Saposin A Mobilizes Lipids from Low Cholesterol and High Bis(monoacylglycerol)phosphate-containing Membranes

PATIENT VARIANT SAPOSIN A LACKS LIPID EXTRACTION CAPACITY

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Saposin A (Sap-A) is one of five known sphingolipid activator proteins required for the lysosomal degradation of sphingolipids and for the loading of lipid antigens onto antigen-presenting molecules of the CD1 type. Sap-A assists in the degradation of galactosylceramide by galactosylceramide-β-galactosidase in vivo, which takes place at the surface of intraendosomal/intralysosomal vesicles. Sap-A is believed to mediate the interaction between the enzyme and its membrane-bound substrate. Its dysfunction causes a variant form of Krabbe disease. In the present study we prepared glycosylated Sap-A free of other Saps, taking advantage of the Pichia pastoris expression system. Using liposomes and surface plasmon resonance spectroscopy, we tested the binding and lipid mobilization capacity of Sap-A under different conditions. Along the endocytic pathway, the pH value decreases, and the lipid composition of intraendosomal and intralysosomal membranes changes drastically. In the inner membranes the cholesterol concentration decreases, and that of the anionic phospholipid bis(monoacylglycero)phosphate increases. Here, we show that Sap-A is able to bind to liposomes and to mobilize lipids out of them at acidic pH values below pH 4.7. Low cholesterol levels and increasing concentrations of bis(monoacylglycero)phosphate favor lipid extraction significantly. Galactosylceramide as a bilayer component is not essential for lipid mobilization by Sap-A, which requires intact disulfide bridges for activity. We also show for the first time that glycosylation of Sap-A is essential for its lipid extraction activity. Variant Sap-A proteins, which cause storage of galactosylceramide in humans (Krabbe disease, Spiegel, R., Bach, G., Sury, V., Mengistu, G., Meidan, B., Shalev, S., Shneor, Y., Mandel, H., and Zeigler, M. (2005) Mol. Genet. Metab. 84, 160–166) and in mutant mice (Matsuda, J., Vanier, M. T., Saito, Y., Tohyama, J., and Suzuki, K. (2001) Hum. Mol. Genet. 10, 1191–1199) are deficient in lipid extraction capacity.

The degradation of plasma membrane-derived glycosphingolipids takes place in the acidic compartments of the cell, the endosomes and lysosomes, on the surface of intraendosomal and intralysosomal vesicles with a diameter in the range of 50–100 nm (1–4). These inner membranes are formed by a lipid-sorting process along the endocytic pathway, during which a decrease of its cholesterol content and an increase of the negatively charged lipid bis(monoacylglycerol)phosphate (BMP) (erroneously also called lysobisphosphaticid acid) takes place (5). In addition, the luminal pH value steadily decreases to achieve optimal conditions for the action of lysosomal enzymes.

Glycosphingolipid degradation starts with the sequential cleavage of monosaccharide residues from glycoconjugates by the action of lysosomal exohydrolases. In vitro, the enzymatic hydrolysis of most membrane-bound sphingolipids is also stimulated by negatively charged lysosomal lipids (6–11). However, the interaction between membrane-bound lipid substrates with four or less sugars (6) and the water-soluble enzymes requires the assistance of membrane perturbing sphingolipid activator proteins. Five sphingolipid activator proteins are known; they are the GM2 activator protein and the four saposins (Sap-A, Sap-B, Sap-C, and Sap-D), which are derived from a common precursor, called Sap-precursor or prosaposin. The deficiency of each has been observed to cause a lysosomal storage disease similar to that produced by the absence of the corresponding enzyme (12–14). The inherited deficiency of the whole Sap-precursor leads to a fatal storage disorder in humans (15, 16) and mice (17). This is accompanied by the accumulation of several sphingolipids and membrane structures within the acidic compartments of the cell.

The saposins A–D (12) are four homologous, not enzymatically active, heat-stable, and protease-resistant glycoproteins of 8–11 kDa. They contain one conserved N-glycosylation site (18), with the exception of Sap-A, which has two, and six highly conserved cysteine residues, which form three disulfide bridges. Although the four Saps share a high degree

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‡ The abbreviations used are: BMP, bis(monoacylglycerol)phosphate; Sap, saposin; gSap-A, glycosylated Sap-A containing a C-terminal His6 tag; nSap-A, nonglycosylated Sap-A containing a C-terminal His6 tag; SPR, surface plasmon resonance; GM2, GaINAcβ1→4Gal(3→4)NeuAcα1→4Glcβ1→1Cer; PC, phosphatidylcholine; MM, minimal methanol; Cyt c, cytochrome c; PBS, phosphate-buffered saline; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
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of homology (19) and similar properties, they act differently and show different specificities.

