The Lysosomal Transport of Prosaposin Requires the Conditional Interaction of Its Highly Conserved D Domain with Sphingomyelin*

Received for publication, January 11, 2002, and in revised form, February 18, 2002
Published, JBC Papers in Press, February 20, 2002, DOI 10.1074/jbc.M200343200

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Lysozomal prosaposin (65 kDa) is a nonenzymic protein that is transported to the lysosomes in a mannose 6-phosphate-independent manner. Selective deletion of the functional domains of prosaposin indicates that the D domain and the carboxyl-terminal region are necessary for its transport to the lysosomes. Inhibitors of sphingolipid biosynthesis, such as fumonisin B1 (FB1) and tricyclodecan-9-yl xanthate potassium salt (D609), also interfere with the trafficking of prosaposin to lysosomes. In this study, we examine sphingomyelin as a direct candidate for the trafficking of prosaposin. Chinese hamster ovary and COS-7 cells overexpressing prosaposin or an albumin/prosaposin construct were incubated with these inhibitors, treated with sphingolipids, and then immunostained. Sphingomyelin restored the immunostaining in lysosomes in both FB1- and D609-treated cells and ceramide reestablished the immunostaining in FB1-treated cells only. n-3-Morpholino-1-propanol (PDMP), which inhibits glycosphingolipids, had no effect on the immunostaining pattern. To determine whether sphingomyelin has the same effect on the transport of endogenous prosaposin, testicular explants were treated with FB1 and D609. Sphingomyelin restored prosaposin immunogold labeling in the lysosomes of FB1- and D609-treated Sertoli cells, whereas ceramide restored the label in FB1 treatment only. Albumin linked to the D and COOH-terminal domains of prosaposin was used as a dominant negative competitor. The construct blocked the targeting of prosaposin and induced accumulation of membrane in the lysosomes, demonstrating that the construct uses the same transport pathway as endogenous prosaposin. In conclusion, our results showed that sphingomyelin, the D domain, and its adjacent COOH-terminal region play a crucial role in the transport of prosaposin to lysosomes. Although the precise nature of this lipid-protein interaction is not well established, it is proposed that sphingomyelin microdomains (lipid rafts) are part of a mechanism ensuring correct intercellular trafficking of prosaposin.

Soluble hydrolases are transported to the lysosomes by the mannose 6-phosphate (M6P)1 receptor. In the endoplasmic reticulum, the newly synthesized hydrolase acquires a preformed oligosaccharide from a lipid-linked intermediate (1) that is subsequently modified in the Golgi apparatus by the addition of phosphomannosyl residues by the UDP-N-acetylglucosamine-1-phosphotransferase. This enzyme catalyzes the transfer of N-acetylglucosamine phosphate to mannose residues in the precursor oligosaccharide chain (2, 3). N-Acetylglucosamine residues are then removed by a phosphodiesterase (4), and the exposed M6P residues are recognized by the the M6P receptor (5).

However, some soluble lysosomal proteins such as prosaposin are transported to the lysosomes in a M6P-independent manner (6). Fibroblasts from patients with mucolipidosis (I-cell disease), caused by a mutation in the UDP-N-acetylglucosamine-1-phosphotransferase that prevents the formation of M6P residues, contain prosaposin in their lysosomes (7). Hepatocytes from these patients have near normal levels of several M6P-dependent soluble lysosomal hydrolases (2, 8, 9). Treatment of cells with the N-glycosylation inhibitor tunicamycin does not block the transport of prosaposin to the lysosomes (10), and when subcellular Golgi fractions from culture cells are permeabilized with a mild detergent, prosaposin remains associated to the Golgi membrane even after competition with free M6P (10).

Prosaposin (65–70 kDa) is a glycoprotein produced in high concentration in the testis, spleen, and brain. The 65- and 70-kDa isomers are encoded by the same gene and arise from post-translational modifications of the same protein (11). The 65-kDa protein is targeted to the lysosome, whereas the 70-kDa isomer is secreted to the extracellular space (11). Prosaposin contains four functional domains, A, B, C, and D (12). In the lysosome, prosaposin is proteolytically cleaved into four 10–15-kDa heat-stable polypeptides termed saposins A, B, C, and D (13). Saposins promote the degradation of sphingolipids by specific lysosomal hydrolases (14). Saposins A and C act in synergy to stimulate the hydrolysis of glucosylceramide and galactosylceramide by activating β-glucosylceramidase and β-galactosylceramidase (15, 16). Deficiency of saposin C causes a variant form of Gaucher’s disease, characterized by mental retardation and distinguished by the accumulation of lipids in the liver and spleen (17). Saposin B activates arylsulfatase A, α-galactosidase, and β-galactosidase (18, 19). Deficiency of saposin B causes a variant form of the lysosomal storage disorder metachromatic leukodystrophy (20). Although saposin D has no known metabolic function, its amino acid sequence along with the adjacent COOH-terminal region is the most conserved region of the molecule. Because of this evolutionary conservation, it was hypothesized that this region of the protein is...
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MATERIALS AND METHODS

Confocal Microscopy

Cell Culture I—CHO cells transfected with a stable expression vector containing a human prosaposin cDNA (21) were incubated in Coon’s F-12 medium (Sigma) supplemented with 10% fetal bovine serum, antibiotics, and 0.1% methotrexate (Sigma) for 72 h. The cells were then trypsinized and resuspended in methotrexate-supplemented media at a concentration of 2 × 10⁴ cells/ml. 2.5 ml of the cell rich medium was added to six wells containing three coverslips each. Six plates were prepared and incubated overnight at 37°C.

