Identification of a Novel Sequence Involved in Lysosomal Sorting of the Sphingolipid Activator Protein Prosaposin*

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Prosaposin is synthesized as a 53-kDa protein, post-translationally modified to a 65-kDa form and further glycosylated to a 70-kDa secretory product. The 65-kDa protein is associated to Golgi membranes and is targeted to lysosomes, where four smaller nonenzymatic saposins implicated in the hydrolysis of sphingolipids are generated by its partial proteolysis. The targeting of the 65-kDa protein to lysosomes is not mediated by the mannose 6-phosphate receptor. The Golgi apparatus appears to accomplish the molecular sorting of the 65-kDa prosaposin by decoding a signal from its amino acid backbone. This investigation deals with the characterization of the sequence involved in this process by deleting the saposin functional domains A, B, C, and D and the highly conserved N and C terminus of prosaposin. The truncated cDNAs were subcloned into expression vectors and transfected to COS-7 cells. The destination of the mutated proteins was assessed by immunocytochemistry. Deletion of the C terminus did not interfere with the secretion of prosaposin but abolished its transport to lysosomes. Deletion of saposins and the N-terminal domain did not affect the lysosomal or secretory routing of prosaposin. A chimeric construct of albumin and the C terminus of prosaposin was not directed to lysosomes. However, albumin connected to the C terminus and one or more functional domains of prosaposin reached lysosomes, indicating that the C terminus and at least one saposin domain are required for this process. In summary, we are reporting a novel sequence involved in the targeting of prosaposin to lysosomes.

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with I-cell disease, which fail to phosphorylate mannose residues on newly synthesized lysosomal proteins have nearly normal levels of prosaposin and saposins in the lysosomes (43, 44).

To identify the sequence responsible for the lysosomal routing of prosaposin, deletions of different domains within this protein were done. We observed that residues 521–557 of the C terminus and at least one saposin domain are necessary for the sorting and targeting of prosaposin to lysosomes.

**EXPERIMENTAL PROCEDURES**

**Materials**—All restriction enzymes and modifying enzymes were purchased from Amersham Pharmacia Biotech, Promega (Madison, WI), Roche Molecular Biochemicals, Life Technologies, Inc., and Stratagene (La Jolla, CA). The pcDNA3.1B vector was bought from Invitrogen (Carlsbad, CA). The albumin cDNA clone was purchased from ATCC (Manassas, VA). Peptatin A (isovaleryl-Val-Val-Ala-Val) and phenylmethylsulfonyl fluoride were purchased from Sigma. Aprotinin, leupeptin, and (+)-brefeldin A (BFA)1 were from Calbiochem. Endoglycosidase H (Endo H) was from Roche Molecular Biochemicals. Dulbecco’s modified Eagle medium (DME), fetal bovine serum, and trypsin were bought from Life Technologies, Inc. DME without methionine and cysteine and t-glutamine, t-glutamine, and glutamine were purchased from Sigma-Aldrich. Nucleo Serum V culture was from Becton Dickinson Labware (Bedford, MA). ECL, Hybond nylon membrane was purchased from Amersham Pharmacia Biotech. Bovine serum albumin was from Roche Molecular Biochemicals, and Kodak X-Omat films were from Eastman Kodak (Rochester, NY). Lowicryl K4M was obtained from MecaLab (Montreal, Canada). LysoTracker Red DND-9 was purchased from Molecular Probes, Inc. (Eugene, OR).

**Antibodies**—Anti-procathepsin B polyclonal antibody was a gift from Dr. John Mort (Shrinor Hospital, McGill University). Mannosidase II antibody was from Dr. Michael D. Griswold (Washington State University). Anti-retroplakin (Carlsbad, CA). Anti-prosaposin polyclonal antibody was a gift from Dr. John Mort (Shriner Hospital, McGill University). Mannosidase II antibody (Endo H) was from Roche Molecular Biochemicals. Thionine and cystine and L-glutamine, L-methionine, L-glutamine, and L-cysteine were purchased from Sigma-Aldrich. The albumin cDNA clone was purchased from Amersham Pharmacia Biotech, Promega (Madison, WI). Lowicryl K4M was obtained from MecaLab (Montreal, Canada). LysoTracker Red DND-9 was purchased from Molecular Probes, Inc. (Eugene, OR).

**Recombinant cDNA Constructs**—A full-length prosaposin cDNA was obtained by screening a mouse testicular cDNA library (Stratagene, CA). Briefly, a PCR-amplified DNA fragment was labeled with 32P and used as a probe. (An aliquot containing 1 million recombinants was screened after transferring the plaques onto nitrocellulose membranes and by hybridizing them with the radioactive probe. Purified positive phages were grown, and the pHBlueScript plasmid containing the insert was excised from the phage according to the manufacturer’s instructions. A full-length 2.6-kb prosaposin cDNA in the plasmid was confirmed after sequencing both DNA strands and used as a template for the different constructs made during this study. The fidelity of individual constructs was confirmed by restriction mapping and DNA sequencing (Sheldon Facility Center, McGill University).