Sap-A is essential for the degradation of galactosylceramide by galactosylceramide-β-galactosidase in vivo (13, 20). The inherited deficiency of this lysosomal enzyme causes Krabbe disease, also called globoid cell leukodystrophy. It is a fatal disorder of myelination with blocks in the catabolism of galactosylceramide and galactosylsphingosine. The inherited deficiency of Sap-A causes an infantile disease similar to globoid cell leukodystrophy, as demonstrated in a human patient carrying a mutation in the Sap-A domain of the Sap-precursor (13). The known mutation is a deletion of three base pairs, which leads to lack of the amino acid 11 (a conserved valine) of Sap-A. In addition, transgenic mice with Sap-A lacking one of the three disulfide bridges develop a phenotype resembling the late-onset form of Krabbe disease (20).

Among the saposins, Sap-A shows the highest amino acid similarity to Sap-C, which is essential for the in vivo degradation of glucosylceramide by glucosylceramide-β-glucosidase (21, 22). In vitro, both activators assist the same enzymes in the same metabolic steps (23–25). The mode of action of saposins A and C has been analyzed in several studies. It has been reported that Sap-A and Sap-C are able to interact directly with the enzymes and to stimulate them (8, 23, 25, 27, 28). Sap-C can also associate with neutral or anionic phospholipid-containing vesicles under acidic conditions (11, 29–31) and trigger membrane reorganization, which may lead to fusion of BMP-containing vesicles (32), formation of patch-like structural domains, and membrane destabilization (11, 30, 33). The capacity of Sap-C to interact with lipids is also shown by its ability to load lipid antigens onto the antigen presenting molecules CD1b (34) and CD1d (35) in the lysosomal compartment. Sap-A is also able to load CD1d molecules with lipid antigens (35). CD1b and CD1d in turn present the lipids to T-lymphocytes on the surface of the cell (1).

So far Sap-A has not been studied as extensively as Sap-C. Interaction of recombinant nonglycosylated Sap-A with phospholipid membranes has been demonstrated by using fluorescence spectroscopy (31). Sap-A prepared from spleen of the Gaucher disease patients has also been shown to destabilize anionic phospholipid-containing vesicles at a pH of 4 but less effectively than Sap-C (30, 36). The destabilizing activity of Sap-A was monitored by the release of a fluorescent dye encapsulated within lipid vesicles.

In previous in vitro studies either nonglycosylated recombinant Sap-A or Sap-A preparations partially purified from tissues have been applied. Both sources have severe drawbacks. Nonglycosylated Sap-A is not present in humans, and the absence of the carbohydrate moiety is expected to change the solubility of the protein and to alter its interaction with membranes. Purification of individual saposins from natural sources results in preparations contaminated with other Saps due to the very similar physicochemical properties shared among them. In the present study we take advantage of the Pichia pastoris expression system, which allows the production of glycosylated Sap-A free of other Saps. To explore the conditions under which Sap-A operates optimally, we mimicked intralysosomal vesicles using liposomes in an acidic environment. To monitor the interaction between the protein and the membrane surface, we used surface plasmon resonance and studied the solubilization of radioactively labeled lipids from liposomal membranes.

EXPERIMENTAL PROCEDURES

Commercial Products—Cholesterol and galactosylceramide (bovine brain) were purchased from Sigma. Phosphatidylcholine (egg yolk) was from Lipoid. Myristoyl-BMP and oleoyl-BMP were from Avanti Polar Lipids. [14C]Phosphatidylcholine was from American Radiolabeled Chemicals, and [14C]cholesterol was from Amersham Biosciences.

Restriction enzymes were from New England Biolabs. Chemicals and solvents were of analytical grade. Expression vectors and P. pastoris strains were obtained from Invitrogen.

Construction of the Sap-A Expression Vector—The cDNA coding for the Sap-A sequence was generated from pSap-PBHE (37) by PCR using the specific primers 5′-ATT GAT TAC GTA TCC CTT CCC TGC GAC ATA TGC-3 and 5′-ATC AAT GAA TTC TTA ATG GTG ATG GTG ATG CTG CGG-3. The forward primer contained an SnaBI restriction site, and the reverse primer incorporated a C-terminal His tag, a stop codon, and an EcoRI restriction site. The PCR product was gel-purified and ligated into the pCR-Skript SK(+) vector (Stratagene) using the pCR-Script cloning kit (Stratagene). The resulting PCR-script-Sap-A vector as well as the expression vector pPIC9K (Invitrogen) were digested with the restriction enzymes SnaBI and EcoRI. After gel purification of both expression vector and Sap-A insert, they were ligated. The resulting plasmid pPIC9K-Sap-A was transformed into competent Escherichia coli DH5α (Promega, Madison, WI), which were cultured in LB-Amp medium (Luria-Bertani medium, 100 μg/liter Ampicillin). The sequence identity and orientation of the insert was confirmed by sequencing.

