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Mannose 6-Phosphate-independent Membrane Association of Cathepsin D, Glucocerebrosidase, and Sphingolipid-Activating Protein in HepG2 Cells*

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The membrane association of the lysosomal enzymes cathepsin D and glucocerebrosidase and its naturally occurring sphingolipid activating protein was studied in HepG2 cells. We differentially permeabilized cells with low concentrations of saponin, at which secretory proteins rinsed out completely, whereas integral membrane proteins were not released. All relevant intracellular compartments were shown to be permeabilized by saponin. Metabolic labeling showed that early precursors of cathepsin D, sphingolipid activating protein, and glucocerebrosidase were completely released from the cells, whereas more than 80% of the high molecular mass intermediates were retained by the cells. Treatment of permeabilized cells with 10 mM mannose 6-phosphate released only 50% of the cell-associated cathepsin D. Glucocerebrosidase remained membrane-associated, but cathepsin D and sphingolipid activating protein were released from the cells after proteolytic processing. Sphingolipid activating proteins and cathepsin D behaved similarly during biosynthesis and showed similar sensitivity to mannose 6-phosphate. The membrane association of the intermediate form of cathepsin D was independent of the presence of *N*-linked oligosaccharides. Subcellular fractionation on sucrose gradients showed that the lysosomal proteins became membrane-associated probably in the Golgi complex, and that both mannose 6-phosphate-dependent and mannose 6-phosphate-independent membrane association occur in the same compartments. We conclude that, in HepG2 cells, cathepsin D, sphingolipid activating protein, and glucocerebrosidase exhibit MPR-independent membrane association which is acquired in the same compartments beyond the rough endoplasmic reticulum.

Lysosomal enzymes are synthesized as glycoproteins at the rough endoplasmic reticulum (rER)[1] and are selectively transported via the Golgi complex to the lysosomes (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). Different mechanisms exist to accomplish this transport selectivity. The best documented is the mannose 6-phosphate receptor (MPR)-mediated transport of most soluble lysosomal enzymes (Creek and Sly, 1984; Dahms *et al.*, 1989).

Cathepsin D is the best studied representative of MPR-directed lysosomal enzymes. Its high mannose oligosaccharide chains aquire mannose 6-phosphate (man6-p) residues early during passage of the Golgi complex at which time complex type oligosaccharides can also be added (Hasilik and von Figura, 1981; Varki and Kornfeld, 1983). The mannose 6-phosphate residues are then recognized by and bound to specific MPR, probably at the *trans* site of the Golgi complex, which prevents secretion of the lysosomal enzyme and results in its delivery to the lysosomes. During transport, the 53-kDa precusor of cathepsin D is proteolytically processed at two sites (Hasilik and von Figura, 1984). A first cleavage results in the 44-kDa intermediate and is thought to take place in a prelysosomal compartment. The second occurs in lysosomes, yielding the 31-kDa and 14-kDa chains of the mature protein (Gieselmann *et al.*, 1983).

Several observations have indicated that lysosomal targetting signals other than man6-p must exist to address proteins to the lysosomes. Lysosomal membrane proteins lack man6-p signals and are directed to the lysosomes by an as yet unresolved targetting system (Kornfeld and Mellman, 1989). Similarly, lysosomal acid phosphatase, which is a transmembrane protein up to lysosomal delivery, lacks man6-p residues (Waheed *et al.*, 1988). Since this protein is transported to the lysosomes via the cell surface and endocytosis, the cytosolic tail is likely to contain information for its proper targetting (Braun *et al.*, 1989). An intermediate position is taken by glucocerebrosidase, which is a membrane-associated lysosomal enzyme lacking a membrane spanning domain (Erickson *et al.*, 1985) but does not acquire man6-p residues (Aerts *et al.*, 1988). Consequently, this enzyme is transported to the lysosomes without involvement of MPR, as is also indicated by the normal amounts present in lysosomes of patients with mucolipidosis II or I-cell disease, in which the phosphorylation of mannose residues is impaired (Neufeld and McKusik, 1983; Nolan and Sly, 1989). Interestingly, lysosomes of certain tissues and cells including liver from these patients also contain normal levels of soluble lysosomal enzymes such as cathepsin D, suggesting that other than MPR-mediated lysosomal targetting systems may be operational for soluble lysosomal enzymes as well (Kornfeld, 1986).