Cell Culture II—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum and antibiotics. The cells were harvested, then plated (5 × 10⁶/100 ml) in NuSerum-supplemented medium overnight in preparation for a transient transfection. The cells were transfected with a wild-type prosaposin construct or an albumin/Sap-D/COOH (Alb-D-COOH) construct as described by Zhao and Morales (25) using a DEAE-dextran/chloroquine phosphate protocol and then plated using Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics overnight.

Inhibitors—The medium was removed from the dishes, and the cells were washed with PBS for 1 min. The CHO and COS-7 cells received fumonisin B1 (FB1, PDMP, and D609) supplemented medium (Biomol Research Laboratories, Inc., PA), Fresh medium was added to other plates to serve as negative controls. The plates were then incubated for 24 h at 37°C.

Esogenous Lipids—CHO and COS-7 cells received treatment with either ceramide, dihydroceramide, or sphingomyelin (Biomol Research Laboratories, Inc., PA) at a final concentration of 5 μM. One set of plates received no lipids. The plates were then incubated for 24 h at 37°C. In the case of the CHO cells, separate plates were also incubated for 12 and 48 h. Incubation at 48 h was of interest because the turnover time of sphingomyelin in vitro at 37°C is 20–26 h.

Immunofluorescent Staining—The cells were washed 3 times in PBS, and, in the case of the COS-7 cells, also incubated in 60 nm Lysotracker (Molecular Probes Inc. Eugene, OR) for 30 min. The cells were then fixed on coverslips with 3.8% paraformaldehyde (Sigma) for 30 min at room temperature and then rinsed twice with PBS and treated with 0.5% Triton X-100 (Roche Molecular Biochemicals) for 10 min at room temperature. The cells were blocked with 100 μl of 10% goat serum for 1 h followed by 100 μl of a-prosaposin antibody for the CHO cells or 100 μl of a-Myc antibody for the COS-7 cells diluted 1:200 in PBS overnight at 4°C. The a-prosaposin antibody was generated in our laboratory against the amino terminus and the functional domains A-B of prosaposin. The characterization and specificity of this antibody was discussed in a previous paper (25). Conversely, a second group of CHO cells treated with the same conditions were incubated with 100 μl of a-carboxyprosaposin antibody (a generous gift from Dr. John S. Mort, Shriner’s Hospital, Montreal, Canada) diluted 1:200 in PBS. The cells were then washed in 0.05% Tween 20 (Sigma) 3 times for 5 min each. The appropriate FITC-conjugated secondary antibody (Sigma) was diluted 1:200, and 100 μl of the antibody solution was placed on each coverslip for 1 h at room temperature. The cells were then washed with 0.05% Tween 20 three times for 5 min each followed by a rinse with distilled water. The coverslips were mounted face down on microscope slides with 90% Mowiol (Calbiochem) in PBS to be viewed on a Zeiss 410 confocal microscope (Carl Zeiss, Germany). The slides were stored in a light proof black box.

Electron Microscopy

Tissue Culture—Three mice were anesthetized using Somnotol (MTC Pharmaceutical Inc.), and their testis were removed under sterile conditions. The tunica albuginea was removed and the seminiferous tubules cut into 2-mm pieces and placed in 10 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics overnight at 37°C.

Cell Culture—COS-7 cells were plated on 100-mm plates and transfected as described above. Some plates did not receive inhibitors or...
Incubation with 5 μM dihydroceramide (G), or 5 μM sphingomyelin (H). Treated cells showed a decreased immunostaining pattern compared with CHO cells not treated with the inhibitor (E). The addition of ceramide, dihydroceramide, or sphingomyelin restored the labeling pattern similar to the untreated control cells (A). CHO cells overexpressing prosaposin were treated with D609 (100 μM) (J–L) followed or not by a 12-h incubation with 5 μM ceramide (J), 5 μM dihydroceramide (K), or 5 μM sphingomyelin (L). All cells displayed a similar immunostaining pattern to untreated control cells (A). CHO cells overexpressing prosaposin were treated with D609 (100 μM) (M–P) followed or not by a 12-h incubation with 5 μM ceramide (M), 5 μM dihydroceramide (O), or 5 μM sphingomyelin (P). The D609-treated cells showed a decreased immunostaining pattern compared with CHO cells not treated with the inhibitor (M). Ceramide or dihydroceramide addition did not modify the weak labeling. Sphingomyelin addition displayed a weak perinuclear reaction. All cells were labeled with α-prosaposin antibody followed by a secondary antibody conjugated to FITC and viewed under a confocal microscope (×400).

Preparation of Lowicryl K4M Sections—The COS-7 cells were trypsinized, pelleted, and fixed for 1 h with 2.5% paraformaldehyde and 0.5% glutaraldehyde in 1 M sodium buffer. The cells were then placed in agarose for structural support and dehydrated with the pieces of the seminiferous tubules in ascending concentrations of cold ethanol and embedded in Lowicryl K4M. Ultrathin sections were cut and placed on Formvar-coated nickel grids.