Construction of the Sap-A Mutant Expression Vectors—Using the site-directed mutagenesis Kit (Stratagene) and the pPIC9K-Sap-A vector as a template, four mutants were constructed. The numbering of the substituted or deleted amino acids on the Sap-A protein corresponds to their positions on the Sap precursors. Sap-A mutant 1 (which corresponds to a variant Sap-A protein carrying the amino acid substitution C106F) was generated using the primers 5′-CGT CCT GGG GAG GTG TCA TTC AAG GAG GAG ATA GTG GAC TCC-3′ and 5′-GGA TTC TTA ATG GTG ATG GTG ATG CTG CCT CGG-3′. Sap-A mutant 2 (which corresponds to a variant Sap-A protein carrying the amino acid substitution C106F) was generated using the primers 5′-CGT CCT GGG GAG ATT GAT TAC GTA TCC CTT CCC TGC GAC ATA TGC-3 and 5′-GGA TTC TTA ATG GTG ATG GTG ATG CTG CGG-3′. Sap-A mutant 3 (which corresponds to a variant Sap-A protein carrying the amino acid substitution C106F) was generated using the primers 5′-CGT CCT GGG GAG ATG TCA TTC AAG GAG GAG ATA GTG GAC TCC-3′ and 5′-GGA TTC TTA ATG GTG ATG GTG ATG CTG CCT CGG-3′. Sap-A mutant 4 (which corresponds to a variant Sap-A protein carrying the amino acid substitution C106F) was generated using the primers 5′-CGT CCT GGG GAG ATT GAT TAC GTA TCC CTT CCC TGC GAC ATA TGC-3 and 5′-GGA TTC TTA ATG GTG ATG GTG ATG CTG CCT CGG-3′.
GTC-3’. The sequence identity of the four mutants was confirmed by sequencing.

Construction of the Expression Vector for Sap-A without Hexahistidine Tag—The cDNA coding for the Sap-A sequence was generated from the pPIC9K-Sap-A vector by PCR using the specific primers 5’-CTC TCG AGA AAA GAG AGG CTG AAG CTT CCC TTC CCT GCG ACA TAT GC-3’ and 5’-GCT CTT AAC CTT TGC GAG TCT CTT CAG TAG GAA TTC GC-3’. The PCR product was digested with the restriction enzymes XhoI and EcoRI and cloned into the vector pPIC9 (Invitrogen). After digestion with the restriction enzymes SacI and Sall, the resulting fragment was ligated with the vector pPIC9K digested with the same enzymes. The sequence identity and orientation of the insert was confirmed by sequencing.

Transformation in P. pastoris Strains and Selection for Multicopy Inserts—Linearized pPIC9K-Sap-A plasmids with and without hexahistidine tag and pPIC9K-Sap-A mutant plasmids were used to transform P. pastoris GS115 strain. The transformation was performed by electroporation (Cellject Electroporation System, Eurogentec) applying an electric pulse of 1500 V, 282 ohms, and 40 microfarads according to the Invitrogen manual. Transformants were selected on minimal dextrose (1.34% yeast nitrogen base with ammonium sulfate, 4 × 10⁻⁵ % biotin, 1% dextrose). Screening for multiple copy inserts was done on YPD plates (1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar) containing increasing amounts of G418 (Geneticin; Invitrogen) up to 4 mg/ml medium.

Genomic DNA of transformed yeasts was isolated using the Easy-DNATM Kit (Invitrogen), and PCR analysis was performed using AOX5’ and AOX3’ primers (Invitrogen). In addition, cells were cultured on minimal dextrose and on minimal methanol (MM; 0.5% methanol instead of dextrose) plates. Using both techniques, the phenotype of most clones producing wild type Sap-A were determined to be Mut⁺ (regular methanol utilization), whereas clones producing mutant Sap-A proteins were Mut⁻ (methanol utilization slow).

Expression and Purification of Sap-A and Variant Proteins—A small culture of a single Mut⁺ (regular methanol utilization) colony was used to inoculate 50–100 ml of buffered glycerol-complex medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% yeast nitrogen base, 4 × 10⁻⁵ % biotin, 1% glycerol). The cultures were grown at 30 °C in a shaking incubator (210 rpm) for 16 h until log phase (A600nm = 2–6) and harvested by centrifugation (10 min, 3000 × g, 4 °C). Secretory expression of the recombinant protein was induced by resuspending the cell pellet in 500 ml of buffered methanol-complex medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% yeast nitrogen base, 4 × 10⁻⁵ % biotin, 0.5% methanol) and maintained by the addition of 0.5% methanol every 24 h for 4–5 days.

Mut⁻ (methanol utilization slow) clones were grown in 2 liters of buffered glycerol-complex medium at 30 °C in a shaking incubator (210 rpm) for 16 h until log phase (A600nm = 2–6) and harvested by centrifugation (10 min, 3000 × g, 4 °C). The cell pellet was resuspended in 500 ml of buffered methanol-complex medium and maintained by the addition of 0.5% methanol every 24 h for 6–7 days.

