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Saposin A: Second cerebrosidase activator protein

(sphingolipid activator protein/β-glucosylceramidase/β-galactosylceramidase/Gaucher disease/Gaucher spleen)

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ABSTRACT **Saposin A, a heat-stable 16-kDa glycoprotein, was isolated from Gaucher disease spleen and purified to homogeneity. Chemical sequencing from its amino terminus and of peptides obtained by digestion with protease from *Staphylococcus aureus* strain V-8 demonstrated that saposin A is derived from proteolytic processing of domain 1 of its precursor protein, prosaposin. Processing of prosaposin (70 kDa) also generates three other previously reported saposin proteins, B, C, and D, from its second, third, and fourth domains. Similar to saposin C, saposin A stimulates the hydrolysis of 4-methylumbelliferyl β-glucoside and glucocerebroside by β-glucosylceramidase and of galactocerebroside by β-galactosylceramidase, mainly by increasing the maximal velocity of both reactions. Saposin A is as active as saposin C in these reactions. Saposin A has no significant effect on other sphingolipid and 4-methylumbelliferyl glycoside hydrolases tested. Saposin A has two potential glycosylation sites that appear to be glycosylated. After deglycosylation, saposin A had a subunit molecular mass of 10 kDa and was as active as native saposin A. However, reduction and alkylation abolished the activation. A three-dimensional model comparing saposins A and C reveals significant sequence homology between them, especially preservation of conserved acidic and basic residues in their middle regions. Each appears to possess a conformationally rigid hydrophobic pocket stabilized by three internal disulfide bridges, with amphipathic helical regions interrupted by helix breakers.**

The lysosomal hydrolysis of sphingolipids is catalyzed by the sequential action of acid hydrolases. Several small heat-stable proteins called sphingolipid activator proteins have been discovered that act as natural nonspecific detergents or stimulate a specific hydrolase, or both. One of these, named saposin B in this report and previously designated by several different terms (1–4), stimulates the hydrolysis of galactocerebroside sulfate by arylsulfatase A (EC 3.1.6.1) (1, 5, 6), GM1 ganglioside by β-galactosidase (EC 3.2.1.23) (2, 7), and globotriaosylceramide by α-galactosidase A (EC 3.2.1.22) (8). This activator protein may have even broader substrate specificity (9). The primary structure of saposin B was determined by sequencing a cDNA encoding it (10), and its physiological significance is underscored by the discovery of its absence in a variant form of metachromatic leukodystrophy (activator-deficient metachromatic leukodystrophy) (11). A second activator protein, called saposin C in this report and also previously designated by several different terms (12–15), stimulates the hydrolysis of glucosylceramide by β-glucosylceramidase (EC 3.2.1.45) (12–15) and that of galactosylceramide by β-galactosylceramidase (EC 3.2.1.46) (16). Unlike saposin B, saposin C appears to interact directly with both enzymes to stimulate activity (17). The primary structure of saposin C was determined by chemical sequencing of its amino acids (18, 19) and by deducing its sequence from nucleotide sequencing of a cDNA encoding prosaposin, its precursor (20). Saposin C has been reported to be deficient in a single patient with a variant form of Gaucher disease (21).

Recently, the complete nucleotide sequence of a cDNA encoding prosaposin, the precursor of saposins B and C, was elucidated (20). Prosaposin is a 511-amino acid glycoprotein, and examination of its amino acid sequence reveals four saposin-like domains, two of which are saposins B and C; these are flanked by two additional domains, which we call saposin A and saposin D. Each domain is approximately 80-amino acid residues long; has nearly identical placement of cysteine residues, glycosylation sites, and helical regions; and is flanked by proteolytic cleavage sites. Molecular models indicate that the proteins derived from each domain can fold to give rise to a conformationally rigid hydrophobic pocket held together by three disulfide bridges. Proteolytic cleavage of prosaposin at each domain boundary was predicted to give rise to four saposin proteins (20). We recently have isolated saposin D, the protein arising from domain four of prosaposin and demonstrated it to be a specific sphingomyelin phosphodiesterase (EC 3.1.4.12) activator (22). In this report we present the isolation and characterization of saposin A, the protein derived from domain one of prosaposin and demonstrate it to be a second activator protein for both β-glucosylceramidase and β-galactosylceramidase.

