procathepsin D and MIB-1 signals by about half, indicating that the procathepsin D-MIB-1 complex is partially sensitive to Man-6-P and Fru-1-P. Other monosaccharide derivatives had almost no effect.
Fig. 3. Endo F and Endo H digestion of membrane-associated procathepsin D and MIB-1. HepG2 cells were pulse labeled for 15 min and chased 1 h. Cultures were then permeabilized in the presence of Man-6-P and processed as described in the legend to Fig. 1. A, fluorograph of Endo F digestion of immunoprecipitates (lane 1, control without digestion; lane 2, Endo F digestion). Arrows indicate procathepsin D. B, fluorograph of Endo H digestion of immunoprecipitates (lane 1, control; lane 2, Endo H digestion). These endoglycosidase digestions showed that MIB-1 was also a glycoprotein with about 4-5 glycosylation sites. Its partial resistance to Endo H suggested that the membrane association occurred in late Golgi or is compartments distal to Golgi.

Fig. 4. Procathepsin D associated with MIB-2 in the soluble phase. HepG2 cells were pulse labeled for 15 min and chased 1 and 5 h. Cultures were then permeabilized with saponin in the presence or absence of Man-6-P (m6p), and the saponin soluble material was cross-linked, immunoprecipitated with anti-cathepsin D antibody, run on a 10% SDS gel, and fluorographed as described under “Materials and Methods.” Lanes 1 and 2, pulse; lanes 3 and 4, 1 h chase; lanes 5 and 6, 5 h chase. The arrow indicates a small amount of MIB-1 was also present in the 1-h chase supernatants (lanes 3 and 4).

autoradiographic films was exceeded to detect the MIB protein signals. Direct quantitation of the isotope in each band before and after Endo F digestion indicated that equivalent percentages of procathepsin D and MIB-1 and MIB-2 were recovered after treatment, thus ruling out the possibility that either MIB-1 or MIB-2 migrated at the position of procathepsin D after the Endo F treatment.

Subcellular Distribution of Procathepsin D Association of MIB-1 and MIB-2 Occurs in Distinct but Overlapping Compartments—The subcellular distribution of procathepsin D-MIB-1 and procathepsin D-MIB-2 complexes was analyzed by Percoll gradient fractionation. Fig. 6 showed that procathepsin D-MIB-1 complex was distributed in two peaks although some MIB-1 containing complex were seen in all fractions except those at the top. The first peak was in fractions 4-6 with light density which overlapped with the peak of galactosyltransferase activity, suggesting it was in the Golgi compartment or perhaps endosomes which also move to this part of the gradient. The second peak of MIB-1 occurred in a dense compartment and co-sedimented with the β-hexosaminidase activity, indicating it was in the dense prelysosomal or lysosomal compartments. Some processing of procathepsin D to the single-chain form could also be detected (arrow, fractions 16-18). Since in this experiment, cross-linking was done at the same time as saponin permeabilization and the whole of each fraction from the Percoll gradient, containing both soluble and membrane proteins, was used for immunoprecipitation, it was expected that the signal of procathepsin D relative to MIB-1 would be much stronger than the relative signal intensity of D-MIB-1 complex.
procathepsin D to MIB-1 in the membrane associated material shown in Fig. 1.

There was only one major peak of MIB-2 containing complex in fractions 4–6 with the highest signal in fraction 5 which also overlapped with the galactosyltransferase activity, indicating that procathepsin D-MIB-2 association was also found in the Golgi. This complex, however, was not associated with the membrane as it can be totally washed out during saponin permeabilization in the presence of Man-6-P (Fig. 4, lanes 3 and 4). The pulse-label experiment in Fig. 4 showed that procathepsin D association with MIB-2 initially formed in the ER (lanes 1 and 2).

**MIB-1 and MIB-2 Are Different Forms of Prosaposin**—Endo F digestion of MIB-1 isolated from membrane bound complex (Fig. 3A) and both MIB-1 and MIB-2 isolated from saponin supernatant (Fig. 5) altered the mobility of both proteins to 58 kDa, suggesting they may be the same protein with different carbohydrate modification. Since membrane association of MIB-1 and procathepsin D were identically sensitive to Man-6-P, these samples were done. Based on the ratio of prosaposin to procathepsin D in the anti-prosaposin immunoprecipitations, it appears that a substantial amount of prosaposin may also be associated with the membrane and not complex with procathepsin D.