After centrifugation (10 min, 3000 × g, 4 °C), sodium azide was added to a final concentration of 0.02%, the pH was adjusted to 7.5, and the supernatant was clarified by filtration or centrifugation (30 min, 8000 × g, 4 °C). The solution was then applied to an affinity column (nickel-nitrilotriacetic acid-agarose, Qiagen). After two wash steps (with 50 mM sodium phosphate, 300 mM sodium chloride, 0.02% sodium azide, pH 7 and 6), the bound protein was eluted using the same buffer system by decreasing the pH to 4.2. Sap-A-containing fractions were pooled, concentrated, and further purified by gel filtration chromatography on a HiLoad Superdex 75 16/60 prep grade column (GE Healthcare). The running buffer was 150 mM sodium phosphate, 0.02% sodium azide, pH 6.8. When fractions containing both glycosylated and nonglycosylated protein were present, a concanavalin A column (Sigma) was used to separate these forms. After equilibration of the material with binding buffer (10 mM sodium phosphate, 0.1 mM sodium chloride, 0.02% sodium azide, pH 7) and loading of the protein sample, the column was washed (10 mM sodium phosphate, 1.5 mM sodium chloride, 0.02% sodium azide, pH 7), and glycosylated protein was eluted (10 mM sodium phosphate, 0.5 mM sodium chloride, 0.75 mM α-methyl-d-mannopyranoside, 0.02% sodium azide, pH 7).

Expression and Purification of Sap-A without the His₆ Tag—For secretory expression of the protein, minimal medium WM VIII (38) was used. For purification, cells were pelleted (10 min, 4000 × g, 4 °C), the supernatant containing the recombinant protein was cleared by centrifugation (30 min, 11000 × g, 4 °C), and the pH of the supernatant was adjusted to 4.0. The filtrated solution was then applied to a HS Macro Prep column (Bio-Rad), washed with sodium citrate buffer (20 mM, pH 4.0), and eluted using a sodium chloride gradient (to a final concentration of 1 M sodium chloride in the same citrate buffer). Sap-A-containing fractions were pooled, concentrated, and further purified by gel filtration chromatography on a HiLoad Superdex 75 16/60 prep grade column (GE Healthcare). The running buffer was 10 mM sodium citrate, 300 mM sodium chloride, pH 4.2.

Characterization of Proteins—The purity and identity of nonglycosylated proteins was confirmed by SDS-polyacrylamide gel electrophoresis followed by silver staining and Western blot analysis (using a rabbit anti Sap-A antibody (37)) and wild type protein by matrix-assisted laser desorption ionization mass spectrometry (matrix, 2,5 dihydroxybenzoic acid; TOF-Spec E, Micromass). The glycoform of the proteins was deglycosylated by treatment with peptide N-glycosidase F and endoglycosidase H (New England Biolabs). Samples of protein before and after deglycosylation were analyzed by SDS-polyacrylamide gel electrophoresis followed by silver staining or Western blot analysis.

Preparation of Liposomes—Large unilamellar vesicles were prepared as described previously (39). During preparation the temperature never exceeded 37 °C. Lipid stock solutions of phosphatidylcholine, cholesterol, BMP, and galactosylceramide were obtained by dissolving them in organic solvents. Appropriate aliquots of lipid stock solutions were mixed. After evaporation of solvents under nitrogen, dried lipids were hydrated to a concentration of 0.5 mM PBS buffer. The suspen-
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The lipids were resolved in 50 μl of chloroform/methanol (1:1, v/v) and sonicated for 1 min, and insoluble salts were pelletized. For separation of lipids, thin layer chromatograms were developed with chloroform/methanol/water (60:25:4, v/v/v). After development, plates were air-dried, sprayed with 8% (w/v) H3PO4 containing 10% (w/v) copper (II) sulfate pentahydrate, and charred at 180 °C for 10 min, and lipids were quantitated by photo densitometry (Shimadzu) at λ = 595 nm. Each kind of liposome was determined in triplicate. The indicated lipid mol % in the figures and in the text corresponds to the content after preparation of liposomes.

Surface Plasmon Resonance (SPR) Spectroscopy—Real-time lipid-protein interaction analysis was performed at 25 °C in a Biacore® 3000 instrument. Liposomes were immobilized on a Pioneer® L1 sensor chip (Biacore), which provides a preimmobilized surface with lipophilic anchors attached to a dextran matrix. For immobilization of liposomes, 60 μl of a liposome solution (0.4 mM in PBS) were injected at a flow rate of 5 μl/min. Unbound liposomes were removed by washing with 10 μl of sodium hydroxide (25 mM) at the same flow rate. To assure complete saturation of the surface, 40 μl of the liposomes solution were newly injected, and the surface was washed with 10 μl of sodium hydroxide (50 mM) at a flow rate of 100 μl/min to remove multilamellar structures and to stabilize the base line, as recommended by the manufacturer instructions (Biacore). After immobilization of liposomes, a signal increase of 5000–8000 response units was achieved. For measurement of the interaction of the protein with the immobilized liposomes, the surface was washed with running buffer (50 mM sodium citrate, pH 4.2, or as indicated), and the signal was adjusted to zero. Protein in running buffer was injected with a flow rate of 20 μl/min for 180 s followed by injection of running buffer. The measurements were repeated at least two times.