MATERIALS AND METHODS

Materials. All 4-methylumbelliferyl (4-MeUmb) substrates, Triton X-100, and protease from *Staphylococcus aureus* strain V-8 were obtained from Sigma. Sephadex G-75 and Con A-Sepharose were purchased from Pharmacia LKB. Affi-Gel 10 and DE 52 cellulose (DEAE-cellulose) were obtained from Bio-Rad and Whatman, respectively. A Vydac protein C4 HPLC column was purchased from Rainin Instruments. N-Glycanase was purchased from Genzyme. [*choline-methyl*-^{14}C]Sphingomyelin and [*glucose*-^{3}H]glucocerebroside were obtained from NEN/DuPont and D. A. Wenger (Jefferson University School of Medicine), respectively. [*galactose*-^{3}H]Galactocerebroside, [*galactose*-^{3}H]ganglioside GM1, and [*galactose*-^{3}H]asialo-GM1 were synthesized in this laboratory as indicated below.

Isolation of Saposin A. Techniques used for the isolation of saposin A were in general similar to those used for the isolation of coglucosidase by Sano and Radin (19). Briefly, frozen spleen (405 g) from a patient with adult-type Gaucher disease was homogenized with 1600 ml of water, and the homogenate was heated at 100°C for 10 min and then

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Abbreviation: 4-MeUmb, 4-methylumbelliferyl.

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centrifuged at 10,000 × *g* for 15 min. The supernatant was made 45% saturated with ammonium sulfate and centrifuged at 10,000 × *g* for 15 min. The supernatant was then made 80% saturated with ammonium sulfate and centrifuged at 10,000 × *g* for 15 min. The pellet was dissolved in 150 ml of 10 mM sodium phosphate buffer (pH 7) and dialyzed against the same buffer overnight. The dialysate was then applied to a 2.5 cm × 18 cm DE 52 column, and a linear gradient of NaCl from 0 to 500 mM was then used to elute the column. Fractions were collected by monitoring enzyme activation of β-glucosidase (23) and by HPLC as described below. Saposin A was eluted by 180–270 mM NaCl (at the top of the column); saposin C, by 270–370 mM NaCl; and saposin D, by 75–180 mM. Saposin B was included in both fractions containing saposins A and C. The fraction containing saposin A was dialyzed against 50 mM Tris·HCl buffer (pH 7.5) and then passed over a column containing 40 ml of Con A-Sepharose 4B. After the column was rinsed with 120 ml of 50 mM Tris·HCl buffer (pH 7.5), saposin A was eluted with 120 ml of the same buffer containing 0.3 M sodium chloride and 0.3 M methyl α-D-mannoside. The fraction was dialyzed against water and then lyophilized. The final purification step was reversed-phase HPLC of the enriched fraction with a Varian model 5000 or Shimadzu LC4A instrument, each equipped with a Vydac C_4 column (4.6 mm × 25 cm) monitoring peaks by absorption at 220 nm. A linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid was used over a 1-hr period to elute saposin A. Up to 0.5 mg of saposin A in 1.5 ml of water was injected with good separation.

Immunological Procedures. Antibodies against saposin A were prepared in a rabbit as described by Crowle (24) and purified with an affinity column that contained purified saposin A linked to an Affi-Gel 10-activated support as described by the manufacturer (Bio-Rad); anti-saposin A was eluted with 2 M $MgCl_2$. Dot blots and immunoblots with the antibodies obtained in this manner were performed as described (10).