Recently, Diment *et al.* (1988) found that in rabbit macrophages 90% of the 53-kDa precursor of cathepsin D was membrane-associated. The authors suggested that this membrane association was not mediated by man6-p residues. We present evidence for a man6-p-independent membrane asso-

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[1] The abbreviations used are: rER, rough endoplasmic reticulum; SAP, sphingolipid activating protein; MPR, mannose 6-phosphate receptor; man6-p, mannose 6-phosphate; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; MEM, minimal essential Eagle's medium; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ciation for glucocerebrosidase, cathepsin D, and SAP in the human hepatoma cell line, HepG2. We combined metabolic labeling with differential permeabilization at low concentrations of saponin. The plasma membrane and endomembranes can be permeabilized in the presence of saponin (Castle and Palade, 1978; Rottier *et al.*, 1984) which is primarily due to complex formation with cholesterol (Schlósser and Wulff, 1969; Bangham and Horne, 1962). Differential permeabilization with saponin can induce release of secretory proteins but not of integral membrane proteins (Wassler *et al.*, 1987; Strous and van Kerkhof, 1989). In our studies, subcellular fractionation suggested that man6-p-independent membrane association is a compartment-specific event and does not depend on the presence of oligosaccharide chains.

MATERIALS AND METHODS

Cells

The human hepatoma cell line HepG2 (Knowles *et al.*, 1980) was cultured in monolayer in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum. 80% confluent cultures were used. Culture media were refreshed 1 day before the experiments. In some experiments, the culture medium was supplemented with 10 μg/ml tunicamycin. Cells were preincubated with the drug for 4 h, and all further incubations were done in the presence of the drug.

Metabolic Labeling and Permeabilization of the Cells

HepG2 cells were grown on 35-mm Petri dishes, washed in phosphate-buffered saline (PBS), and preincubated in MEM without methionine (Gibco Laboratories). Cells were then labeled for 15 min with 60 μCi/ml [^{35}S]methionine (800–1200 Ci/mmol, Radiochemical Center, Amersham) and chased for various periods of time in 1 ml of the culture medium. For differential permeabilization, cells were kept on ice, washed in ice-cold PBS, and incubated in 1 ml of PBS containing 2 mg/ml saponin (Merck, Darmstadt) for 30 min on a rocking platform. After 30 min, the saponin-containing medium was removed, and cells were washed with PBS and solubilized in 1 ml of PBS containing 1% Triton X-100 and 1 mM phenylmethanesulfonyl fluoride (PMSF). Aliquots of the cell extracts and culture media were used for immunoprecipitation.

Proteinase K Treatment—After pulse-chase labeling, cells were washed with ice-cold PBS and incubated at 0 °C with PBS containing 0.1 mg/ml proteinase K (Boehringer Mannheim) and 1 mM EDTA. After 30 min, the protease was inactivated by adding 1 ml of PBS containing 1 mM PMSF.

Antisera—Anti-glucocerebrosidase antiserum, prepared as reported previously (Aerts *et al.*, 1986), was used. The antisera against the asialoglycoprotein receptor and against sphingolipid activating protein 2 were kind gifts of Dr. A. L. Schwartz (Washington University, St. Louis) and by Dr. D. Wenger (University of Colorado, Denver), respectively. The antiserum against cathepsin D, isolated from human placenta, was prepared in our laboratory. The antiserum against α_1-antitrypsin was obtained from Dakopatts.

Immunoprecipitation

Aliquots of Triton X-100 extracts of [^{35}S]methionine-labeled cells were immunoprecipitated, and IGSORB (New England Enzyme Center) was used to isolate the immune complexes (Strous and Lodish, 1980). The immune precipitates were washed three times with ice-cold PBS, containing 1% bovine serum albumin, 1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% human serum albumin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) and 1 mM PMSF. After a final wash with 10-fold diluted PBS, immune complexes were analyzed by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE). For glucocerebrosidase, we used 8% acrylamide; for cathepsin D, asialoglycoprotein receptor and α_1-anti-trypsin-10% acrylamide; and for SAP gradient, gels from 7 to 17% acrylamide. For SDS-PAGE of cathepsin D, nonreducing conditions were used. Fluorograms of these gels were quantified by scanning full lanes in an LKB Ultroscan XL Enhanced Laser Densitometer, within the linear range of the film and the densitometer.

Cell Fractionation and Sucrose Density Gradient Centrifugation

HepG2 cells were grown on 9-cm Petri dishes and pulse-labeled with [^{35}S]methionine as described above. Cells were incubated with ice-cold PBS for 30 min which in some experiments contained 2 mg/ml saponin with or without 10 mM mannose 6-phosphate. Cells were harvested by scraping in 1 ml of low salt buffer (10 mM Hepes, 15 mM KCl, 1.5 mM $MgCl_2$) after incubation for 10 min. Cells were homogenized in 50 strokes in a Dounce homogenizer with a tight fitting pestle (Kontes). The homogenate was centrifuged for 5 min at 300 × *g* to remove nuclei. 750 μl of the postnuclear supernatant was layered on top of an 11-ml linear 0.7 to 1.5 M sucrose gradient, containing 20 mM Tris, pH 7.5, and 1 mM EDTA. Intracellular compartments were separated by centrifugation in a Beckman ultracentrifuge at 100,000 × *g* in an SW 41 rotor for 3 h at 4 °C. Subsequently, fractions were immunoprecipitated in the presence of 0.5% Triton X-100 and subjected to SDS-PAGE. The sucrose concentration of the fractions was measured in a refractometer. β-Hexosaminidase and galactosyltransferase marker enzyme activities were measured according to Galjaard (1980) and Strous and Berger (1982), respectively.