Column Experiment of Radioactive Lipid Mobilization—A volume of 250 μl of a liposome solution (0.4 mM in PBS) labeled either with [14C]PC (2 mol %, 1 μCi/ml liposomes) or with [14C]cholesterol (3.8 mol %, 1 μCi/ml liposomes) was applied to a 250 μl of octyl-Sepharose Fast Flow (Sigma) column. After 30 min of incubation at room temperature, the column was successively washed with PBS and running buffer (50 mM sodium citrate, pH 4.2). When a constant radioactivity value in the elution fractions was reached, 250 μl of protein solution (cytochrome c (5 μM in running buffer) or glycosylated Sap-A containing a C-terminal His6 tag (5 μM in running buffer)) were loaded onto the column and incubated for 5 min. This process was repeated three times. Finally, the column was washed with running buffer, and the remaining lipids were completely eluted with chloroform/methanol (1:1, v/v). Radioactivity was quantified by counting the eluting fractions in a liquid scintillation analyzer (Packard).

RESULTS

Basic Experiments—To mimic the in vivo conditions on the surface of in vivo endosomal and intralysosomal vesicles, liposomes with an average diameter of 100 nm were presented to a Sap-A solution. Except for the cases indicated (corresponding to the data presented in Fig. 2D), all glycosylated and nonglycosylated, wild type, and variant Sap-A proteins analyzed contain a C-terminal hexahistidine tag. For most experiments, glycosylated recombinant Sap-A containing a C-terminal His6 tag, designated as gSap-A, was used. A highly purified preparation of this protein (>95% purity as determined by silver staining) has been obtained and characterized as described under "Experimental Procedures." The interaction of the protein with the lipid bilayer in the absence of detergents were monitored by SPR, one of the optical, surface-oriented biosensing techniques that can be used to monitor biomolecular interactions between two binding partners. It requires the immobilization of one of them at a solid surface. For interaction measurements a solution of the analyte flows over the immobilized binding partner. In our case liposomes containing 61 mol % of PC, 14 mol % myristoyl-BMP, and 25 mol % cholesterol (standard liposomes) were immobilized on a Pioneer® L1 sensor chip (Biacore). It has been shown by electron microscopy (40), by fluorescence microscopy (41), or by analyzing the release of a fluorescent marker from liposomes while loading the chip (42, 43) that the sensor chip captures mostly intact liposomes. With this system the following control experiments were performed.

Interaction of Control Substances with Immobilized Liposomes—When the running buffer (50 mM sodium citrate, pH 4.2) was used without any protein addition as a negative control, no alteration of the base line was observed (Fig. 1). On the other hand, cytochrome c (Cyt c, 2.5 μM in running buffer) rapidly associated with the immobilized liposomes and dissociated incompletely within the time interval measured (Fig. 1). In contrast, the detergent Chaps (20 mM in running buffer) led to a drop of the signal far below the base line. This indicates complete removal of lipids from the chip (Fig. 1), since the final...
response unit value that was reached corresponded exactly to the signal obtained before immobilizing the liposomes on the chip (data not shown).


gSap-A Mobilized Lipids from Immobilized Liposomes—The curves generated when gSap-A was used as the analyte are similar to a combination of the curves given by Cyt c and Chaps (Fig. 1). During the period of protein injection, the increase of the signal indicates binding of the protein to the liposomes. The following drop of the signal far below the base line at protein concentration of 1 μM or higher indicates a release of lipids from liposomes. This process continued and intensified when protein-free running buffer was injected. Increasing concentrations of gSap-A caused an intensification of both binding and extraction of lipids (Fig. 2A). Extraction of lipids by gSap-A (2.5 μM in 50 mM sodium citrate) was optimal at pH 4.2 and decreased drastically when the pH was increased (Fig. 2B). At pH values above 5, no extraction of lipids was observed. The increase of sodium citrate concentration also resulted in a decrease of the effect of lipid extraction by 2.5 μM gSap-A at pH 4.2 (Fig. 2C). Taking into account these results, we adjusted our standard conditions to a protein concentration of 2.5 μM, a sodium citrate concentration of 50 mM, a pH of 4.2, and the composition of liposomes as given above.

Glycosylated Sap-A Lacking the His6 Tag Mobilized Lipids from Immobilized Liposomes and Reproduced the Tendency Observed by gSap-A—To exclude the possibility that the His6 tag at the C terminus of the recombinant protein influences the results observed for gSap-A significantly, we expressed gSap-A lacking the His6 tag (gSap-Aw/o His) in the P. pastoris expression system. Purified protein was used to test its interaction with standard liposomes at different pH values (Fig. 2D) and with liposomes in which the BMP and cholesterol content were varied at pH 4.2 (data not shown). We got similar results to those described for gSap-A with a His6 tag.