**Pro-WT** is the wild type prosaposin cDNA subcloned into a mamma
lion expression vector pcDNA3.1B. A pair of primers, Fc (5′-ggacagagtatcattgagttggtgct-3′) and Rc (5′-ggacagagtatcattgagttgtctt-3′), was designed to amplify a 1.6-kb fragment consisting of the whole open reading frame of prosaposin by PCR with vent DNA polymerase. A Kozak sequence GCCACC was added to primer Fc at the 5′-end. An EcoRI restriction site was added at the 5′-end of primer Rc for suitable subcloning. The pcDNA3.1B vector was digested with BamHI and filled in with a Klenow fragment to create a blunt end. The vector was then digested with EcoRI and the sequence and the DNA concentration was estimated. PCR fragments were digested with EcoRI restriction enzyme, purified, and subcloned into the prepared pcDNA3.1B vector. The resulting plasmid was designated Pro-WT.

**ΔN-term** and **Δc-term** are prosaposin constructs lacking part of the N terminus (residues 17–59) and C terminus (residues 520–556). For the **ΔN-term** truncated construct, part of its N-terminal region in front of the domain for saposin A was eliminated. An upstream primer Nf (5′-ggacagagtatcattgagttggtgct-3′) and downstream primer Nt (5′-ggacagagtatcattgagttgtctt-3′) were designed according to the sequence of prosaposin cDNA. Two pairs of primers, Fc/Nt and Rc/Nt, were used in the first run of PCR. Fc/Rc primers were utilized in the second run of PCR using amplified Fc/Nt fragment and Rc/Nt fragment as templates. PCR fragment (1.6 kb) was digested by EcoRI restriction enzyme and ligated into the prepared pcDNA3.1B vector. The resulting plasmid construct was called ΔN-term.

For the **Δc-term** truncated construct, the same upstream Fc primer and a new downstream primer COOHr (5′-ggacagagtatcattgagttggtgct-3′) were used to omit a sequence (LLLLGTEKCWGGWSPWYQNMET-AARCNAVHDKHRHHVIN) encoding the C terminus after the saposin D domain and subcloned into the pcDNA3.1B vector.

**ΔA** (residues 57–147), **ΔB** (residues 189–278), **ΔC** (residues 309–396), and **ΔD** (residues 434–522) mutant plasmids are prosaposin constructs lacking individual saposin functional domains. Three pairs of primers were used for each construct. The external primers Fc/Rc were the same as for the Pro-WT construct. The two internal primers were Af (5′-acagagaactggctggcaaacc-3′) and Ar (5′-ggacagagtatcattgagttggtgctg3′) for ΔA; primers Bf (5′-acagagaactggctggcaaacc-3′) and Br (5′-ggacagagtatcattgagttggtgctg3′) for ΔB; primers Cf (5′-ggacagagtatcattgagttggtgctg3′) and Cr (5′-ggacagagtatcattgagttggtgctg3′) for ΔC; and primers Df (5′-ggacagagtatcattgagttggtgctg3′) and Dr (5′-ggacagagtatcattgagttggtgctg3′) for ΔD. All of these primers were backwarded to each other. The pairs of primers Fa/Cr and Ar/Rc, Fa/Br and Cr/Rc, Fa/Rc and Dr/Rc, and Fa/Dr and Br/Rc were used in the first run of PCR for ΔA, ΔB, ΔC, and ΔD constructs, respectively. Primers Fa/Rc were used in the second run of PCR using the amplified Fa/Ar constructs, Fa/Br fragments, Fa/Br and Rc/Rc fragments, Fa/Rc and Dr/Rc fragments, and Fa/Dr and Br/Rc fragments as templates for each construct. The final PCR products were purified and digested with EcoRI and then subcloned into the prepared pcDNA3.1B vector.

**Alb/Pro-c-term**, **Alb/(Pro-D+C-D+Pro-c-term)**, and **Alb/WT-Constructs**—The first three fusion protein constructs contain the full-length albumin and partial prosaposin sequences, i.e., the C terminus of prosaposin (Alb/Pro-c-term), the domain D plus the C terminus of prosaposin (Alb/Pro-D+C-term), and the domains C and D plus the C terminus of prosaposin (Alb/Pro-C+D+Pro-c-term). Alb-WT is wild type albumin. Three pairs of primers were used for Alb-WT, in which one pair of primers (Alb/F/Alb-R) prepared according to the nucleotide sequence of albumin. Primers Alb-F (5′-ccagcagcagccctcgccctc-3′) and Alb-R (5′-cacgccccaactcgtgggcctggaccagat-3′) were used for Alb/Pro-B-f: Alb-WT (5′-ccagcagcagccctcgccctc-3′) and Alb/Pro-D+Pro-c-term: Alb-WT (5′-ccagcagcagccctcgccctc-3′).