RESULTS

We examined changes in membrane association of the lysosomal proteins cathepsin D, sphingolipid activating protein (SAP), and glucocerebrosidase during biosynthesis. Cathepsin D and SAP are both soluble proteins when mature, but only with cathepsin D has the MPR dependence for transport to lysosomes been documented. On the other hand, glucocerebrosidase does not contain man6-p and is a membrane-associated protein in the lysosome. To study the membrane association of biosynthetic intermediates of these lysosomal proteins in HepG2 cells, the cells were pulse-labeled for 15 min with [^{35}S]methionine and chased for various periods of time. The proteins were immunoprecipitated and analyzed by SDS-PAGE. In parallel to the pulse-chase experiments, cells were differentially permeabilized with saponin in the absence or presence of man6-p.

Biosynthesis and Membrane Association of Glucocerebrosidase—As seen in Fig. 1 (*top section*), glucocerebrosidase is synthesized as a 62.5-kDa precursor, which is converted to a 68-kDa intermediate after 2 h. The mature 59-kDa enzyme is clearly visible only after 24 h of chase. The enzyme was not

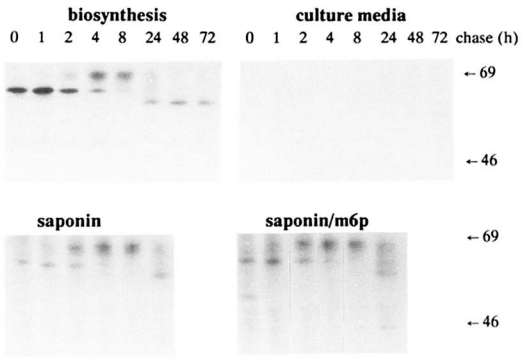

FIG. 1. **Membrane association of glucocerebrosidase.** Cell cultures were pulse-labeled for 15 min with [^{35}S]methionine and chased for the times indicated. Cells were immediately solubilized after the chase period, and aliquots of the cell lysates (*top section, left*) as well as of the culture media (*top section, right*) were taken, and glucocerebrosidase was immunoprecipitated and subjected to SDS-PAGE. In the *lower section* incubations with 2 mg/ml saponin in PBS in the absence (*left*) or the presence (*right*) of 10 mM man6-p are shown. After the chase periods were completed, cells were washed with PBS (0 °C) and incubated with 2 mg/ml saponin for 30 min at 0 °C in the absence (*left lower section*) or the presence (*right lower section*) of 10 mM mannose 6-phosphate. Cells were washed with PBS and solubilized. Glucocerebrosidase was immunoprecipitated from the lysates and subjected to SDS-PAGE. Apparent molecular mass markers are shown on the *right*.

secreted into the culture media. This maturation pattern is identical with that seen in human fibroblasts (Jonsson *et al.*, 1987), albeit that maturation in HepG2 cells is somewhat faster. After pulse-chase labeling, the cells were treated for 30 min at 0 °C with 2 mg/ml saponin, either with or without 10 mM man6-p. For immunoprecipitation and SDS-PAGE, aliquots of cell-lysates and media were used for all three conditions (Fig. 1). As can be seen in Fig. 1 (*left bottom section*), about 90% of the 62.5-kDa precursor form of glucocerebrosidase was released upon treatment with saponin, regardless of the chase period. In contrast, the 68-kDa and 59-kDa species remained associated with the cells. Quantitation of the signals by scanning of the fluorograms revealed that 80 to 85% of both species was retained by the differentially permeabilized cells. Addition of 10 mM man6-p during treatment with saponin had no effect on the amounts of these species retained after saponin treatment (*right bottom section*). Thus, glucocerebrosidase is synthesized as a soluble protein that acquires membrane association at the time the 62.5-kDa form is converted to the 68-kDa form. This membrane association is not mediated by man6-p and is maintained after the conversion of the 68-kDa form to the 59-kDa form.