Extraction of Membrane Lipids by gSap-A

As described above, SPR measurements using Chaps or gSap-A as analytes resulted in curves that reach values below the base line. This observation can be explained by the mobilization of lipids out of the lipid bilayer. To confirm this hypothesis, we tried to recover and analyze the solution eluted from the SPR flow cell after the measurement. Because quantification of the recovered material by mass spectrometry was not successful and the use of radioactivity at the Biacore instrument is not allowed in our laboratory, we performed an alternative experiment. Radioactively labeled liposomes were bound to octyl-Sepharose columns, and radioactivity in eluted fractions after applying gSap-A or Cyt c was determined. Fig. 3A shows the effect of Cyt c (5 μM in 50 mM sodium citrate buffer, pH 4.2) on liposomes labeled with [14C]cholesterol and [14C]PC. In this case only a minor extraction of radioactive lipids could be observed that was indistinguishable from background radioactivity. In contrast, when gSap-A (5 μM in 50 mM sodium citrate buffer, pH 4.2) was applied to columns loaded with both kinds of liposomes, radioactivity (0.83% of bound [14C]PC and 0.13% of bound [14C]cholesterol) was eluted, supporting our hypothesis (Fig. 3B).
Influence of Different Lysosomal Lipids on the Membrane-perturbing Properties of gSap-A

BMP Enhanced the Extraction of Membrane Lipids by gSap-A—BMP is a unique anionic phospholipid that concentrates in the inner membranes of endosomes and lysosomes (5, 44–47) and distinguishes them from the plasma membrane or limiting membranes of cellular organelles. To investigate how BMP affects the lipid extraction capacity of gSap-A, we prepared liposomes with different BMP concentrations. These liposomes were immobilized on an L1 chip, and the effect of gSap-A under standard conditions was tested using the SPR technique (Fig. 4). Increasing concentrations of BMP in the liposomes did not significantly affect the binding of the protein to the membranes but resulted in an increasing drop of the signal, indicating lipid mobilization. We also prepared liposomes containing different concentrations of oleoyl-BMP, which is more abundant in human cells than the myristoyl-BMP used in the present study. gSap-A was able to mobilize the same proportion of lipids out of standard liposomes prepared with both kinds of BMP. The tendencies observed regarding binding and lipid extraction under cholesterol and BMP variation could also be confirmed using liposomes containing oleoyl-BMP.

Cholesterol Inhibits the Extraction of Membrane Lipids by gSap-A—Cholesterol is especially concentrated in the plasma membrane and in membranes of early endocytic organelles (48). Inner membranes show a cholesterol concentration gradient decreasing from early and late endosomes. In the inner membranes of lysosomes cholesterol is almost absent (5). To explore whether the cholesterol content influences the action of gSap-A, we tested its interaction with liposomes of different cholesterol concentrations using the SPR technique. Increasing concentrations of cholesterol caused a reduction of the signal drop (Fig. 4). The binding of the protein remained unaffected. This effect could be observed with all BMP concentrations tested.
Galactosylceramide Does Not Influence the Extraction of Membrane Lipids by gSap-A—Galactosylceramide is a characteristic glycolipid for the myelin sheaths of nerve cells. During its degradation by the enzyme galactosylceramide-β-galactosidase, the presence of Sap-A is essential. To determine the influence of galactosylceramide on the lipid mobilization capacity of gSap-A, we introduced 4 mol % galactosylceramide in liposomes containing 14 mol % BMP and cholesterol ranging from 4 to 38 mol %. No significant difference could be observed between curves with and without galactosylceramide (data not shown).

Analysis of Membrane-perturbing Properties of Variant Sap-A Proteins

Nonglycosylated Sap-A Showed a Different Capacity to Bind to the Membrane and to Mobilize Lipids—To investigate whether the presence of the carbohydrate moiety affects the binding and lipid mobilization capacity of Sap-A, we purified nonglycosylated Sap-A (nSap-A) and compared its effects with those produced by glycosylated protein using the SPR technique (Fig. 5, A and B). Under standard conditions several differences could be observed. When nSap-A was injected (Fig. 5B), a strongly increased binding signal was observed. At the end of the first interval of 180 s the signal began to drop slowly. This decrease continued after injection of running buffer. The curves reached and passed the base line before the measurement was finished. In contrast, the curves corresponding to gSap-A (Fig. 5A) showed a drop of the signal shortly after beginning the measurement, and the curves dropped below the base line within the first 180 s.

The effect of cholesterol on the lipid mobilization capacity of the proteins was comparable in both cases; the lower the cholesterol concentrations, the faster and more pronounced the drop of the signal. In addition, Fig. 2E shows the effect of increasing salt concentrations on the action of nSap-A. The binding of nSap-A to the lipid bilayer decreased with increasing sodium citrate concentrations, whereas the extraction of lipids increased. At high salt concentrations the behavior of nSap-A resembles that of gSap-A.
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The Loss of One Disulfide Linkage in Sap-A Caused a Drastic Decrease of Its Lipid Mobilization Effect—With the aim of testing how the failure of one disulfide linkage affects the action of Sap-A, we prepared three protein variants. Variant 1 carries the amino acid substitution C106F that impaired the formation of one disulfide bridge. This variant corresponds to the one present in the Sap-A knock out mouse (20). Variant 2 carries the amino acid substitution C132F present in the homologous protein Sap-C that was originally observed in a Sap-C-deficient patient (49). Variant 3 carries the substitution C132A. In the last two cases the formation of the second disulfide linkage is impaired.