Immunocytochemistry—40-μl drops of 10% goat serum were placed on a rubber mat within a Petri dish, and the grids were incubated on the drops tissue-side down for 15 min. The grids were then placed on drops of α-prosaposin antibody diluted 1:20 in TBS at a concentration of 50 μg/ml (seminiferous tubules) or α-Myc (1:10) antibodies (COS-7 cells) and incubated for 1 h. Four washes of 5 min each with 0.1% Tween 20 were followed by a second 15-min incubation with goat serum. The grids were then incubated on secondary α-rabbit antibody conjugated to 15-nm gold (COS-7 cells) or α-Myc (1:10) antibodies (COS-7 cells) and incubated for 1 h. Four washes of 5 min each with 0.1% Tween 20 were followed by a second 15-min incubation with goat serum. The grids were then incubated on secondary α-rabbit antibody conjugated to 15-nm gold and α-mouse conjugated to 10 nm gold (COS-7 cells). Four 5-min washes with 0.1% Tween 20 in Tris-buffered saline and two washes in distilled water were followed by counterstaining with uranyl acetate for 2 min and lead citrate for 30 s. The grids were then viewed on a Philips 400 electron microscope.

Preparation of Epon Sections—The COS-7 cells were trypsinized, pelleted, and fixed for 1 h with 2.5% glutaraldehyde in 0.1 M phosphate buffer. After embedding in 1% agarose, the cells were post-fixed with osmium tetroxide. Increasing concentrations of ethanol were used for subsequent dehydration. The cells were then embedded in Epon. Semithin sections were cut and mounted on 200 mesh copper grids. Staining of the grids was done with uranyl acetate for 5 min followed by lead citrate for 2 min. The grids were viewed on a Philips 400 electron microscope.

Statistical Analysis—Quantitative analysis was performed to determine the colloidal gold density of prosaposin labeling in lysosomes. Lysosomes were selected in a manner that included the following criteria; lysosomes had to be spherical, range from 0.2 to 0.4 μm in diameter, and have moderate electron density (10). Thirty lysosomes per condition were examined from three different grids that came from three mice. The 15-nm gold particles in each lysosome were counted, and the area of each lysosome was determined using a MOP-3 instrument (Carl Zeiss, Germany). The average lysosomal density was measured by dividing the number of gold particles by the lysosomal area. The mean density and the S.D. were calculated for each group, and the results were analyzed statistically using Student’s t test.

RESULTS

Confocal Microscopy

In a previous study sphingomyelin was singled out to conditionally interact with the D domain of prosaposin before its transport to the lysosomes. However, the evidence for the role of sphingomyelin in this process was based on the use of sphingolipid inhibitors. Thus, the first objective of the present investigation was to examine the direct effect of sphingomyelin and sphingomyelin precursors in the transport of prosaposin to the lysosomes using sphingomyelin-depleted cells.

Control Cells—To determine whether the incubation of CHO cells with sphingomyelin, dihydroceramide, and ceramide altered or not the distribution of prosaposin to the lysosomes, CHO cells were incubated with these lipids for 12, 24, and 48 h and stained with α-prosaposin antibody. The immunostaining yielded a perinuclear Golgi-like staining and a granular reaction typical of lysosomes comparable with CHO cells that did not receive the lipid supplementation (21) (Figs. 2, A–D, 3, A–D, and 4, A–D).

Effect of Dihydroceramide, Ceramide, and Sphingomyelin in CHO Cells Treated with Fumonisin B1—Treatment of cells with the sphingolipid inhibitor fumonisin B1 showed a decrease in immunostaining with α-prosaposin antibody (Fig. 2, panel E). Incubation of FB1-treated cells with ceramide, dihydrocer-
amide, or sphingomyelin for 12, 24, and 48 h increased the immunostaining pattern similar to that of the control cells (Figs. 2–4, panels F–H). FB1 blocks the synthesis of dihydroceramide but does not interfere with the conversion of exogenous dihydroceramide or ceramide to sphingomyelin. Hence, the addition of sphingomyelin or the restoration of the sphingomyelin pathway restituted the cytoplasmic staining in CHO cells.

**Fig. 3.** CHO cells overexpressing prosaposin were incubated with 5 μM ceramide (B), 5 μM dihydroceramide (C), or 5 μM sphingomyelin (D) for 24 h. Untreated cells served as a control (A). All cells displayed a similar immunostaining pattern, showing a perinuclear reaction. CHO cells overexpressing prosaposin were treated with FB1 (25 μg/ml) (E–H) followed or not by a 24-h incubation with 5 μM ceramide (F), 5 μM dihydroceramide (G), or 5 μM sphingomyelin (H). The FB1-treated cells showed decreased immunostaining compared with CHO cells not treated with the inhibitor (E). The addition of exogenous ceramide, dihydroceramide, or sphingomyelin restored the immunostaining similar to the untreated control cells (A). CHO cells overexpressing prosaposin were treated with GSL synthesis inhibitor PDMP (25 μg/ml) (I–L) followed or not by a 24-h incubation with 5 μM ceramide (J), 5 μM dihydroceramide (K), or 5 μM sphingomyelin (L). All cells displayed a similar immunostaining pattern to untreated control cells (A). CHO cells overexpressing prosaposin were treated with D609 (25 μg/ml) (M–P) followed or not by a 24-h incubation with 5 μM ceramide (N), 5 μM dihydroceramide (O), or 5 μM sphingomyelin (P). The D609-treated cells showed a decreased immunostaining compared with CHO cells not treated with the inhibitor (M). Ceramide or dihydroceramide additions did not modify this low labeling pattern. In contrast, the addition of sphingomyelin restored the labeling pattern to one similar to the untreated control cells (A). All cells were labeled with α-prosaposin antibody followed by a secondary antibody conjugated to FITC and viewed under a confocal microscope (×400).