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**Cell Culture and Transfections**—COS-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO2 at 37 °C. Cells (60% confluence) grown on coverslips (12 mm in diameter) in six-well dishes were transfected with LipofectAMINE according to the manufacturer’s instructions. Cells grown in 10× 106 CFU of dishes were transfected according to the DEAE-dextran method (76). Briefly, COS-7 cells (5 × 105) were seeded into DMEM containing 10% Nu-Serum overnight. 5 μg of plasmid DNA was mixed with 40 μl of 5% DEAE-dextrin, 5 μl of 0.1 M chloroquine phosphate in 5 ml of DMEM with 10% Nu-Serum for 4 h. Cells were washed with 10% MeSO for 1 min, rinsed with PBS, and replaced with DMEM containing 10% fetal bovine serum for at least 48 h.
**Metabolic Labeling, Immunoprecipitation, and Endo H Digestion—**

Cells were washed twice with PBS after 48 h of transfection with different plasmid constructs and starved for 1 h in starvation medium (DME/F lacking methionine and cysteine but containing 5% dialyzed fetal bovine serum and 200 μM glutamine). Cells were then pulsed in starvation medium supplemented with Trans-35S-label (300 μCi/ml) for the times indicated. For experiments including chase periods, the radiolabeled medium was removed, and cells were washed twice with PBS and incubated with cell growth medium supplemented with unlabeled methionine (1.5 mg/ml) and cysteine (2.4 mg/ml) for the chase time indicated. When specified, cells were incubated with brefeldin A (20 μg/ml) for 1 h prior to the addition of medium containing the radiolabeled and fresh brefeldin A.

Monolayers of cells and media were collected. Cells were lysed by boiling in 50 μl of 1% SDS and 200 mM dithiothreitol for 5 min. An equal volume of 100 mM citric acid buffer (pH 5.5) and 1 unit of endoglycosidase H was added to the immunoprecipitates and incubated at 37 °C for 16 h. The processed samples were resolved by 10% SDS-PAGE and detected by fluorography. After treating with primary anti-Myc antibody or anti-prosaposin antibodies bound to anti-mouse IgG-agarose or protein A-agarose, the immunoprecipitates were washed three times with 1× lysis buffer. Samples were then resolved by SDS-PAGE, and radioactive signals were visualized by fluorography. A 15% Tricine-SDS-PAGE was performed to resolve smaller molecular weight proteins.

To digest the immunoprecipitates with endoglycosidase H, they were released from agarose and antibodies first, by boiling in 50 μl of 1% SDS and 200 mM dithiothreitol for 5 min. An equal volume of 100 mM citric acid buffer (pH 5.5) and 1 unit of endoglycosidase H was added to the immunoprecipitates and incubated at 37 °C for 16 h. The processed samples were resolved by 10% SDS-PAGE and detected by fluorography.

**Immunofluorescent and LysoTracker Staining and Confocal Microscopy—**

For immunofluorescent staining, the cells transfected with the wild type and mutated constructs were fixed with 3.8% paraformaldehyde buffer (5 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 0.5% Nonident P-40, 10 μg/ml pepstatin A, 10 μg/ml aprotime, 10 μg/ml leupeptin). After treating with primary anti-Myc antibody or anti-prosaposin antibodies bound to anti-mouse IgG-agarose or protein A-agarose, the immunoprecipitates were washed three times with 1× lysis buffer. Samples were then resolved by SDS-PAGE, and radioactive signals were visualized by fluorography. A 15% Tricine-SDS-PAGE was performed to resolve smaller molecular weight proteins.

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**Immunogold Labeling—**

For immunofluorescent staining, the cells transfected with the wild type and mutated constructs were fixed with 3.8% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature and washed with PBS. Cells were permeabilized with 0.5% Triton X-100 in PBS for 30 min at room temperature and then blocked with 10% goat serum in PBS for 1 h. After washing with PBS, the cells were incubated with monoclonal antibody against Myc (1:100) and polyclonal antibody against mannosidase II (1:500) at 4 °C overnight. Following three washes in 0.05% Tween 20 in PBS, cells were incubated with a 1:100 dilution of FITC-conjugated goat anti-mouse and tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit secondary antibodies. After three washes in 0.05% Tween 20 in PBS, the cells were rinsed with double distilled H2O. Subsequently, the coverslips were mounted on slides using Mowiol (Calbioche). Staining of acidic compartments was done on live COS-7 cells grown on coverslips by adding LysoTracker (50 nM) to culture medium for 30 min at 37 °C, followed by incubation in normal medium without LysoTracker for 15 min. Cells were then immunostained as described above. Cells were examined with a Zeiss Axiovert confocal microscope with a ×63 objective and the appropriate filter set. Fluorescent images were then resized using Adobe Photoshop.

**Deletion of Prosaposin Functional Domains—**

To assess the overlap between the two antibodies in all constructs, including the N-term and C-term mutants with Myc antibody and secondary FITC-conjugated goat anti-mouse antibody.

**RESULTS**

**Analysis of Prosaposin Domains—**

Alignment of amino acid residues for saposins as well as the N and C terminus of prosaposin showed a similarity of 70–90% among different species (2, 3, 45, 46). Each saposin contains 80 amino acids, 6 conserved cysteine residues that form three internal disulfide bonds, an N-glycosylation site, a conserved proline residue, and 14 or 15 hydrophobic amino acid residues at the same position (46). Alignment between the N (residues 1–60) and C termini (residues 521–557) of prosaposin showed a 40% identity and 66% similarity. A GenBank data base search revealed a 70% similarity between 29 amino acids of the N terminus of the human surfactant protein B (SP-B) and the C terminus of prosaposin. This surfactant protein B sequence is involved in the transport of this protein to the lamellar bodies of pneumocyte type II (47). A unique characteristic of the lysosomal nature of lamellar bodies is the content of hydrolases and a lysosomal membrane glycoprotein (48, 49).