Assays for Lysosomal Activities. Sphingomyelinase activity was assayed as described by Wenger (25) with [*choline-methyl*-^{14}C]sphingomyelin as substrate. A Triton X-100 extract of a particulate fraction of human liver as described by Radin and Berent (23) was used as the enzyme source. β-Galactosylceramidase and β-glucosylceramidase were assayed as described by Wenger *et al.* (16) with [^{3}H]galactosylceramide prepared as described (26) and [^{3}H]glucosylceramide obtained from D. A. Wenger. Human spleen was homogenized with 2 volumes of water and centrifuged at 12,500 × *g* for 10 min, and the supernatant obtained was used as the enzyme source of β-galactosylceramidase. The same human liver preparation used for sphingomyelinase activity was used as a crude enzyme source for β-glucosylceramidase activity. Pure β-glucosylceramidase was obtained from human placenta as described (27). GM1 ganglioside β-galactosidase activity was assayed as described (28) by using [*galactose*-^{3}H]ganglioside GM1 synthesized as described (29) and purified human liver acid β-galactosidase prepared in this laboratory. Assays for enzymes that hydrolyze 4-MeUmb β-D-glucoside or 4-MeUmb β-D-galactoside were performed as described (30). Hexosaminidase activity (EC 3.2.1.30) against [*galactosamine*-^{3}H]asialo-GM2 was assayed as described (31).

Peptide Sequencing. Peptide sequencing of intact and of reduced and alkylated saposin A was accomplished by using an Applied Biosystems model 470 A gas-phase sequencer equipped with a model 120A on-line phenylthiohydantoin analyzer under standard conditions specified by the manufacturer. Reduction and alkylation of saposin A (825 μg) were performed essentially as described by Waxdall *et al.* (32). The protein was dissolved in 500 μl of 6 M guanidine hydrochloride/2 mM EDTA/0.5 M Tris·HCl, pH 8.1, and heated at 50°C for 30 min, after which 3 mg of dithiothreitol was added. After heating again at 50°C for 5 hr, 3.9 mg of iodoacetic acid in 100 μl of 1 M NaOH was added. After incubation for 15 min in the dark at room temperature, the mixture was dialyzed against water, and the product was repurified by HPLC as above. The reduced and alkylated saposin A was treated with protease from *S. aureus* strain V-8 as follows: 623 μg of reduced and alkylated saposin A was incubated with 20 μg of protease as described by Houmard and Drapeau (33). The hydrolysate was chromatographed by HPLC as above, and five major peaks were collected for amino acid sequencing.

Other Analytical Methods. Protein concentration and carbohydrate content were determined by the method of Lowry *et al.* (34) and the phenol/sulfuric acid method (35), respectively. Deglycosylation of saposin A and saposin C was accomplished by using N-Glycanase under conditions described by the manufacturer (Genzyme). Enzyme kinetic constants (K_m and V_{max} values) were calculated from Lineweaver–Burk plots.

RESULTS

Isolation and Characterization of Saposin A. Saposin A was isolated from Gaucher spleen by taking advantage of its thermal stability, acidity, Con A-Sepharose affinity, and hydrophobicity. The yield of pure saposin A was 16.1 mg from 405 g of frozen tissue. Saposin A was judged to be pure by several analytical criteria, including a single diffuse band at 16 kDa on sodium dodecyl sulfate/polyacrylamide gels (17.5% acrylamide) (Fig. 1), a single sharp band at 10 kDa after deglycosylation in the same system (data not shown), a single peak by HPLC (Fig. 2), and single peaks of amino acids obtained upon automated sequencing of the amino terminus of purified saposin A (data not shown). Saposin A contains two glycosylation sites and was found to contain ≈30% carbohydrate by phenol/sulfuric acid analysis. Amino acid sequence analysis of the native saposin A failed to identify the asparagine at position 21. From comparison with the primary structure deduced from the cDNA for prosaposin (20), this residue must be glycosylated—i.e., Asn(CHO)-Ala-Thr. Similarly, asparagine 42 from peptide a (see Fig. 4) was not directly identified by automated Edman degradation. However, sequence analysis of peptide a after N-Glycanase digestion identified an aspartate at position 42 as expected after deglycosylation (data not shown). The molecular mass of native saposin A was determined by polyacrylamide gel electrophoresis to be 16 kDa; after deglycosylation, its molecular mass was 10 kDa. Under the same conditions saposin C, which contains a single potential glycosylation site, had a molecular mass of 13 kDa before deglycosylation and 10 kDa after deglycosylation. Antibodies raised against saposin A did not cross-react with saposin B, C, or D (Fig. 3), and antibodies against saposin B, C, or D did not cross-react with saposin A (data not shown). Chemical