**Fig. 4.** CHO cells overexpressing prosaposin were incubated with 5 μM ceramide (B), 5 μM dihydroceramide (C), or 5 μM sphingomyelin (D) for 48 h. Untreated cells served as a control (A). All cells displayed a similar immunostaining pattern, showing a perinuclear reaction. CHO cells overexpressing prosaposin were treated with FB1 (25 μg/ml) (E–H) followed or not by a 48-h incubation with 5 μM ceramide (F), 5 μM dihydroceramide (G), or 5 μM sphingomyelin (H). The FB1-treated cells showed a decreased immunostaining pattern compared with CHO cells not treated with the inhibitor (E). All cells displayed a similar immunostaining pattern to untreated control cells (A). CHO cells were treated with GSL synthesis inhibitor PDMP (25 μg/ml) (I–L) followed or not by a 48-h incubation with 5 μM ceramide (J), 5 μM dihydroceramide (K), or 5 μM sphingomyelin (L). All cells displayed similar immunostaining pattern to untreated control cells (A). CHO cells overexpressing prosaposin were treated with D609 (100 μg/ml) (M–P) followed or not by a 48-h incubation with 5 μM ceramide (N), 5 μM dihydroceramide (O), or 5 μM sphingomyelin (P). The D609-treated cells showed a decreased immunostaining pattern compared with CHO cells not treated with the inhibitor (M). The addition of ceramide, dihydroceramide, or sphingomyelin did not modify this low labeling pattern. (×400).
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**Effect of Dihydroceramide, Ceramide, and Sphingomyelin in CHO Cells Treated with D609**—Compared with non-treated cells, D609 significantly decreased the cytoplasmic immunostaining with α-prosaposin antibody (Figs. 2–4, panels M). The addition of ceramide or dihydroceramide for 24 h did not modify this weak staining (Figs. 3, N–P, and 4), whereas the addition of exogenous sphingomyelin for 24 h increased the staining to a level similar to that of the control cells (Fig. 2A). D609 inhibits the conversion of ceramide to sphingomyelin. Therefore, exogenous ceramide and dihydroceramide cannot restore the sphingomyelin pathway due to the distal action of this inhibitor. When the cells were incubated with ceramide, dihydroceramide, and sphingomyelin for 12 h (Fig. 2, N–P), the immunostaining pattern of α-prosaposin was weak, indicating that exogenous sphingomyelin did not have sufficient time to function. When the cells were treated with D609 followed by a 48-h incubation with ceramide, dihydroceramide, and sphingomyelin, no labeling was detected with α-prosaposin antibody (Fig. 4, N–P). Incubation at 48 h was of interest because the turnover time of sphingomyelin in vitro at 37 °C is 25–26 h. Hence, at 48 h exogenous sphingomyelin should be degraded, explaining the lack of immunostaining found in these cells. The cells incubated with sphingolipid precursors, including sphingomyelin, for 48 h after FB1 treatment were labeled (Fig. 4, F–H). Although sphingomyelin may be broken down in these cells, they are capable of synthesizing sphingomyelin by using ceramide from catabolized sphingolipids due to the location of the FB1 blockage (Fig. 1).

**Treatment of the CHO Cells with PDMP**—Incubation of CHO cells with PDMP that selectively blocks the production of glycosphingolipids but not of sphingomyelin (22) (Fig. 1) yielded a similar immunostaining to the control cells (Figs. 2–4, panels I–L). This suggests that the transport of prosaposin to the lysosomes did not require the presence of glycosphingolipids. The addition of ceramide, dihydroceramide, or sphingomyelin for 12, 24 and 48 h did not change the immunostaining of the cells reacted with α-prosaposin antibody.

**Cathepsin B Staining of CHO Cells**—The inhibitors and the exogenous sphingolipids were also added to CHO cells labeled with α-cathepsin B antibody (Fig. 5, A–L). Cathepsin B is a soluble lysosomal protein that is transported from the trans-Golgi to the lysosomes by the M6P receptor (26). As expected, inhibition of lipid synthesis with the inhibitors FB1 or D609 yielded a labeling pattern similar to that of untreated control cells labeled with α-cathepsin B antibody. The addition of exogenous ceramide, dihydroceramide or sphingomyelin alone for 24 h or in conjunction with any of the inhibitors did not change the immunostaining of cells labeled with α-cathepsin B antibody. This indicated that the treatment with the inhibitors and the addition of exogenous lipids did not affect the protein synthetic machinery or other cell biological functions such as M6P lysosomal targeting.