Biosynthesis and Membrane Association of Cathepsin D—Fig. 2 shows the biosynthesis and membrane association of cathepsin D in HepG2 cells. The protein is synthesized as a 51-kDa precursor of which some gives rise to a 53-kDa form after 1 h of chase, probably due to conversion of one of the two high mannose oligosaccharide chains to a complex type chain on part of the 51-kDa precursor (Hasilik and von Figura, 1981). A small amount of a 55-kDa form is also detectable at this time. It was impossible to properly quantify these three species separately. The amount of the 55-kDa species was estimated to be less than 10% of the 51-kDa species at each time point. Secretion of all three species into the culture medium was first detected after 1 h of chase. The secretion leveled off after 2 h and did not exceed 20% of initially synthesized precursor. The precursor molecules were converted to a 44-kDa intermediate form after 1.5 h of chase. This 44-kDa form was slightly trimmed before being processed to the 31-kDa mature form. The mature 31-kDa form was first detected after 2 h of chase. Most of the 51-kDa and all of the 44-kDa intermediate and the 31-kDa mature species of cathepsin D were released upon treatment of the cells with saponin (Fig. 2, *left bottom section*). This result is in agreement with the current opinion that mature cathepsin D is a soluble lysosomal protein. As soon as the higher molecular mass species appeared all three species (51, 53, and 55 kDa) became cell-associated. From the fluorograms in Fig. 2, it is clear that the 51-kDa form accounted for most of the release of cathepsin D. The relative amounts of the 51- and 53-kDa species that became cell-associated were estimated to be equal at each time point. Analysis by isoelectric focussing indicated that all of the initially synthesized precursor was released after incubation with saponin, whereas precursor isoforms with a lower pI, appearing after 1 h of chase, became completely cell-associated (not shown). Surprisingly, about half of the retained 51- to 55-kDa species still remained cell-associated when cells were treated with both 10 mM man6-p and saponin (Fig. 2, *right bottom section*). A 3-fold higher man6-p concentration did not increase the release of cathepsin D (not shown). All three species were sensitive to man6-p to the same extent as indicated in Fig. 2. The highest sensitivity for man6-p was measured after 1 h of chase. At all chase times, the relative amount of man6-p-independent cathepsin D present in the cells was constant. When we used glucose 6-phosphate instead of man6-p, no additional release of cathepsin D was found, indicating the specificity for man6-p (not shown). The sensitivity of the man6-p-independent membrane association to acidification was tested by changing the pH of the saponin solution in the presence of man6-p. In the pH range between 5.5 and 8.0, no effect on the amount of man6-p-independent membrane association of the 51- to 55-kDa species of cathepsin D was found (not shown). From these results we conclude that cathepsin D is synthesized as a soluble protein and becomes membrane-associated at the time that the 51-kDa species is converted into the higher molecular mass species. Most or all of the cathepsin D intermediates released after differential permeabilization by saponin was the 51-kDa form, indicating that the 51-kDa form became membrane-associated by the time the 53-kDa and 55-kDa species also appeared. This membrane association is only partially mediated by man6-p. In fact, almost 50% of the membrane-associated 51- to 55-kDa species was insensitive to addition of man6-p or acidification. The membrane association had disappeared after proteolytic processing of the 51- to 55-kDa species to the 44-kDa form.

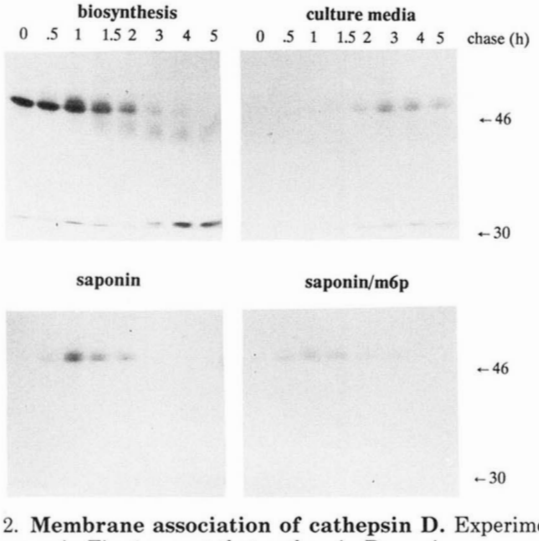

FIG. 2. **Membrane association of cathepsin D.** Experimental details are as in Fig. 1 except that cathepsin D was immunoprecipitated instead of glucocerebrosidase.

Biosynthesis and Membrane Association of SAP—In parallel, membrane association was studied of the common precursor of four sphingolipid activating proteins (SAPs), naturally occurring soluble activator proteins one of which interacts intralysosomally with glucocerebrosidase (Barranger and Ginns, 1989). This protein is encoded by a single gene (Furst *et al.*, 1988; O'Brien *et al.*, 1988), and the individual SAPs are derived by proteolytic processing. In HepG2 cells, SAP precursor was synthesized as a 68-kDa form which was converted to a 73-kDa intermediate after 30 min of chase (Fig. 3, *left top section*). A small amount of the 73-kDa form was secreted into the culture medium (Fig. 3, *right top section*). A 50-kDa intermediate form was present after 1 h of chase. Finally, mature SAP was detectable as a protein of 12 kDa after 1.5 h of chase. The 68-kDa precursor, the 50-kDa intermediate, and the 9-kDa mature form of SAP were released after treatment with saponin (Fig. 3, *left bottom section*). The 73-kDa intermediate, however, was almost completely retained in the cells. When 10 mM man6-p was added with the saponin, half of the 73-kDa forms remained associated with the cells (Fig. 3, *right bottom section*). This additional release did not occur when the man6-p was replaced by glucose 6-phosphate (not shown). These results show that SAP probably acquires man6-p residues and can be translocated to the lysosomes by the MPR