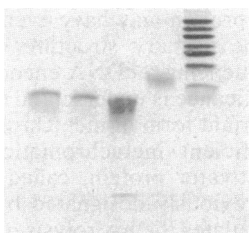

FIG. 1. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of saposin A. The purified saposin A (7.3 μg; lane A) was electrophoresed together with saposin B (5 μg; lane B), saposin C (4.5 μg; lane C), and saposin D (5 μg; lane D). Lane S contained a protein standard mixture. The lowest protein marker is α-lactalbumin (14,200 Da). The gel was stained with Coomassie brilliant blue.

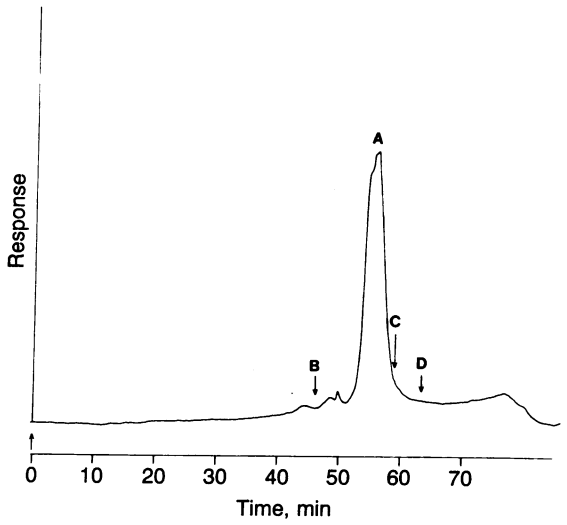

FIG. 2. HPLC of saposin A. Purified saposin A was analyzed by HPLC as described. Under the same conditions, saposin B, C, and D appear with retention times as indicated by arrows.

sequencing of the N-terminal amino acids of saposin A and protease cleavage peptides of reduced-alkylated saposin A revealed colinearity between 69 chemically sequenced amino acids (Fig. 4) and the polypeptide predicted to arise from proteolysis of domain 1 of prosaposin (amino acids 47–130), with cleavage apparently occurring at lysine residues at the domain 1 boundaries (20).

Stimulation of β-Glucosylceramidase and β-Galactosylceramidase by Saposin A. Purified saposin A stimulated enzymatic hydrolysis of 4-MeUmb β-D-glucopyranoside, glucocerebroside, and galactocerebroside. When the purified preparation of β-glucosylceramidase was used, the activation of 4-MeUmb β-D-glucoside hydrolysis was maximum at pH 4.5 (data not shown). The extent of activation by saposin A at this pH was similar to that given by saposin C, the first saposin identified as an activator of 4-MeUmb β-D-glucoside hydrolysis (30), over a wide concentration range. In the presence of 0.05% Triton X-100 and 2 mM 4-MeUmb β-D-glucoside, maximal activation (10-fold higher than the control value) occurred at a concentration of 3.15 μM for each saposin (Fig. 5). Although the activation by both saposins was nearly identical at low concentrations, activation by saposin A was maximal at a lower concentration than activation by saposin C. As reported for saposin C (11–13), phosphatidylinositol also stimulated the hydrolysis of 4-MeUmb β-D-glucoside to an extent slightly higher than saposin A (Table 1). However, the addition of phosphatidylinositol to the assay mixture that contained saposin A did not increase the rate of hydrolysis. The activation by phosphatidylinositol was virtually unchanged by increasing its concentration up to 120 μM. A similar effect of saposin A and phosphatidylinositol was also