**Wild-type Construct-transfected COS-7 Cells**—The second objective of this investigation was to determine whether an albumin-prosaposin chimeric construct, to be used as a dominant negative competitor in this study, uses the same lysosomal pathway as prosaposin. To this effect cultured cells transfected with a prosaposin wild-type construct or with an albumin-prosaposin chimeric construct were incubated with sphingolipid inhibitors, supplemented with sphingomyelin, and immunostained with an Myc-tag antibody. As expected, cells transfected with the full-length prosaposin cDNA, linked to a Myc tag, showed a strong immunoreaction to the α-Myc antibody (Fig. 6, A–C). The antibody yielded an intense perinuclear reaction as well as a cytoplasmic punctate staining. LysoTracker (red fluorescence), which is known to stain acidic compartments (trans-Golgi, endosomes, and lysosomes), also produced a Golgi-like intense reaction in the perinuclear region and a punctate staining in the cytoplasm of the transfected cells. Several overlapping images, appearing as yellow fluorescence, could be seen, demonstrating that recombinant prosaposin and the LysoTracker dye shared the same compartment. Cells treated with D609 lost the green prosaposin staining (Fig. 6D). The LysoTracker staining was decreased but still present in punctate structures (Fig. 6E). No overlapping was found under this experimental condition (Fig. 6F). When these cells were supplemented with exogenous sphingomyelin, both the green prosaposin staining in the lysosomal compartment and the red LysoTracker staining of the Golgi and lysosomal compartment returned (Fig. 6, G–I). Several overlapping structures could be seen suggesting the return of prosaposin into the lysosomal compartment, substantiating the hypothesis that sphingomyelin is required for the transport of prosaposin to the lysosomes.

**COS-7 Cells Transfected with the Albumin-Prosaposin Chimeric Construct**—Cells transfected with this construct designated albumin/SAP-D/COOH/Myc tag yielded a strong immunostaining in the perinuclear region and in cytoplasmic punctate structures (Fig. 7, A–I). Thus, albumin linked to the D domain plus the COOH-terminal region of prosaposin appeared...
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Treatment of Seminiferous Tubules—To examine the transport and distribution of endogenous prosaposin, we used isolated seminiferous tubules from mouse testes. In this model system two cell types are present, the germinal cells and the Sertoli cells. Sertoli cells are the somatic components of the seminiferous tubules, which have lysosomes containing prosaposin (27). Immunogold labeling of seminiferous tubules with the α-prosaposin antibody specifically labeled the lysosomes of Sertoli cells (Fig. 8). The quantitative analysis of immunogold labeling of the lysosomes of Sertoli cells from seminiferous tubules incubated in culture medium alone yielded an average density of 14.57 gold particles/μm² (Fig. 9). Sertoli cell lysosomes from seminiferous tubules supplemented with ceramide yielded a density of 16.88 gold particles/μm², whereas the tissue treated with sphingomyelin produced a density of 15.87 gold particles/μm². These results were not statistically significantly different when compared with untreated control seminiferous tubules (Figs. 8 and 9).

Tissue treated with 25 μg/ml of FB1 displayed a reduction of lysosomal immunogold labeling compared with control tissue. Still, few grains were localized in the lysosomes, and background was minimal. Statistical analysis supported these observations with the average density decreased to 3.79 gold particles/μm². This represented a reduction of 74% in comparison to the untreated control cells. This reduction was shown to be statistically significant using a Student’s t test. Seminiferous tubules treated with FB1 and then supplemented with sphingomyelin showed a green staining pattern similar to untreated control cells. Cytoplasmic green punctate structures as well as intense Golgi perinuclear reactions were seen in the transfected cells. LysoTracker staining frequently overlapped with the green immunostaining, demonstrating that the albumin/SAP-D-COOH/Myc tag construct reached the lysosomal compartment. This result provided additional evidence that sphingomyelin is required for prosaposin transport to the lysosomes.

FIG. 6. COS-7 cells were transfected with a full-length wild-type prosaposin construct. The prosaposin construct was localized with an α-Myc antibody and FITC-conjugated secondary antibody. LysoTracker, a red fluorescent acidic marker, was used to identify the lysosomal compartments of the cells. The control untreated cell (A) showed a perinuclear reaction with a strong punctate reaction. The LysoTracker staining (B) confirmed that the punctate structures were lysosomes. The construct was found in these structures as demonstrated by the overlaid image (C and inset). The addition of D609 eliminated most of the perinuclear staining and all of the punctate structures (D). As was verified by the LysoTracker stain (E) and overlaid image (F and inset), none of the constructs are in the lysosomal compartments. The addition of sphingomyelin (SM) to the D609-treated cells restored the perinuclear and punctate staining (G). The LysoTracker staining confirmed that the targeting of the construct was restored in the lysosomes, as demonstrated by the overlaid image (I and inset) (>1000).

FIG. 7. COS-7 cells were transfected with an albumin-prosaposin (Alb-D-COOH) construct. The construct was localized with an α-Myc antibody and localized with an FITC-conjugated secondary antibody. LysoTracker, a red fluorescent acidic marker, was used to identify the lysosomal compartments of the cells. The control untreated cell (A) showed a perinuclear reaction with a strong punctate reaction. The LysoTracker staining (B) confirmed that the punctate structures were lysosomes and that the constructs were found in these structures as demonstrated by the overlaid image (C and inset). The addition of D609 eliminated most of the perinuclear staining and all of the punctate structures (D). As is verified by the LysoTracker stain (E) and overlaid image (F and inset), none of the construct was in the lysosomal compartment. The addition of sphingomyelin (SM) to the D609-treated cells restored the perinuclear reaction and punctate reaction (G). The LysoTracker staining confirmed that the targeting of the construct was restored in the lysosomes as demonstrated by the overlaid image (I and inset) (>1000).
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Tissue treated with D609 exhibited a marked decrease in lysosomal labeling compared with control Sertoli cells. Quantitative studies supported the qualitative analysis, registering the average density of immunogold grains to be 3.16 gold particles/μm². This represented a reduction of 78% in comparison to untreated control Sertoli cells and was shown to be statistically significant using Student’s t test. The mouse testis treated with FB1, showed a visible reduction in lysosomal immunogold staining compared with control lysosomes of Sertoli cells. The FB1-treated tissue supplemented with 5 μM of ceramide (FB1 + C2) or sphingomyelin (FB1 + Sm) restored the immunogold labeling in the lysosomes of these cells. Sertoli tubules treated with GSL inhibitor (PDMP). The immunolabeling pattern in the lysosomes of Sertoli cells was similar to that of untreated control cells. Cells treated with D609 displayed a marked decrease in lysosomal labeling compared with control cells. The addition of ceramide (D609 + C2) did not restore the labeling of the lysosomes in these cells, whereas the addition of exogenous sphingomyelin (D609 + Sm) did (×40,000).