SPR measurements of glycosylated and nonglycosylated forms of variants 1–3 showed similar curves (Fig. 5, C–H). The protein variants associated with the immobilized liposomes. They show a higher binding than wild type Sap-A. In addition nonglycosylated variants have an enhanced binding capacity compared with glycosylated ones. After running buffer was injected a slow dissociation was observed for the nonglycosylated variants. The curves remained almost above or close to the base line, indicating the absence of or a strongly reduced capacity of all three variants to extract lipids.

Patient Variant Sap-A Was Unable to Mobilize Lipids Out of Membranes—Recently, a mutation on the Sap-A domain of the Sap precursor, causing the deletion of Val-11 of Sap-A, has been shown to be a cause of an infantile form of Krabbe disease in a human patient (13). We prepared the corresponding glycosylated and nonglycosylated variant Sap-A (variant 4) and tested both using the SPR technique. Our data (Fig. 5, I and J) were similar to that obtained for the other variant proteins described above. Under all cholesterol concentrations tested, the binding capacity of variant 4 proteins was enhanced, but they were unable to mobilize lipids out of the membrane.

DISCUSSION

Sphingolipids and other compounds of mammalian cell membranes are degraded within the acidic compartments of the cell (50). They reach the lysosomal compartment on inner membranes either by endocytosis or autophagy. These inner membranes containing an increased concentration of BMP and a decreased level of cholesterol are digested by water-soluble hydrolases with the help of membrane active lipid transfer proteins. Among them are the saposins A, B, C, and D, which are very important since their combined inherited deficiency leads to a fatal membrane storage disease with pronounced accumulation of a multitude of sphingolipids. To shed light on the mode of action of Sap-A at the membrane-water interphase, we took advantage of the SPR technique. SPR has been used for real-time interaction analysis to determine the concentration of biomolecules, kinetic constants, binding specificity, etc. In our case, liposomes with an average diameter of 100 nm, simulating intralysosomal vesicles, were immobilized on the surface of the sensor chip. In comparison with the controls, gSap-A (2.5 μM in running buffer) shows a short binding to the lipid membrane and a partial mobilization of lipids out of it (Fig. 1.). Lipid mobilization, in the sense of extraction of lipids from the sensor chip fixed membrane, became evident when the curve reached negative response units values. Thus, the action of gSap-A could be compared with that of a weak detergent.

Binding and Lipid Extraction Capacity of gSap-A Depends on Its Concentration, the pH Value, and Ionic Strength—Both binding and lipid extraction capacity are dependent on protein concentration and pH (Fig. 2, A and B). The pH value within the lysosomes has been shown to be in the range 4–5.5 (51, 52). Accordingly, gSap-A shows maximal binding and lipid mobilization capacity at pH 4.2 (Fig. 2B). The optimal pH of galactosylceramide-β-galactosidase, the enzyme assisted by Sap-A, has been described to be at 4.5, also showing a high degree of activity at pH 4.2 (53). In addition, the extraction of lipids is negatively affected by increasing the sodium citrate concentration of the buffer. Therefore, local changes of salt concentration in the lysosomes might modulate the lipid mobilization capacity of gSap-A.

The mobilization of lipids out of liposomes caused by gSap-A could be confirmed using radioactively labeled liposomes attached to octyl-Sepharose (Fig. 3). In addition, these experiments provide information about the kind of lipids extracted. We found that at least PC and cholesterol are mobilized out of standard liposomes by gSap-A at pH 4.2.

The amount of radioactivity released corresponds to about 0.83% (for [14C]PC liposomes) and to 0.13% ([14C]cholesterol liposomes) of the total radioactivity bound. The original ratio of PC:cholesterol in liposomes was of 2.4:1 (61 mol % PC and 25 mol % cholesterol). However, the eluted PC was of 0.51 mol % of total liposomal lipids, and the eluted cholesterol was of 0.032 mol % of total liposomal lipids. Consequently the ratio of eluted PC:cholesterol was of 16:1. Because the proportion of PC:cholesterol before and after elution of lipids from the column changed, it is possible to speculate that lipids are eluted by Sap-A as molecules rather than as intact liposomes.

The proportion of released radioactivity when using this method contrasts with the 30–50% of mobilized lipids when the SPR technique is used. This apparent difference between both systems is explained by the observation that the proportion of protein to lipid is about 250 times higher using the SPR technique than using the column experiment of radioactive lipid mobilization. Because the volume of the columns, which in turn determines the amount of lipid bound, cannot be reduced infinitely, a high amount of about 19 mg of protein would be needed to reproduce the conditions and proportions in the SPR experiment, which is virtually impossible. The column experiment, however, allowed us to confirm the occurrence of lipid extraction by gSap-A.

BMP Enhances and Cholesterol Reduces the Lipid Extraction Capacity of gSap-A—The lipid composition of intraendosomal and intralysosomal membranes differs substantially from that of plasma membrane or the limiting membrane. BMP is a major component of intralysosomal vesicles, whereas phosphatidylserine, which has been used in previous studies (27–30, 36, 54–56), is a minor component of the lysosomes (0–3% of the phospholipid composition (43)). Our results show that BMP enhances the lipid extraction capacity of gSap-A without altering its binding to the membrane (Fig. 4).