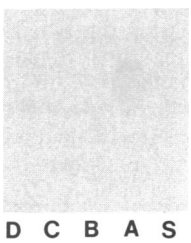

FIG. 3. Immunoblot of saposin A. Saposin A (14.6 μg; lane A), saposin B (10 μg; lane B), saposin C (9 μg; lane C), saposin D (10 μg; lane D), and a protein standard mixture (lane S) were electrophoresed as described in the legend of Fig. 1, but for a shorter time. The gel was immunoblotted with anti-saposin A antiserum as described.

Ser Leu Pro Cys Asp Ile Cys Lys Asp Val 10
Val Thr Ala Ala Gly Asp Met Leu Lys Asp 20
Asn Ala Thr Glu Glu Glu Ile Leu Val Tyr 30
Leu Glu Lys Thr Cys Asp Trp Leu Pro Lys 40
Pro Asn Met Ser Ala Ser Cys Lys Glu Ile 50
Val Asp Ser Tyr Leu Pro Val Ile Leu Asp 60
Ile Ile Lys Gly Glu Met Ser Aug Pro Gly 70
Glu Val Cys Ser Ala Leu Asn Leu Cys Glu 80
Ser Leu Gln Lys 84

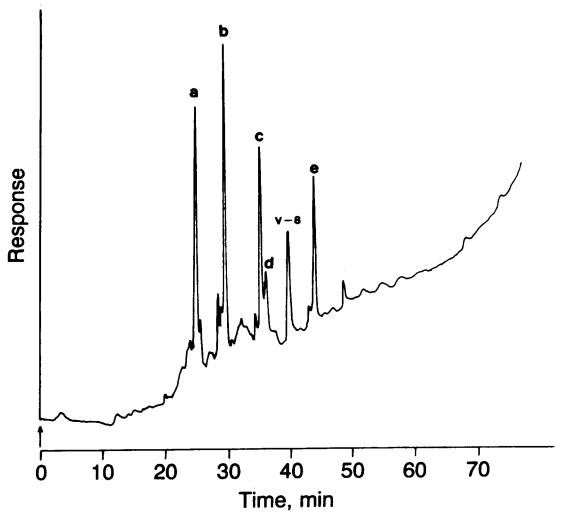

FIG. 4. (*Upper*) Amino acid sequence of saposin A. The amino acid sequence predicted from the cDNA for prosaposin is given. The polypeptides that are underlined were confirmed by chemical sequencing of the amino terminus of intact saposin A (amino acids 1–25) and the protease V-8 cleavage products of purified reduced and alkylated saposin A. (*Lower*) HPLC of protease V-8 cleavage products used in chemical sequencing of saposin A peptides. Peaks: a, d, and e, amino acid residues 33–48, 1–11, and 50–60, respectively; b, three fragments, each containing amino acids 50–54, 66–74, and 33–39 from the N terminus, respectively; c, four fragments, each containing residues 1–4, 50–53, 66–74, and 27–32, respectively, from the N terminus.

observed when Triton X-100 was removed from the assay system, although the extent of activation was smaller. However, as noted by Glew *et al.* (36) using saposin C, a synergic effect of saposin A and phosphatidylinositol was observed in the hydrolysis of the natural substrate, glucocerebroside, even in the presence of Triton X-100 (Table 1).