Plasmid Only—As a negative experimental control, COS-7 cells were transfected with the plasmid only. The immunogold labeling of these cells mimicked that of the Alb-D construct cells, with a high number of endogenous protein gold particles (15 nm) localized to the lysosomes. Small gold particles (10 nm) were absent from the lysosomes (Fig. 10A).

Alb-D Construct—In COS-7 cells transfected with the Alb-D construct, 10-nm gold particles representing the constructs were absent from the lysosomes. Instead, these cells showed a strong reaction with 15-nm gold particles, representing endogenous prosaposin labeling (Fig. 10B).

Alb-D-COOH Construct—Immunogold labeling in the lysosomes of cells transfected with the Alb-d-COOH construct showed an overwhelming majority of 10-nm construct-associated gold particles and weak labeling of endogenous-associated 15 nm particles (Fig. 10C). This observation was further confirmed by a quantitative analysis that demonstrated a statistically significant decrease of endogenous prosaposin immunogold labeling and an increase in the immunogold labeling of the chimeric construct (Fig. 10D).

Morphological Phenotype of Dominant Negative Competitors—The purpose of this experiment was to examine the morphological effects on the lysosomes of COS-7 cells transfected with the dominant negative competitor. The rationale was that if the Alb-D-COOH construct competes out the endogenous prosaposin, it should act as a dominant negative competitor that inhibits the transport of prosaposin to the lysosomes. Prosaposin-deficient lysosomes should exhibit accumulation of undigested membranes due to the inability of these organelles to digest sphingolipids (28).
Plasmid Only—As a negative experimental control, some COS-7 cells were transfected with the plasmid only. The morphology of multivesicular bodies and mature lysosomes in these cells were similar to that of the Alb-D construct cells and wild-type cells (Fig. 11, A and B).

Alb-D Construct—The multivesicular bodies and mature lysosomes of COS-7 cells transfected with Alb-D constructs were similar to the wild-type morphology (Fig. 11C).

Alb-D-COOH Construct—In cells transfected with Alb-D-COOH, lysosomal morphology was compromised. Specifically, no electron-dense mature lysosomes were observed. In the perinuclear region of these cells there was an accumulation of abnormal multivesicular bodies, which contain large quantities of undegraded membrane (Fig. 12, A and B).

DISCUSSION

In this investigation we present direct evidence that sphingomyelin is an essential sphingolipid for the transport of prosaposin to the lysosomes. Sphingolipids are membrane components containing a ceramide moiety linked to a carbohydrate (glycosphingolipids (GSLs)) or phosphocholine (sphingomyelin) (22). GSLs are found in the plasma membrane as integral components of the outer leaflet facing the extracellular space (29–31). Sphingomyelin is also found on the outer leaflet of the plasma membrane and on the luminal aspect of membranes enclosing intracellular organelles (31). GSLs and sphingomyelin are synthesized in the Golgi apparatus (22). Sphingolipids and cholesterol may be found in microdomains called lipid rafts that are implicated in sorting and vesicle formation (24). Degradation of sphingolipids occurs in lysosomes by the concerted activity of hydrolases and sphingolipid activator proteins (30, 31). Saposins A, B, C, and D are four activators derived from partial proteolysis of a common precursor, prosaposin (13). Studies in our laboratory demonstrated the existence of two forms of prosaposin, a lysosomal isomer (65 kDa) and a secreted form (70 kDa) found in extracellular fluids (10, 27). Genetic deficiency of lysosomal prosaposin leads to an accumulation of undigested sphingolipids in the lysosomes (13). Therefore, prosaposin follows two distinct trafficking pathways, (a) a direct delivery of prosaposin from the Golgi apparatus to the lysosomes and (b) a secretory routing from the Golgi apparatus to the extracellular space.

This investigation encompasses three objectives. The first one deals with the role of sphingomyelin in the targeting of prosaposin to the lysosomes. Lysosomal prosaposin (65 kDa) is a Golgi membrane-bound glycoprotein that is transported to the lysosomes in a mannose 6-phosphate-independent manner.
The use of inhibitors of sphingolipid synthesis, PDMP, FB1, and D609 suggested that prosaposin does not depend on GSLs but on sphingomyelin for its transport to the lysosomal compartment (21). However, the analysis of these data was based on the suppression of sphingolipid synthesis and, therefore, on negative results. The use of these inhibitors alone did not provide direct evidence since these compounds may have side effects on the treated cells. To overcome these hurdles we decided to examine the role of sphingolipids and, more specifically, sphingomyelin in the transport of prosaposin to the lysosomes. This objective was accomplished by altering endogenous lipid synthesis in CHO cells, COS-7 cells, and testis explants followed by the addition of exogenous lipids.