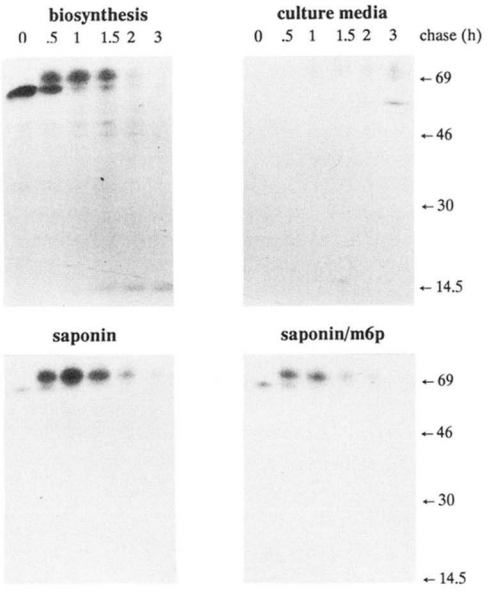

FIG. 3. **Membrane association of sphingolipid activating protein.** Experimental details are as in Fig. 1 except that SAP was immunoprecipitated instead of glucocerebrosidase.

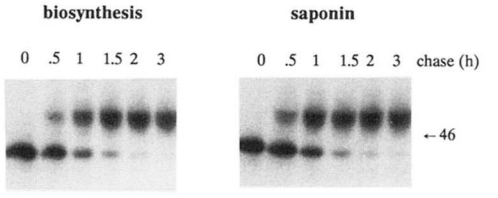

FIG. 4. **Recovery of the asialoglycoprotein receptor.** Experimental details are as in Fig. 1 except that the asialoglycoprotein receptor was immunoprecipitated instead of glucocerebrosidase.

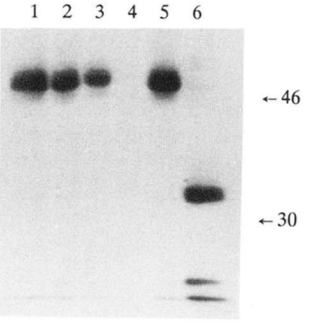

FIG. 5. **Accessibility of membrane-associated cathepsin D for digestion by proteinase K.** Cells were pulse-labeled with [^{35}S] methionine for 1 h, washed with PBS, and incubated for 30 min at 0 °C with either PBS (*lanes 1* and *2*), 2 mg/ml saponin in PBS (*lanes 3* and *4*), or 1% Triton X-100 in PBS (*lanes 5* and *6*). 0.5 mg/ml Proteinase K was added to the incubations in *lanes 2*, *4*, and *6*. The digestion was stopped by 1 mM PMSF. After cells from *lanes 1*, *2*, *3*, and *4* were washed with PBS prior to solubilization, all lysates were immunoprecipitated for cathepsin D and subjected to SDS-PAGE. Apparent molecular mass markers are shown on the *right*.

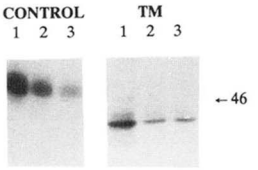

FIG. 6. **Membrane association in cells cultured in the presence of tunicamycin.** Cell cultures were preincubated for 4 h with 10 μg/ml tunicamycin (*TM*). Cells were pulse-labeled with [^{35}S] methionine and chased for 1 h in the presence of the drug. Control cells are shown in the *left section*. After the chase period, cells were incubated for 30 min at 0 °C with either PBS (*lanes 1*) or 2 mg/ml saponin in PBS in the absence (*lanes 2*) or the presence (*lanes 3*) of 10 mM man6-p.

system. These results are very similar to those found for cathepsin D, suggesting that man6-p-insensitive membrane association is not unique for cathepsin D.

Membrane Association Is Restricted to Lysosomal Proteins—To exclude the possibility that whole cells or their membrane structures were lost during the treatment with saponin, an integral membrane protein, the asialyglycoprotein receptor, was analyzed by immunoprecipitation. No loss of either the precursor or the mature asialoglycoprotein receptor was detected at the concentration of saponin used (Fig. 4). To examine whether the membrane association also occurred with secretory proteins in HepG2 cells, α_1-antitrypsin was immunoprecipitated. From the time α_1-antitrypsin was synthesized until it was secreted as the 54-kDa mature protein, no membrane association was detected (data not shown). These results are in agreement with the effect of saponin on the release of secretory proteins (Wassler *et al.*, 1987; Strous and van Kerkhof, 1989).