**Deletion of Prosaposin Functional Domains—**

Based on the analysis of prosaposin, saposin functional domains A, B, C, and D and the N and C terminus of prosaposin were deleted. The truncated constructs were designated ΔA, ΔB, ΔC, ΔD, N-term, and ΔC-term to indicate the deleted region of the protein. Wild-type prosaposin was designated Pro-WT (Fig. 1A). The constructs were subcloned into a mammalian expression vector, pcDNA3.1B, which harbors a Myc epitope tag before the stop codon. Pro-WT and all truncated mutants were transiently transfected into COS-7 cells. The intracellular distribution of the constructs was assessed by immunofluorescent staining with primary anti-Myc monoclonal antibody and secondary FITC-conjugated goat anti-mouse antibody.

The two forms of wild type prosaposin (i.e., the 65-kDa lysosomal form and the 70-kDa secretory form) were found by Western blot analysis with antibody against Myc in Pro-WT (Fig. 1B). All mutants also produced two forms of prosaposin with lower molecular weights due to the truncation of their corresponding domains (Fig. 1B). Confocal immunofluorescence of COS-7 cells transfected with wild-type prosaposin Pro-WT and ΔA, ΔB, ΔC, ΔD, and ΔN-term mutants with Myc antibody yielded both a perinuclear and a granular reaction. Cells transfected with the ΔC-term mutant did not yield any granular staining, but the reaction persisted in the perinuclear region (Fig. 1C).

To confirm whether the granular structures observed in Pro-WT and ΔA, ΔB, ΔC, ΔD, and ΔN-term were lysosomes, the lysosomal marker LysoTracker was used as a probe. This marker is sensitive to low pH and stains all acidic vesicles including the trans-Golgi region. The LysoTracker (red fluorescence) showed overlap with the punctate structures stained with Myc antibody (green fluorescence) in Pro-WT, ΔA, ΔB, ΔC, ΔD, and ΔN-term (Fig. 2A). In ΔC-term mutant, although the LysoTracker detected numerous lysosomes, Myc antibody staining was negative in these granules (Fig. 2A).

Since the Myc antibody yielded a perinuclear reaction reminiscent of the Golgi apparatus in all of the constructs including the ΔC-term mutant, an anti-mannosidase II antibody was used as a Golgi marker. Confocal immunostaining revealed an overlap between the two antibodies in all constructs, including in the ΔC-term mutant (Fig. 2B).

To further confirm the immunofluorescence results, a double immunogold labeling was conducted on COS-7 cells transfected with the Pro-WT, ΔA, ΔB, ΔC, ΔD, ΔC-term, and ΔN-term
FIG. 1. Effect of truncation on the lysosomal transport of prosaposin. A, schematic representation of wild type and truncated prosaposin constructs subcloned into the pcDNA3.1B expression vector. Pro-WT is the wild type prosaposin containing the full-length cDNA of prosaposin encoding the N terminus (N-term), the saposin A, B, C, and D domains, and the C terminus (C-term). The truncated constructs were designated ΔN-term (deletion of residues 17–59), ΔA (deletion of residues 57–147), ΔB (deletion of residues 189–278), ΔC (deletion of residues 309–396), ΔD (deletion of residues 404–522), and ΔC-term (deletion of residues 520–556) to indicate the specific regions deleted from prosaposin (shown by dotted lines). B, expression of wild type and truncated prosaposins after transfection into COS-7 cells. Approximately 50 μg of whole cell lysates were
constructs and analyzed by electron microscopy. To accomplish this objective, goat anti-mouse IgG conjugated to 10-nm gold was used to detect the monoclonal Myc antibody, and goat anti-rabbit IgG conjugated to 15-nm gold was used to detect the rabbit procathepsin B antibody. The results showed a strong labeling with 10-nm gold particles and a weak labeling with the 15-nm gold particles of electron-dense membrane-bound structures in COS-7 cells transfected with the Pro-WT, ∆A, ∆B, ∆C, ∆D, and ∆N-term (Fig. 3). These structures were considered to be lysosomes. However, in cells transfected with the ∆C-term construct, no labeling was found with anti-Myc antibody in the lysosomes.

Metabolic Labeling Study—To study the biosynthetic processing of the wild type and truncated prosaposin in vivo, to examine whether they were properly folded within the endoplasmic reticulum (ER) and therefore transported to their destinations, pulse-chase experiments were conducted using Tran<sup>35</sup>S-label. COS-7 cells transfected with pcDNA3.1B vector alone or containing the Pro-WT and the truncated constructs were incubated with Tran<sup>35</sup>S-label for 30 min and chased for 30 min and 2 h. Cell lysates and samples from the medium were immunoprecipitated with anti-Myc antibody and then subjected to SDS-polyacrylamide gel electrophoresis and fluorography. As shown in Fig. 4A, the 65-kDa form was visualized after 30 min in Pro-WT and in each of the truncated mutants including the ∆C-term. After chasing for 2 h, the amount of 65-kDa protein decreased in cell lysates, while the 70-kDa secretory protein increased in the spent media. In the mutants, the equivalents of the 65- and 70-kDa proteins were slightly smaller due to their truncation. In the case of the ∆C-term mutant, the increase of the secretory form in the medium was more prominent as confirmed by densitometry (Fig. 4B). These data suggest that cells transfected with the Pro-WT and the truncated mutants including ∆C-term produced properly folded proteins within the ER and that the transport to the Golgi apparatus and secretion in the media was not altered.