Treatment of prosaposin-transfected CHO cells with each of the inhibitors alone yielded results that were consistent with those reported in previous studies (21). Supplementation with exogenous sphingomyelin restored the prosaposin immuno-

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**FIG. 10.** Representative lysosome showing the presence of endogenous prosaposin (15 nm colloidal gold particles (arrows) and the absence of α-myc labeling in COS-7 cells transfected with an empty plasmid (Plasmid) or with Alb-D constructs (Alb-D). Representative lysosome showing a strong presence of Alb-D-COOH construct (encircled 10-nm colloidal gold particles) and a weak presence of endogenous prosaposin in lysosomes of transfected COS-7 cells (Alb-D-COOH) (×40,000). Panel D represents the density of gold particles per μm² of lysosome in each of the conditions listed above. The black bars represent the average density of endogenous prosaposin, whereas the gray bars demonstrate the Alb-D-COOH construct. In the plasmid-only and Alb-D-transfected cells, note the high concentration of endogenous prosaposin (14 and 16 particles/μm²) and the very low amount of construct labeling (1 and 2 particles/μm²). However, in the Alb-D-COOH, the construct labeling is very high (15 particles/μm²), whereas endogenous prosaposin is very low (3 particles/μm²). *, values statistically different according to Student’s t test.

**FIG. 11.** Representative multivesicular bodies (MVB) of COS-7 cells transfected with the Alb-D construct (A) or with the plasmid-only construct (B). The organelles show normal, wild-type morphology. Representative lysosome (L) of COS-7 cells transfected with the Alb-D construct is shown in panel C. This organelle also shows normal, wild-type morphology (×40,000).
staining. Although it was difficult to determine which compartment was stained at the resolution of the confocal microscope in CHO cells, the results suggest that sphingomyelin was required to restore the immunostaining pattern observed in control cells. This observation was supported by electron microscopic results, showing an inhibition of lysosomal prosaposin transport in the presence of D609. FB1 caused a decrease in the immunostaining pattern of CHO cells when labeled with α-prosaposin antibody. PDMP, a glycosphingolipid inhibitor that does not affect sphingomyelin synthesis, was also used in this investigation to serve as a negative control for CHO cells. Confocal microscopic images of cells treated with PDMP followed or not with supplementation of exogenous lipids displayed a similar immunostaining pattern to the untreated control cells when reacted with α-prosaposin antibody. These results indicate that glycosphingolipids are not required for the transport of prosaposin to the lysosomes.

Cathepsin B is a soluble lysosomal protein that is known to be targeted from the Golgi to the lysosomes. Confocal microscopy showed an unchanged immunostaining pattern of cathepsin B in cells treated with FB1 or D609. Because the half-life of cathepsin B is less than 24 h (32), the strong immunostaining with the α-cathepsin B antibody indicates that this pathway was not altered by FB1 or D609. Thus, our results indicate that none of the inhibitors used in this experiment nor the exogenous lipids interfered with the mannose 6-phosphate receptor system.

Prosaposin-transfected CHO cells were also incubated with exogenous sphingolipid precursors at various time intervals. The optimal incubation time was determined to be 24 h since the turnover rate of sphingomyelin in vitro was determined to be 25–26 h (33). Incubation at 12 h yielded similar results in the confocal microscope as the results obtained from cells incubated with the sphingolipid precursors for 24 h. The intensity of labeling was in some cases fainter than the intensity observed in the 24-h incubation. This was attributed to the optimal time required for the exogenous lipids to enter the different compartments of the cells. The 48-h incubation yielded varying results. The control cells and the PDMP-treated cells exhibited a similar immunostaining pattern as cells treated for 24 h. Cells treated with FB1, followed by the addition of exogenous lipids for 48 h displayed similar immunostaining pattern to their 24-h counterparts, including the case of sphingomyelin supplementation. The restoration of labeling seen when sphingomyelin was added to FB1-treated cells in the 48-h incubation was attributed to the de novo synthesis of endogenous sphingomyelin from breakdown products of exogenous sphingomyelin, which is metabolized in 25–26 h. On the other hand, D609-treated cells incubated with any of the exogenous lipids for 48 h showed no fluorescent immunostaining under the confocal microscope. The addition of sphingomyelin did not maintain the perinuclear labeling because the lipid was metabolized within the first 26 h, and the resulting ceramide from sphingomyelin breakdown could not be converted back to sphingomyelin due to the inhibition of sphingomyelin synthase by D609. In fact, exogenous ceramide or dihydroceramide added to the medium could not be converted to sphingomyelin due to the sphingomyelin synthase enzymatic blockage.

The data generated in the CHO cell line demonstrated a relationship between trafficking of prosaposin and the presence of sphingomyelin. However, the exact compartment in which prosaposin was targeted was difficult to determine by confocal microscopy. In a previous study using the same transfected CHO cell line in conjunction with electron microscopy, it was demonstrated that the final destination of recombinant prosaposin was the lysosomal compartment (21). In this investigation we used an additional model system, the seminiferous tubules from the mouse testes, to verify the role of sphingomyelin in the targeting of prosaposin to the lysosomes. Sertoli
cells, the somatic components of the seminiferous epithelium lining these tubules, are professional phagocytes that have large amounts of lysosomes containing endogenous prosaposin (27). Using a similar experimental approach (i.e. depletion and supplementation of sphingolipid precursors) and immunogold labeling, it was possible to study the effects of sphingomyelin on the targeting of prosaposin to lysosomes. This was best accomplished using the electron microscope and quantitative analysis on the lysosomes of Sertoli cells. The results confirmed the confocal data. Although PDMP did not decrease the immunogold labeling of the Sertoli cell lysosomes, fumonisin B1 and D609 produced a significant reduction in labeling. Dihydroceramide, ceramide, and sphingomyelin restored the labeling in fumonisin B1-treated tubules, and only sphingomyelin did in D609-treated tubules.