A possible explanation for the partial release of the lysosomal proteins from the permeabilized cells is that saponin does not open all intracellular compartments. To test this possibility, proteinase K was added during the incubation with saponin after 1 h of chase (Fig. 5). When cells were not treated with saponin, no cathepsin D was accessible to proteinase K although some cells were lost (*lanes 1* and *2*). In the presence of saponin, all retained cathepsin D was degraded by the proteinase K (*lanes 3* and *4*). When 1% Triton X-100 was added during the incubation with proteinase K, a partially degraded remnant of cathepsin D was detected in the lysate (*lane 6*). This was absent from cells treated with saponin, indicating that this remnant was released like the soluble mature cathepsin D during permeabilization (*lane 4*). This result demonstrates that all compartments that contain cathepsin D were permeabilized during treatment with saponin.

Membrane Association Is Independent of the Presence of N-linked Oligosaccharides—To further specify the mechanism of membrane association, cells were cultured in the presence of tunicamycin to prevent *N*-linked glycosylation of cathepsin D (Rosenfeld *et al.*, 1982; Erickson *et al.*, 1981). After pulse labeling, cells were incubated in the absence or the presence of saponin and man6-p, after which cathepsin D was immunoprecipitated. In control cells (Fig. 6), the 51- to 55-kDa species were retained similarly as seen above (Fig. 2) after permeabilization with saponin in the absence or the presence of man6-p. Synthesis of cathepsin D in the presence of tunicamycin reduced the apparent molecular mass to 43 kDa (Fig. 6, *TM*, *lanes 1–3*). Tunicamycin also reduced protein synthesis to a level of about 70% of control cells. However, a quarter of the unglycosylated cathepsin D had become membrane-associated in a similar fashion as the cathepsin D in the control experiment (*TM*, *lane 2*). As expected, addition of man6-p had no effect on the membrane association (*TM*, *lane 3*). Therefore, we conclude that membrane association of cathepsin D is independent of the presence of *N*-linked oligosaccharides. No effect of tunicamycin on membrane association was observed in a similar experiment for glucocerebrosidase or SAP (not shown).

Distribution of Cathepsin D on Sucrose Density Gradients—We next characterized the compartment in which cathepsin D becomes membrane-associated. Pulse-chase-labeled cells were incubated in the absence or the presence of saponin with and without man6-p. The cells were then homogenized, and

a postnuclear supernatant was fractionated on a sucrose density gradient. Cathepsin D was immunoprecipitated and subjected to SDS-PAGE. Golgi and lysosomal fractions were marked by galactosyltranferase and β-hexosaminidase enzyme activities, respectively (Fig. 7*E*). To localize the rER on the gradient, albumin was immunoprecipitated after fractionation of cells that were only pulse-labeled with [^{35}S]methionine for 5 min on an identical gradient (Fig. 7*E*). The distribution of the precursor of the asialoglycoprotein receptor, an integral membrane protein, in the rER peak was not changed by the use of saponin (not shown), indicating that saponin did not change the localization of the rER on the gradient. The peak of galactosyltransferase shifted only one fraction to a higher density (not shown). When cells were chased for 1 h (Fig. 7*A*), three major peaks of cathepsin D were found. The first peak (fractions 1–5) co-fractionated with the rER marker at the bottom of the gradient and contained only the 51-kDa form. The 53-kDa and 55-kDa forms fractionated together with some 51-kDa in a second peak in fractions (6–13) at medial densities. These fractions also contained the highest level of galactosyltransferase enzyme activity. The fractions (14–17) in the third peak at the top of the gradient contained mostly the 51-kDa species.