Although the ∆C-term mutation abolished the transport of prosaposin to the lysosomes, it did not interfere with its secretion. Since the ∆C-term mutant protein did not reach the lysosomes, it was predicted that COS-7 cells transfected with this construct would not yield mature saposins as opposed to cells transfected with the Pro-WT construct. To confirm if this was the case, cells transfected with ∆C-term and Pro-WT were subjected to a longer pulse labeling experiment with Tran<sup>35</sup>S-label (Fig. 4C) to detect the proteolytically cleaved saposins. After 5 h of labeling, cell lysates were immunoprecipitated with anti-prosaposin antibody, which recognizes both prosaposin and mature lysosomal saposins. The immunoprecipitates were resolved in a 15% Tricine-SDS-PAGE and fluorographed. Wild type 65–70-kDa and ∆C-term slightly smaller prosaposins were detected in cell lysates (Fig. 4C). As expected, mature saposins were not observed in cells transfected with the ∆C-term construct but present in the cells transfected with the Pro-WT construct.

The Addition of Prosaposin Functional Domains to a Secretory Protein—To determine whether the C terminus alone was sufficient for intracellular targeting, a chimeric protein encoding the full-length cDNA of albumin plus the C terminus of prosaposin (Alb/Pro-c-term) was constructed and subcloned into the pcDNA3.1B vector and transfected into COS-7 cells. Wild type albumin cDNA was also prepared as a control (Alb). Albumin is a secretory protein that reaches the extracellular space by a constitutive secretory pathway. After transfection, cells were immunostained with anti-Myc antibody, followed by a secondary FITC-conjugated goat anti-mouse antibody. Some cells were simultaneously stained with LysoTracker. Myc antibody (green fluorescence) produced a perinuclear Golgi-like reaction (Fig. 5A). However, the punctate structures stained by LysoTracker (red fluorescence) did not react with anti-Myc antibody. This suggested that the C terminus alone was insufficient to drive albumin to lysosomes. Based on this result and on the mutational analysis of prosaposin, it was hypothesized that one or more saposin domains should also be present along with the C terminus to direct albumin to the lysosomes.

To test this hypothesis, a chimeric protein was engineered by fusing an albumin cDNA with nucleotide sequences encoding domain D and the C terminus of prosaposin (Alb/Pro-D, c-term). In addition, an albumin fusion protein containing domains C and D plus the C terminus of prosaposin (Alb/Pro-C, D, c-term) was also constructed. Anti-Myc antibody yielded a punctate reaction in both chimeras (Fig. 5A), which overlapped with LysoTracker staining. Western blotting with Myc antibody confirmed the expression of wild type and chimeric albumin proteins (Fig. 5B).

Endo H Treatment of Prosaposin—The difference between the 65-kDa and 70-kDa prosaposins is due to their glycosylation state (55). To determine the structures of their N-linked carbohydrates and to identify the potential sorting compartment for the lysosomal precursor within the Golgi apparatus, endoglucosidase H digestion was performed on immunoprecipitated proteins. Anti-Myc antibody was used to immunoprecipitate recombinant prosaposins from lysates of COS-7 cells transfected with wild type Pro-WT and mutant constructs. Results revealed that in all cases, the 65-kDa band shifted to a 53-kDa band, which corresponds to the molecular weight of the native form of prosaposin (55). The less prominent protein band corresponding to the 70-kDa protein was resistant to Endo H treatment (Fig. 6A). Similarly, the 70-kDa secretory protein immunoprecipitated from the medium was Endo H-resistant (Fig. 6B). This result suggested that the 65-kDa lysosomal form of prosaposin exits from the cis/medial compartment of the Golgi, while the 70-kDa protein traversed the Golgi apparatus, completing its terminal glycosylation.