The effect of saposin A on the Michaelis–Menton constant of 4-MeUmb β-D-glucoside was determined with pure β-glucosylceramidase. Addition of 8 μM saposin A reduced the K_m value from 2.94 to 1.61 mM and increased the V_{max} from 9.5 nmol/hr to 50 nmol/hr (Lineweaver–Burk plot not shown). After complete deglycosylation, repurified saposin A activated purified β-glucosylceramidase activity to the same extent as did native saposin A. A similar result has been reported for saposin C by Sano and Radin (37). Saposin A was inactive after reduction and alkylation.

Saposin A also stimulated the hydrolysis of galactocerebroside by partially purified β-galactosylceramidase in the presence of phosphatidylserine. Its specific activity appeared to be slightly lower than saposin C as shown in Fig. 6; saposin A stimulated activity of the crude enzyme 6-fold, while saposin C stimulated it 8-fold, at maximal concentrations. A similar stimulation was obtained with sodium taurocholate.

Saposin A did not stimulate the activities of GM1-β-galactosidase or asialo-GM1-β-*N*-acetylhexosaminidase and inhibited by 30% the activity of sphingomyelinase (data not shown). It also did not stimulate activities for hydrolysis of

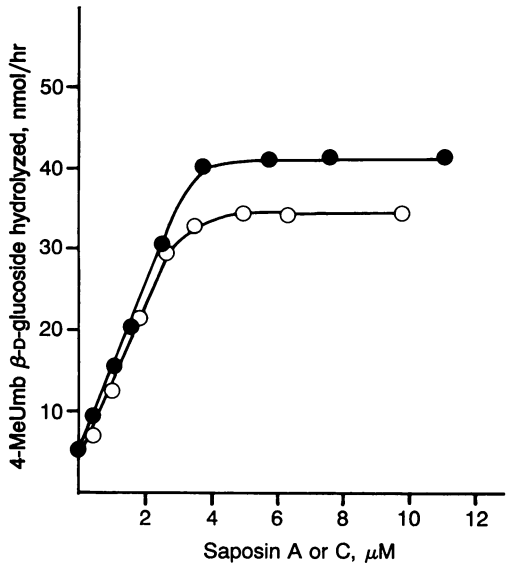

FIG. 5. Effect of saposin A and saposin C on 4-MeUmb β-D-glucosidase activity. Various amounts of saposin A were added to the activation assay, and the effect was compared with that of saposin C. The assay mixture contained 2 mM 4-MeUmb β-D-glucosidase, 0.05% Triton X-100, and 50 mM acetate buffer (pH 4.5) in 200 μl. Purified glucosylceramidase from human placenta mixed with 20 μg of bovine serum albumin was used as the enzyme source. ○, Activity obtained with saposin A; ●, activity obtained with saposin C.

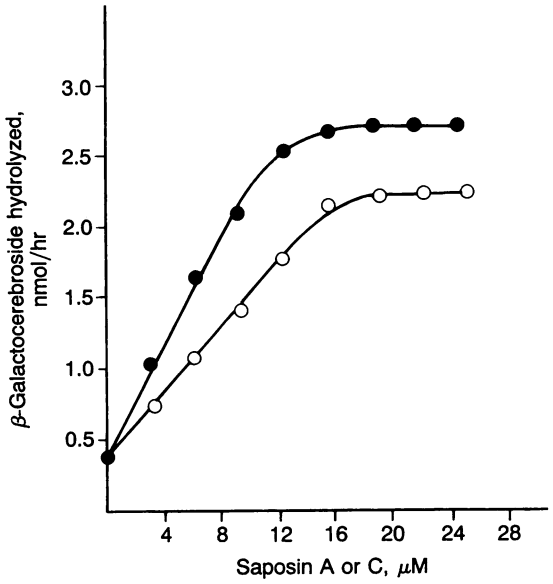

FIG. 6. Effect of saposin A and saposin C on β-galactosylceramidase activity. Various amounts of saposin A were added to the activation assay and the effect was compared with that of saposin C. The assay mixture contained 20 nmol of [*galactose*-^{3}H]galactocerebroside (523 cpm/nmol) and 20 μmol of citrate/phosphate buffer (pH 4.2) in 200 μl. Human liver particulate preparation solubilized by 0.1% Triton X-100 was used as the enzyme source. ○, Activity obtained with saposin A; ●, activity obtained with saposin C.