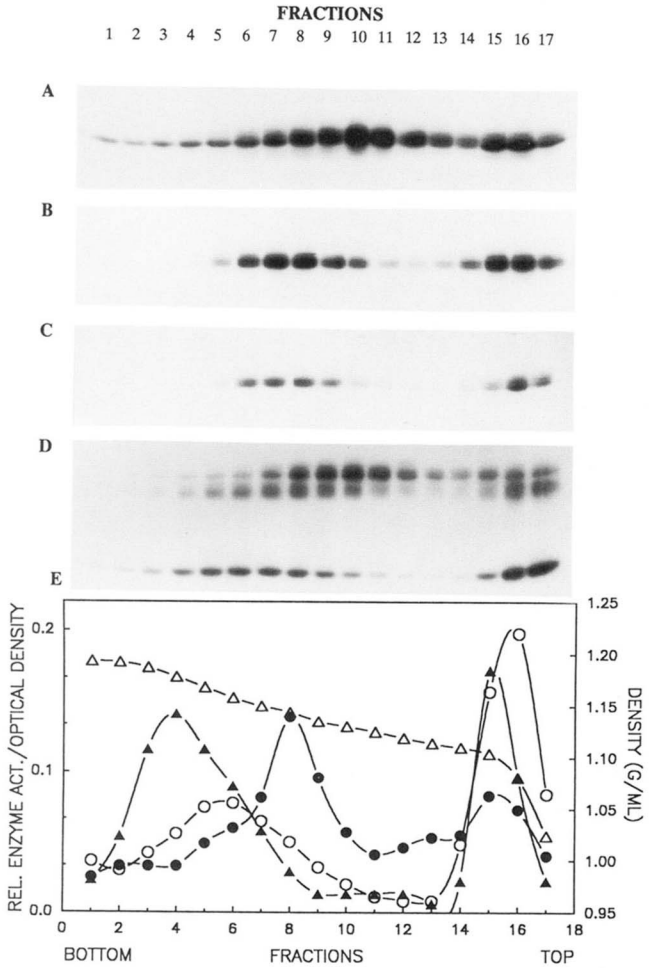

FIG. 7. **Subcellular distribution of cathepsin D on sucrose density gradients.** Cell cultures were pulse-labeled with [^{35}S]methionine for 15 min and chased for 1 h (*A*, *B*, *C*) or 2.5 h. (*D*). After the chase period, cells were incubated for 30 min at 0 °C with either PBS (*A* and *D*) or 2 mg/ml saponin in PBS in the absence (*B*) or the presence (*C*) of 10 mM man6-p. Cells were then homogenized and applied to 0.7 to 1.5 M sucrose gradients for analysis of cathepsin D distribution. Fractions were processed for immunoprecipitation of cathepsin D, and the immunoprecipitates were subjected to SDS-PAGE. Density (*open triangles*), enzyme activities for galactosyltransferase (*solid circles*), and β-hexosaminidase (*open circles*), and the distribution of albumin after pulse labeling with [^{35}S]methionine (*solid triangles*) are shown in *E*.

Incubation of the cells with saponin prior to the homogenization caused the 51-kDa form to disappear from the rER peak (Fig. 7*B*). However, this form remained present in comparable amounts with the 53-kDa form in both other peaks. The 51- to 55-kDa species had the same distribution as they had without saponin treatment, although they fractionated at a slightly higher density (one fraction). Some of the 51- to 55-kDa species that fractionated in light fractions (10–12) (Fig. 7*A*) were lost upon treatment with saponin. Whether this was due to a density change caused by the presence of saponin or to release from different compartments is not clear. Addition of 10 mM man6-p to the saponin yielded the identical distribution as obtained with saponin alone; only the amounts of radioactive material diminished (Fig. 7*C*).

Chasing the cells for 2.5 h resulted in two additional peaks. While the same distribution for the 51- to 55-kDa species was obtained as after 1 h of chase, the 44-kDa intermediate form appeared in fractions of densities slightly higher than the peak containing the Golgi fractions (Fig. 7*D*). The 31-kDa form co-fractionated with β-hexosaminidase at densities slightly higher than the 44-kDa form. Upon saponin treatment, both the 44- and 31-kDa species were released from the cells whereas the 51- to 55-kDa species maintained their localization (not shown). The 44- and 31-kDa species present on top of the gradient were likely from leaky vesicles. Similarly, the 62.5-kDa precursor of glucocerebrosidase fractionated with the rER peak, whereas the 68-kDa intermediate had a similar distribution as the 51- to 55-kDa species of cathepsin D in both of the other peaks; mature 59-kDa glucocerebrosidase co-fractionated with the 31-kDa cathepsin D, thus with β-hexosaminidase.

Based on these fractionation studies, we conclude that cathepsin D becomes membrane-associated as soon as it leaves the rER, as was already suggested by the release of most of the 51-kDa form in the studies on biosynthesis and membrane association (Fig. 2). Whether arrival in the Golgi complex occurs prior to membrane association or vice versa could not be determined. Addition of man6-p to the saponin reduced the amount of the 51- to 55-kDa species but had no effect on their distribution, indicating both man6-p-dependent and man6-p-independent membrane association occur in the same compartments.