Treatment with BFA—To verify whether the Golgi apparatus was the site where the 65-kDa lysosomal precursor was sorted, BFA was used prior to Tran<sup>35</sup>S-labeled pulse-chase experiments. BFA is a fungal metabolite that causes rapid redistribution of the components of the Golgi apparatus to the ER (50, 51). COS-7 cells transfected with the wild type prosaposin (Pro-WT) were incubated with BFA 1 h prior to Tran<sup>35</sup>S-labeling. In the presence of BFA, the amount of intracellular protein in cell lysates remained unchanged after chasing for 4 h, while in cells not treated with BFA, the amount of protein decreased after chasing for 2 h (Fig. 7). In the medium of cells incubated subject to a 10% SDS-PAGE, transferred onto a nylon membrane, and immunoblotted with anti-Myc monoclonal antibody. Two bands representing the 65- and 70-kDa wild type prosaposins (WT) are indicated in the membrane. Mutants (∆N-term, ∆A, ∆B, ∆C, ∆D, and ∆C-term) also produced two slightly lower bands depending on the size of the truncated sequences. Nontransfected COS-7 cells (COS-7) and COS-7 cells transfected with the vector alone (V) were used as negative controls. C, immunofluorescent staining of COS-7 cells after transfection with expression vectors containing wild type (Pro-WT) and truncated prosaposin (∆N-term, ∆A, ∆B, ∆C, ∆D, and ∆C-term). Primary anti-Myc monoclonal antibody was visualized with a FITC-conjugated goat anti-mouse antibody. Pro-WT (WT) and the mutant constructs ∆N-term, ∆A, ∆B, ∆C, and ∆D yielded Golgi-like perinuclear and granular reactions. Cells transfected with ∆C-term show a Golgi-like perinuclear staining, but the granular reaction is seemingly absent.
with BFA, there was a very low amount of the 70-kDa protein after chasing for 30 min, 2 h, and 4 h. This indicated that the 70-kDa secretory protein present before the supplementation of BFA was beyond the proximal compartment of the Golgi apparatus. In contrast, in cells incubated without BFA, the amount of protein in the medium increased after 30 min and 2 h (Fig. 2).

**Fig. 2. Localization of wild type and truncated prosaposin in COS-7 cells.** A, colocalization of wild type and truncated prosaposins after immunostaining with anti-Myc antibody and LysoTracker (red fluorescence) overlapped with punctate structures stained with anti-Myc monoclonal antibody (green fluorescence). A partial overlap was detected on a Golgi-like perinuclear region in wild type, Pro-WT (WT), and ΔN-term, ΔA, ΔB, ΔC, and ΔD mutants. No overlap of punctate structures stained by LysoTracker was found in ΔC-term mutant due to the absence of granular staining with anti-Myc antibody. B, colocalization of ΔC-term mutant and mannosidase II. Anti-mannosidase II polyclonal antibody was used as a Golgi marker and visualized with a tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit antibody. ΔC-term mutant was stained with anti-Myc monoclonal antibody and visualized with a FITC-conjugated goat anti-mouse antibody. An overlap between the Myc (green fluorescence) and mannosidase II (red fluorescence) antibodies was observed in a Golgi-like perinuclear region of COS-7 cells transfected with the ΔC-term construct. This result demonstrated that prosaposin mutations did not affect the transit of the truncated protein from the ER to the Golgi apparatus.
7. Taken together, these data suggest that the 65-kDa lysosomal precursor contains mainly high mannose sugar residues and that it is present in the proximal aspect of the Golgi apparatus. On the other hand, the 70-kDa secretory form is glycosylated with complex/hybrid sugar residues and appear to exit from the distal region of the Golgi apparatus.
Prosaposin exists as a 65-kDa lysosomal precursor that is routed to the lysosomes and a 70-kDa secretory form that is secreted to the extracellular space (27, 41, 52–54). Time course experiments demonstrated that the 65-kDa form is derived from post-translational modification of a 53-kDa native protein and that is the precursor of the 70 kDa secretory prosaposin (55). Prosaposin is highly conserved from avian to mammals (45, 46). Conserved regions of prosaposin include four saposin domains designated A, B, C, and D (1) and its N and C termini. Little or no conservation exist among the linker regions found between domains A and B, B and C, and C and D (45, 46). In lysosomes, the 65-kDa protein is cleaved into four sphingolipid activator proteins termed saposins A–D, which are essential cofactors for the hydrolysis of glycosphingolipids with short oligosaccharide chains by specific hydrolases (56, 57).

The dichotomy of prosaposin's lysosomal and secretory pathways raises a number of interesting questions such as how a fraction of the 65-kDa protein escapes terminal glycosylation and what is the mechanism utilized by the lysosomal prosaposin to leave the Golgi apparatus.

Prosaposin, unlike most lysosomal hydrolases, is targeted to lysosomes by a mannose 6-independent mechanism (41–43). The transport of soluble hydrolases to lysosomes depends on their mannose 6-phosphate residues that are recognized by the mannose 6-phosphate receptor in the trans-Golgi region (29, 34, 58). The ligand-receptor complex is then carried to lysosomes via a clathrin-coated vesicle. The 65-kDa protein has been shown to be associated with Golgi membranes (41).