The second objective of this investigation was to determine whether an albumin-prosaposin chimeric construct used the same lysosomal pathway as prosaposin and consequently employ this construct as a dominant negative competitor. Thus, COS-7 cells were transfected with a prosaposin cDNA or with a chimeric construct composed of albumin, the prosaposin D domain, and its adjacent COOH-terminal region (termed albumin/SAP-D/COOH). Both recombinant proteins were linked to a Myc tag and displayed targeting to cytoplasmic punctate structures (green fluorescence) that also reacted with LysoTracker (red fluorescence), a dye specific for acidic organelles that also reacted with Lyso-Tracker (green fluorescence). Both recombinant proteins were linked to chimeric construct composed of albumin, the prosaposin D domain, and its adjacent COOH-terminal region (termed albumin/SAP-D/COOH). The results confirmed the confocal data. Although PDMP did not decrease the immunogold labeling of the Sertoli cell lysosomes, fumonisin B1 and D609 produced a significant reduction in labeling. Dihydroceramide, ceramide, and sphingomyelin restored the labeling in fumonisin B1-treated tubules, and only sphingomyelin did in D609-treated tubules.

The third and final objective was to determine the role of the prosaposin D domain by testing the hypothesis that the Alb-D-COOH chimeric construct uses the same lysosomal targeting mechanism as, and competes with, endogenous prosaposin. This competition should result in the depletion of prosaposin from the lysosomes of COS-7 cells.

Immunogold labeling of cells transfected with the Alb-D-COOH construct linked to an Myc tag showed well labeled lysosomes with the α-Myc antibody and negligible lysosomal labeling with the α-prosaposin antibody. Conversely, COS-7 cells transfected with the Alb-D construct without the COOH-terminal region showed no immunostaining with the α-Myc antibody and strong labeling with the α-prosaposin antibody. This type of immunogold labeling was also observed in control cells transfected with the plasmid only. These results indicate that the dominantly expressed chimeric protein (Alb-D-COOH construct) established preferential use of the targeting mechanism of endogenous prosaposin. The prosaposin antibody was raised to recognize domains A and B of prosaposin. Hence, the antibody does not cross-react with the albumin constructs, which contain the D domain and the COOH region, but do recognize mature saposins A and B. Thus, the weak immunogold labeling of the prosaposin antibody is attributed to the competitive effect of the construct and the presence of residual saposins. Along with the adjacent COOH-terminal region, the D domain is the most conserved region of prosaposin (34), and it has been suggested to interact with sphingomyelin (16) and acidic phospholipids (35). It is tempting to speculate that the role of the prosaposin D domain is to bind to sphingomyelin in the membrane of the cis-Golgi compartment. This interaction is probably required to allow the binding of the COOH-terminal domain of prosaposin to a targeting protein yet to be identified (Fig. 13).

Alb-D-COOH-transfected cells presented prominent accumulation of perinuclear multivesicular bodies and an absence of mature lysosomes. It appears that lysosomal progression becomes arrested at this stage of maturation. The resulting multivesicular bodies presented multi-layered membranes and prominent accumulation of undegraded lipid matter. These morphological results support the immunostaining data, demonstrating that the endogenous prosaposin is absent from lysosomes of cells transfected with Alb-D-COOH.

In conclusion, the Alb-D-COOH construct used the same mechanism of transport as endogenous prosaposin and acted as a dominant negative competitor that displaced prosaposin from the lysosomes, inducing the retention of undigested membranes in multivesicular bodies. These results support the notion of a novel mechanism of lysosomal targeting, involving a simultaneous interaction of the prosaposin D domain with sphingomyelin, and the COOH-terminal region of prosaposin with an unknown targeting protein (21, 25). A putative compartment where this interaction could occur is the cis/medial region of the Golgi apparatus, which has been implicated in the synthesis of sphingomyelin (36). Furthermore, a recent study demonstrated that the 65-kDa lysosomal prosaposin is endoglycosidase H-sensitive, whereas the 70-kDa secretory isoform is endoglycosidase H-resistant. Because the processing pathway within the Golgi apparatus is highly ordered, this result suggests that a significant fraction of the 65-kDa isoform is sorted in the Golgi apparatus before it reaches the distal stacks, where it becomes fully glycosylated and endoglycosidase H-resistant (25).

Interestingly, the COOH-terminal region of prosaposin is 66% similar to the NH2-terminal of region of surfactant B, which has been implicated in the targeting of this protein to the lamellar bodies of pneumocyte type II (37). Like prosaposin, surfactant B requires a prosaposin domain to be targeted to the lamellar bodies. Taken together, our investigation suggests the existence of a new targeting pathway between the Golgi apparatus and the lysosome.

REFERENCES

Role of Sphingomyelin in the Transport of Prosaposin

The Lysosomal Transport of Prosaposin Requires the Conditional Interaction of Its Highly Conserved D Domain with Sphingomyelin

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doi: 10.1074/jbc.M200343200 originally published online February 20, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M200343200

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