Changes in cholesterol concentration also do not affect binding of gSap-A to the membrane. Nevertheless, decreasing cho-
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Lesterol concentrations favor the extraction of lipids by gSap-A. Thus, cholesterol tends to stabilize membranes and to minimize the lipid extraction capacity of gSap-A. Niemann-Pick type C disease is characterized by a failure in cholesterol transport out of the endosomes and lysosomes (57, 58). As a consequence, cholesterol accumulates within these organelles. In addition, storage of glycosphingolipids in spleen (59) and brain has been described. In the white matter of several patients, a severe loss of myelin lipids has been reported (26). Our results indicate that accumulation of cholesterol might impair the release of lipids by Sap-A or other Saps, which causes a slower degradation and accumulation of the respective lipid substrate.

The changes that take place along the endocytic pathway include lowering of luminal pH as well as decreasing cholesterol and increasing BMP concentrations of internal membranes. They are destined to create ideal conditions for membrane disintegration and digestion within the acidic compartments of the cell.

**Variant Sap-A Proteins Show Increasing Binding and Decreasing Lipid Extraction Capacity**—The physiological role of glycosylation in Sap-A is still not well understood. An in vitro study showed that glycosylation of Sap-A has no influence on the activation of galactosylceramide-β-galactosidase (23). However, it should be expected that glycosylation affects the interaction of Sap-A with membranes. This interaction has been investigated using nonglycosylated recombinant Sap-A (31). In this case, the N and C termini of Sap-A as well as its central region have been observed to insert into the lipid bilayer. Nevertheless, the presence of glycosylation in the mentioned central region of Sap-A might render its incorporation into the membrane more difficult. Using Sap-A prepared from spleen of Gaucher’s disease patients and phosphatidylserine-containing liposomal vesicles, it has been shown that Sap-A associates poorly with the membrane but can still perturb it (30). Our data (Fig. 5, A and B) show that at all cholesterol concentrations used, nonglycosylated Sap-A (nSap-A) binds better to the membranes than glycosylated Sap-A. In addition, lipid extraction is reduced and is only observed when protein-free buffer is applied. This buffer flow does not represent a physiological situation since proteins are always present in the lysosomal lumen. Hence, under physiological conditions (represented by the first 180 s of measurement) nSap-A is almost unable to mobilize lipids from the membrane. In contrast, gSap-A shows a short binding period after which mobilization of lipids occurs, all this under almost physiological conditions. Glycosylation seems to have a negative effect on binding of gSap-A to the membrane. However, the oligosaccharide moiety probably increases the solubility of the molecule. Thus, gSap-A might be able to bind or insert into the membrane and extract lipids out of it. This correlates with the fact that at high salt concentrations the behavior of nSap-A resembles that of gSap-A (Fig. 2E). The presence of charged molecules might increase the solubility of nSap-A, imitating the effect of the oligosaccharide moiety on gSap-A.

The formation of three disulfide bridges is indispensable for the activity of saposins, as demonstrated for Sap-A in vitro (23) and in vivo (20). Our results (Fig. 5, C–H) show that variant proteins lacking one disulfide bridge have an increased capacity to bind to the lipid membrane but lose the ability to remove lipids from it. Therefore, our data may explain at least in part the phenotype displayed by the Sap-A knock-out mouse carrying a C106F mutation (20). The Sap-A variant present in the tissues of the mouse not only lost its conformational stability but also its ability to mobilize lipids from the intraluminal membranes. Substitution of Cys by Phe or Ala in variants 2 and 3, respectively, leads to similar results, excluding a mere steric effect of Phe on the action of Sap-A.

Sap-A lacking Val-11, a valine conserved among the Saps, shows similar properties as variant Sap-A lacking one disulfide linkage. The loss of lipid mobilization capacity of this variant protein might explain the phenotype of the human patient carrying this abnormal Sap-A. Thus, the intact lipid mobilization capacity of Sap-A seems to be essential for its normal activity in vivo.

In summary, the results of our experiments allow us to conclude that glycosylated Sap-A is able to bind to the lipid membrane, to extract lipids out of it, and to return rapidly into solution, optimally at the lysosomal pH of 4.2. In contrast, nonglycosylated saposin A binds to the membrane and remains there. It is unable to return into the aqueous phase and to release lipids from the membrane. Evidently, the oligosaccharide moiety of the protein stabilizes the protein in aqueous solution. High amounts of BMP and low amounts of cholesterol in the liposomes enhance the release of lipids by gSap-A, whereas the increase of ionic strength reduces this ability. Sap-A variants that lack one of the three disulfide bridges or carry the deletion of Val 11 are unable to mobilize lipids from membranes and to disintegrate them. This behavior could explain the phenotypes of the Sap-A knock-out mouse and of the human patient suffering from Krabbe disease.

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