**FIG. 5.** **Expression and targeting of chimeric constructs.** A, targeting of chimeric proteins to the lysosomes. A typical Golgi-like reaction (green fluorescence) was observed in COS-7 cells transfected with Alb/Pro-c-term as well as with the wild type albumin (Alb-WT). A Golgi-like reaction plus a prominent lysosome-like staining was achieved with the Alb/(Pro-D + Pro-c-term) and Alb/(Pro-C, D + Pro-c-term) constructs (green fluorescence). LysoTracker staining (red fluorescence) was seen in COS-7 cells transfected with the different constructs. The punctate reaction obtained with anti-Myc (green fluorescence) in Alb/(Pro-D + Pro-c-term) and Alb/(Pro-C, D + Pro-c-term) overlapped with LysoTracker staining (red fluorescence). This experiment demonstrated that the prosaposin domain plus the C terminus are required for the transport of albumin to the lysosomes. B, Western blot analysis of wild type and albumin chimeric proteins. COS-7 cells transfected with wild type albumin (Alb-WT) and chimeric constructs Alb/Pro-c-term, Alb/(Pro-D + Pro-c-term), and Alb/(Pro-C, D + Pro-c-term) were lysed and subjected to a 10% SDS-PAGE, transferred onto a nylon membrane, and immunostained with anti-Myc monoclonal antibody. The molecular mass of wild type albumin (Alb-WT) is indicated by an arrow (66 kDa). Other bands with higher molecular masses correspond to the chimeric proteins.
FIG. 6. Treatment with Endo H of COS-7 cells transfected with wild type Pro-WT (WT) and ΔN-term, ΔA, ΔB, ΔC, ΔD, and ΔC-term prosaposin constructs. After transfection with Pro-WT and truncated constructs, COS-7 cells were labeled with Tran35S-label (300 μCi/ml) for 30 min and chased for 2 h. Cell lysates and culture medium were immunoprecipitated with anti-Myc antibody and incubated with Endo H (1 unit) at 37°C for 16 h. Immunoprecipitated proteins were resolved in a 10% SDS-PAGE followed by fluorography. A, in cell lysates, the 65-kDa protein band shifted to a 53-kDa band, and the less prominent 70-kDa band remained unchanged (Endo H+). Similar band patterns were also observed with the truncated prosaposins (ΔN-term, ΔA, ΔB, ΔC, ΔD, and ΔC-term) with slightly smaller molecular masses due to the truncations. B, in culture medium, the 70-kDa (WT) band, representing the secretory form of prosaposin, was not modified after incubation with Endo H. Similar results were obtained with truncated prosaposins (ΔN-term, ΔA, ΔB, ΔC, ΔD, and ΔC-term). COS-7 cells transfected with the vector alone (Vector) were used as a negative control.

FIG. 7. Pulse-chase of COS-7 cells transfected with Pro-WT treated with BFA. Cells were incubated with BFA (20 μg/ml) (+BFA) 1 h prior to the addition of Tran35S-label (300 μCi/ml). Replicas of COS-7 cells were grown in absence of BFA and used as controls. After labeling for 30 min, cells were chased for an additional 30 min, 2 h, and 4 h. Immunoprecipitates of cell lysates and culture medium were resolved in a 10% SDS-PAGE and fluorographed. BFA caused intracellular retention of the 65-kDa protein. In control cells, the 65-kDa protein decreased intracellularly, while the 70-kDa protein increased in control medium.

ratus might be dependent on a protein-protein or protein-lipid interaction. To test this hypothesis and to determine whether or not a specific domain of prosaposin is involved in this process, a wild type prosaposin cDNA and several truncated constructs were subcloned into a mammalian expression vector and transfected into COS-7 cells. The recombinant constructs contained a Myc epitope tag that allowed their identification from endogenous prosaposin during the course of our immunological studies. The lysosomal marker LysoTracker was also used to identify lysosomes by confocal microscopy. Confocal immunofluorescence demonstrated that deletion of individual domains encoding saposin A, B, C, or D or the N terminus did not interfere with the targeting of prosaposin to the lysosomal compartment. On the other hand, deletion of the C terminus abolished the targeting of prosaposin to lysosomes. To check if these deletions caused retention of mutated prosaposin in the ER, COS-7 cells were metabolically labeled with Tran35S-label and chased. Cell lysates and culture medium were immunoprecipitated and fluorographed. The results demonstrated the presence of the equivalent 65- and 70-kDa proteins of prosaposin in the cell lysates and of the 70-kDa secretory protein in the medium. In summary, our results clearly indicated that deletions of the ΔA, ΔB, ΔC, ΔD, ΔN-term, and ΔC-term did not cause retention of prosaposin in the ER and that the presence of the C terminus of prosaposin was essential for the sorting and targeting of prosaposin to the lysosomes.

To verify if the C terminus alone was sufficient for driving prosaposin to the lysosomes, a chimeric fusion protein between albumin and the C terminus of prosaposin was expressed in COS-7 cells using the same vector. A vector containing the cDNA of albumin alone was used as a control. As expected for a secretory protein, wild type albumin was detected in the culture medium. However, chimeric albumin fused with the C terminus of prosaposin was not found within lysosomes. Instead, it was secreted into the culture medium.

We rationalized then that truncated constructs ΔA, ΔB, ΔC, ΔD, and ΔN-term, which were targeted to lysosomes, contained the C terminus and at least three saposins. Since saposins display, among themselves, similar structure and play similar roles in vivo and in vitro, we decided to test the hypothesis that one or more saposin domains plus the C terminus are required for the transport of prosaposin to lysosomes. Thus, two new fusion plasmids were made. The first one was composed of albumin plus domain D and the C terminus of prosaposin. The second construct was made of albumin plus domains C and D and the C terminus of prosaposin. As predicted, confocal immunostaining demonstrated that albumin was routed to the lysosomal compartment when it was connected to one saposin domain plus the C terminus of prosaposin.