Taken together, these data demonstrated that two lysosomal enzyme precursors, prosaposin and procathepsin D, associated into a complex within a few minutes after synthesis and that this complex became membrane associated in the Golgi comitant with complex carbohydrate modification. After arrival in the lysosome and subsequent proteolytic processing, they dissociated from each other and from the membrane.

**DISCUSSION**

Our results showed that a portion of procathepsin D interacted with a portion of prosaposin to form a protein complex immediately after their synthesis in the ER. This complex, however, did not bind to the membrane until delivery to Golgi apparatus, where this complex associated with the membrane both in Man-6-P dependent and independent manner (Fig. 1, lanes 3 and 4). The complex was delivered to the lysosome and after 5 h of chase, when procathepsin D had been proteolytically processed, the complex dissociated, and both proteins were released from the membrane. This time pattern of membrane binding and intermolecular interaction and the pattern of Man-6-P sensitivity of procathepsin D and prosaposin are exactly the same as that reported by Rijnboutt et al. (1991a), suggesting that Man-6-P dependent membrane association of procathepsin D and prosaposin may actually occur as a protein complex. It is possible that Man-6-P independent lysosomal targeting might need preassembly of these lysosomal proteins as complexes in the ER. Since the membrane binding of the procathepsin D-prosaposin complex is both Man-6-P dependent and independent in HepG2 cells, the Man-6-P dependent pathway may also contribute to the transport of this complex to the heavy lysosome.

Procathepsin D is synthesized as a soluble protein lacking transmembrane or hydrophobic domains, so, it is improbable that procathepsin D alone binds to the membrane through direct interaction with the lipid bilayer. Prosaposin is a precursor of four polypeptides with similar domains (saposin A, B, C, and D) (O’Brien et al., 1988; Fujibayashi and Wenger, 1986a, 1986b; Nakano et al., 1989). Prosaposin is also synthesized as a soluble protein lacking a transmembrane domain, however, each saposin domain has three potential amphipathic helices with cysteine residues properly positioned to give a trihelical hydrophobic domain stabilized by disulfide bonds. Proteins with such domains possibly interact directly with the lipid bilayer (Wynn, 1986; O’Brien and Kishimoto, 1991). At least saposin B is known to directly bind to phospholipids (Neufeld and Mckusik, 1983). If prosaposin has some of the combined properties of each saposin, it may also have lipid binding capability. In fact, it has been shown that prosaposin could bind and transport gangliosides from donor liposomes to acceptor membrane (Hirawa et al. 1992). The binding seems also to be dependent on the structure of gangliosides, with longer carbohydrate chains or a higher number of sialic acid residues having higher bind-
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sorting machinery (e.g. Ioannou et al., 1992), it is possible that either or both sorting systems prefer to recognize quanta of pre-associated lysosomal constituents represented, for example, by the procathepsin D-prosaposin complex detected in our experiments. Sorting of such quanta may provide a mechanism for maintaining a balanced composition of the hydrolytic compartment.

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ing affinity. Prosaposin also has sequence similarity to the precursor of pulmonary surfactant-associated protein SP-B (Patty, 1991), a protein which readily binds phospholipids. Taken together, it may be possible for prosaposin to interact directly with the lipid bilayer of the membrane with preference for membranes with higher content of complex glycolipids. If this occurs, it could also explain the Man-6-P independent procathepsin D binding to membrane since procathepsin D is associated with prosaposin. Loss of membrane binding of procathepsin D in the lysosome may be simply due to the loss of interaction with prosaposin when the propeptide is cleaved off in the lysosome, or due to the processing of prosaposin to mature saposins in the lysosome. Although binding to the membrane occurred after acquisition of endoglycosidase H resistance, this modification is apparently not necessary for membrane association since the Man-6-P independent membrane-bound complex was detected in Lec 1 Chinese hamster ovary cells which are unable to form complex N-linked carbohydrate on proteins (Stanley et al., 1979). Grassel and Hasilik (1992) have found that procathepsin D is associated with a 60-kDa glycoprotein by cross-linking human promonocytes U937. Since they did the cross-linking with whole un-extracted cells, it is not known if any part of this complex is associated with the membrane. We do not know if this protein is prosaposin or if it represents a different protein that associates with procathepsin D and which can only be detected by cross-linking whole cells.

Targeting of proteins to the lysosome involves segregation at some point in the common area of the secretory pathway. In the case of the Man-6-P mediated pathway, recognition occurs early in the Golgi by the enzymes responsible for synthesis of the Man-6-P marker (Reitman and Kornfeld, 1981; Varki and Kornfeld, 1981; Waheed et al., 1993). Unlike these enzymes, the func-