such 4-MeUmb derivatives as pyrophosphate, α-galactoside, β-galactoside, α-glucoside, α-mannoside, β-fucoside, β-glucuronide, β-N-acetylgalactosaminide, and palmitate (data not shown).

DISCUSSION

Some of us have predicted that proteolytic processing of prosaposin generates four saposin proteins, which stimulate lysosomal hydrolysis of sphingolipids (20). In view of this observation, we have named these proteins the saposins A, B, C, and D and proposed a new systematic nomenclature (22). In addition to previously known saposins B, C, and D, as described above, the isolation of saposin A reported here completes the isolation of the four mature saposin proteins and identifies a second activator protein for lysosomal enzymes that hydrolyze glucosylceramide and galactosylceramide. Among the four saposins, only saposin A and saposin C activate the same enzymes, β-glucosylceramidase and β-galactosylceramidase. The fact that both β-glucosylceramidase and β-galactosylceramidase are activated to a similar extent may suggest that they possess regions of homology.

Table 1. Effect of saposin A and phosphatidylinositol (PtdIns) on hydrolysis of 4-MeUmb β-D-glucoside and glucocerebroside

Addition			Hydrolysis,* nmol/hr	
Triton X-100, %	Saposin A, μM	PtdIns, μM	4-MeUmb β-D-glucoside	Gluco-cerebroside
0.05	0	0	4.3 ± 0.2	0.09 ± 0.01
0.05	6.3	0	20.2 ± 0.7	0.12 ± 0.02
0.05	0	6.5	31.6 ± 1.0	0.37 ± 0.01
0.05	6.3	6.5	25.4 ± 0.3	0.96 ± 0.03
0	0	0	1.8 ± 0.1	ND
0	6.3	0	5.9 ± 0.1	ND
0	0	6.5	8.7 ± 0.1	ND
0	6.3	6.5	5.1 ± 0.3	ND

*Average ± SD of triplicate assays as described. ND, not determined.

Numerous amino acid sequence homologies are evident when saposin A and saposin C are compared (Fig. 7). Both contain acidic residues that are not present in saposins B and D; one cluster occurs between amino acids 21 and 45. Especially noteworthy is the identical placement of glutamic, proline, and cysteine residues in this 24-amino acid stretch. The two proteins are 50% homologous in this region, suggesting that this conserved region may be involved in enzyme activation. Tertiary structural models in which helical wheels (38) are used to depict the three-dimensional structure of saposins A and C indicate a similar structure for each (Fig. 7 *Upper* and *Lower*); at least three internal sequences at approximately amino acid residues 4–19, 23–37, and 43–54 are capable of forming amphipathic helices separated by helix breakers (glycosylation at positions 21 in each and 42 in saposin A and conserved proline residues), with cysteine residues being properly positioned to form three internal disulfide bridges in the hydrophobic interior. Noteworthy is that many of the residues facing the hydrophobic pocket are identical in both saposin A and saposin C. Perhaps this hydrophobic region is important in substrate interaction since cerebrosides are hydrophobic. In addition, the exposure of conserved aqueous glutamic and lysine residues (positions 25, 26, 33, and 49) may create a charged domain on the outside of the helix at the same position in each saposin. Physical studies comparing the two saposins should aid in understanding their three-dimensional structures.