Each saposin has a high degree of interspecies similarity of hydrophobic amino acids (45, 46). The percentage of hydrophobic amino acids is 36% in saposin A, 37% in B, 28% in C, and 39% in D (45). This high degree of conservation of hydrophobic residues and their alignment occupying equivalent positions with their side chains buried in the folded structure (46) un-
nderlines the capacity of saposins to interact with lipids. In fact, the interaction between prosaposin and its derived saposins with sphingolipids has been extensively documented (22, 59, 61). Saposins C and D have also been shown to exhibit a high affinity for phospholipids. Furthermore, the presence of acidic phospholipids such as phosphatidylycerine in membrane bilayers greatly favors their binding to saposin D (61). The interaction with phospholipids is a characteristic of several other saposin-like proteins (62), such as SP-B (63). Others members of the saposin-like protein family include the pore-forming peptide of *Entamoeba histolytica* (64, 65), NK-lysin (66), acid sphingomyelinase (67), acylcoyacyl hydrolase (68), and plant aspartic proteinases (69, 70). Saposin-like proteins differ widely in function, but the activity of most of them is mediated via lipid interactions. Interestingly, the saposin-like domain within aspartic proteinase has been implicated in the vacuolar targeting of this protein by means of its interaction with membrane phospholipids (69).

Lysosomal prosaposin was initially found to be targeted to lysosomes in a mannose 6-phosphate-independent manner (40, 41, 43). When isolated Golgi fractions were permeabilized with a mild detergent (41), lysosomal prosaposin remained associated to the membrane, while secretory prosaposin (70 kDa) was released into the incubation medium. This suggests that the interaction of lysosomal prosaposin with phospholipid or sphingolipids may play a role in sorting and targeting this protein to the lysosomes. Recently, sphingomyelin, a membrane sphingolipid manufactured in the cis/medial region of the Golgi apparatus (71), was demonstrated to be involved in the transport of prosaposin to lysosomes (72). Cultured cells incubated with fumonisin B1, an inhibitor of sphingolipid synthesis that competes with sphinganine as a substrate of ceramide synthase (73), produced a dramatic decrease in the immunogold labeling of lysosomes with anti-prosaposin antibody. To examine if the mannose 6-phosphate receptor-mediated pathway was affected by this treatment, cells treated or not treated with fumonisin B1 were labeled with anti-cathepsin A antibody. The results showed no significant differences in the immunogold labeling of the lysosomal compartment of the treated and nontreated cells, indicating that the effect of fumonisin B1 on the transport of prosaposin to the lysosomes was specific. The effect of DL-threo-1-phenyl-2-decanoyl-amino-3-morpholino-1-propanol-HCl, a compound that selectively inhibits the synthesis of glycosphingolipids but not of sphingomyelin and/or ceramide, and the effect of tricyclodecan-9-y1 xanthate potassium (D609), which specifically blocks the formation of sphingomyelin (74), were also examined. The results showed that only D609 blocked the transport of prosaposin to the lysosomes, suggesting that sphingomyelin was the main sphingolipid implicated in the association with prosaposin to the lysosomes (72).

During the course of this investigation we showed that the 65-kDa lysosomal prosaposin is Endo H-sensitive, whereas the 70-kDa secretory form is Endo H-resistant. Since the processing pathway within the Golgi apparatus is highly ordered, the treatment with this enzyme was used to distinguish complex from high mannose oligosaccharide linked to prosaposin (29, 75). Glycoproteins are modified in successive stages as they move through the Golgi-processing compartment. Thus, a significant fraction of the 65-kDa form must escape from the Golgi apparatus before it reaches the distal stacks, where it becomes fully glycosylated and Endo H-resistant. This notion was confirmed in cells transfected with the wild type prosaposin after incubation in BFA. BFA is a fungal antibacterial drug that causes rapid redistribution of proteins located in the Golgi apparatus but not of proteins residing in a post-Golgi compartment. Our results showed an intracellular retention of the 65-kDa protein, indicating that the lysosomal form only reaches the cis/medial region of the Golgi apparatus before sorting. The presence of negligible amounts of the 70-kDa protein in the medium represented secretory prosaposin that already passed the distal region of the Golgi prior to the addition of BFA.

Based on this evidence, a model, in which the interaction of prosaposin with phospholipids and/or sphingolipids may play an important role in bringing prosaposin close to the Golgi membrane near a “receptor protein,” was proposed. According to this model, the C terminus of prosaposin may contain the binding site for this receptor protein, creating a receptor-ligand-like complex, which is then packed into cargo vesicles and transported to the endosomes, multivesicular bodies, and/or lysosomes. Interestingly, the C terminus of prosaposin is significantly similar (66%) to the N terminus of SP-B, which has been implicated in the targeting of this protein to the lamellar bodies in pneumocyte type II (47). Similar to prosaposin, which requires the C terminus and at least one saposin domain, SP-B also requires the presence of the N-terminal region and a yet unidentified sequence within the backbone of this protein that contains a saposin-like domain. Taken together, all of these results indicate that an alternative mechanism of sorting and transport of prosaposin between the Golgi apparatus and the lysosomes may exist. In conclusion, we demonstrated that the C terminus and at least one saposin domain may be sufficient for the lysosomal sorting and targeting of this sphingolipid activator protein.

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