DISCUSSION

We have studied the membrane association of lysosomal glucocerebrosidase, cathepsin D, and SAP during their biosynthesis. Glucocerebrosidase is a membrane-associated enzyme lacking both a membrane-spanning domain and man6-p residues, the common recognition signal for the MPR. Biosynthesis of glucocerebrosidase in HepG2 cells was similar to earlier studies (Erickson *et al.*, 1985; Jonsson *et al.*, 1987). Maturation of glucocerebrosidase comprises co-translational attachment of four *N*-linked oligosaccharide chains that are predominantly converted to the sialylated complex type (Erickson *et al.*, 1985; van Weely *et al.*, 1990). No proteolytic events are involved during maturation of the protein. Differential permeabilization of pulse-labeled cells with saponin showed that glucocerebrosidase was synthesized as a soluble protein and acquired its membrane association as soon as the high molecular mass species appeared. As expected, this membrane association was insensitive to man6-p.

The results on the biosynthesis of cathepsin D are in good agreement with studies in other cell types (Erickson *et al.*,

1981; Rosenfeld *et al.*, 1982; Gieselmann *et al.*, 1983). Cathepsin D was synthesized as a 51-kDa protein and underwent processing of both the polypeptide chain and the oligosaccharide chains. A small amount (<20%) of the 51- to 55-kDa species was secreted. Like most soluble lysosomal enzymes studied so far, cathepsin D targetting to lysosomes is mediated by MPR. We show that, in addition to MPR, cathepsin D shows a man6-p-independent membrane association in HepG2 cells. Cathepsin D became membrane-associated as soon as the higher molecular mass species appeared. Almost 50% of the membrane-associated species of cathepsin D was not released in the presence of an excess of man6-p. There was no difference between the high molecular mass species with regard to membrane association, man6-p sensitivity, or secretion into the culture media. Membrane association was independent of *N*-linked oligosaccharide chains. Part of the 51-kDa cathepsin D precursors was lost upon saponin permeabilization, suggesting that membrane association takes place after the protein has left the rER. This could also hold for the release of the precursor of glucocerebrosidase. The membrane association was not unique for cathepsin D. The high molecular mass intermediate of SAP behaved identically with cathepsin D. That membrane association of SAP was to some extent sensitive to man6-p suggests that a portion of SAP is transferred to the lysosomes by MPR. The synthesis of SAP in HepG2 cells was similar to that shown in fibroblasts (Fujibayashi and Wenger, 1986). Cell fractionation of differentially permeabilized cells on sucrose density gradients confirmed that these molecules became membrane-associated after they left the rER. Both man6-p-dependent as well as man6-p-independent membrane association of cathepsin D coincided with galactosyltransferase in fractions of medial density. Both types of membrane associations had the same relative distributions and apparently occurred in the same compartments. We conclude that cathepsin D is membrane-associated in the Golgi complex. It is possible that this membrane association continues in endosomes since the membrane association appears to be maintained until proteolytic processing occurs. This finding is in agreement with the recent observations of Diment *et al.* (1988) that in the endosomes of rabbit macrophages 90% of the 53-kDa precursor of cathepsin D could be solubilized only in the presence of 0.5% Triton X-100. These authors conclude that the 53-kDa precursor of cathepsin D is membrane-associated in endosomes and presumably in the Golgi complex.

Although cathepsin D and SAP share some properties of membrane association with glucocerebrosidase, it remains to be elucidated whether the same mechanisms are involved. The sequence of a cDNA encoding glucocerebrosidase has shown hydrophobic regions for formation of possible membrane anchors in the polypeptide chain (Barranger and Ginns, 1989). Since *N*-linked oligosaccharide chains are not a prerequisite for this membrane association, it seems likely that such hydrophobic regions are involved. For cathepsin D, membrane association has disappeared at the time of proteolytic processing. This is different from glucocerebrosidase where no proteolytic processing occurs during maturation. These results together with those with tunicamycin (Fig. 6) suggest that cleavage of precursors of cathepsin D involved detachment of the polypeptide membrane anchor or a conformational change of the protein, resulting in the dissociation from the membranes. On the other hand, it is possible that membrane association was caused by an interaction with an unknown receptor that prevented proteolytic processing until the protein was released.

It has been acknowledged for some time that MPR-independent targetting of lysosomal enzymes must exist (Kornfeld, 1986; von Figura and Hasilik, 1986). First of all, patients with mucolipidosis-II or I-cell disease, in which newly synthesized soluble lysosomal enzymes are secreted from the cells because phosphorylation of mannose residues does not occur, are known to contain normal amounts of glucocerebrosidase and lysosomal transmembrane proteins in all tissues. More strikingly, some tissues and cells of these patients, like liver, contain near normal levels of soluble lysosomal hydrolases, although these tissues and cells are impaired by the disease and cannot synthesize man6-p for targetting to the lysosomes by MPR (Neufeld and McKusick, 1983; Kornfeld, 1986; Nolan and Sly, 1989). It is possible that the man6-p-independent membrane association of soluble lysosomal enzymes observed in human hepatoma cells in the experiments presented in this paper is involved in MPR-independent targetting of lysosomal enzymes to lysosomes in such tissues as the liver.

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