An accumulation of saposin C up to 37 times normal has been reported to occur in the spleen of Gaucher patients (17). Our recent investigations (ref. 22 and present study) show that saposins A and D also accumulate in this organ. We calculate that this organ contains saposins A, C, and D in concentrations of 40, 27, and 70 μg/g of fresh tissue, respectively. The content of saposin B in Gaucher disease spleen is lower at ≈3 μg/g of tissue. The mechanism of the accumulation is not clear. Saposin A is not only present in Gaucher disease splenic tissue but also present in smaller amounts in normal liver and other human tissues as determined by immunoblotting and HPLC. We have isolated saposin A from human liver and found it to possess

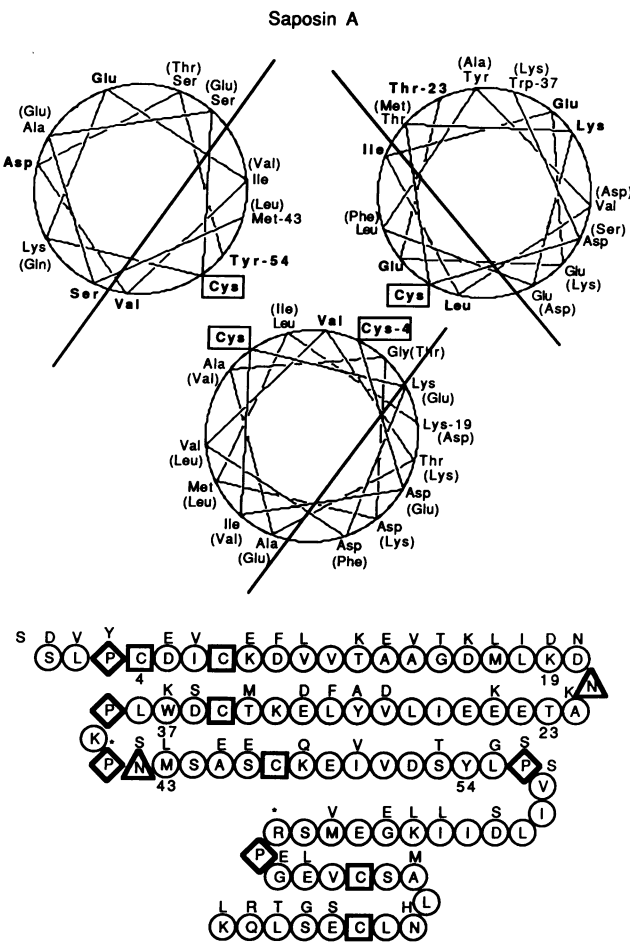

FIG. 7. Helical wheel (38) and two-dimensional depictions of saposin A and saposin C. (*Upper*) Three wheels depicting three potential amphipathic helices of saposin A including residues 4–19, 37–23 (residues 23–37 are inverted), and 43–54 are shown. Cysteine residues are boxed, and the hydrophobic interior faces are indicated by lines through each wheel. The amino acid residues in bold type represent residues that are identical in saposins A and C. The amino acids in parentheses are the corresponding residues in saposin C, with residues 5–20, 38–24, and 43–54 used in the helices. (*Lower*) A two-dimensional model of saposin A indicating the six cysteines (boxes), two potential glycosylation sites (triangles), and conserved proline residues (diamonds). The letters above the model show the amino acid sequence of saposin C. Those residues that have no letter above them are identical in both sequences. An asterisk indicates that there is no corresponding residue in saposin C.

stimulating activity for 4-MeUmb β-D-glucoside hydrolysis, identical to saposin A isolated from Gaucher disease spleen (unpublished data). This observation indicates that saposin A is involved in the normal metabolism of gluco- and galactocerebrosides.

Evolution has generated two saposin proteins (A and C) similar in structure encoded by the same genetic locus and derived from the same precursor, both of which activate the first step in the lysosomal hydrolysis of glucocerebroside and galactocerebroside. Homologies of protein and nucleotide sequence between saposins A and C indicate that they are derived from a common ancestral gene segment that was duplicated in the saposin gene.

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