Differential Membrane Interactions of Saposins A and C:
Implications for the Functional Specificity*

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ABBREVIATIONS: PS, phosphatidylserine; PC, phosphatidylcholine; EPC, egg PC; BPS, brain PS; DPS, dansyl-PS; SLPC, 1-palmitoyl-2-stearoyl(n-doxy)-sn-glycero-3-phosphocholine; TEMPO, 2,2,6,6-tetramethyl-piperidine-N-oxyl; 4MU-Glc, 4-methyl-umbelliferyl-β-D-glucopyranoside; CD, circular dichroism; FRET, fluorescence resonance energy transfer.
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SUMMARY

Saposins are small, heat-stable glycoprotein activators of lysosomal glycosphingolipid hydrolases that derive from a single precursor, prosaposin, by proteolytic cleavage. Three of these saposins (B, C and D) share common structural features including: lack of tryptophan, a single glycosylation sequence, the presence of three conserved disulfide-bonds, and a common multi-amphipathic helical bundle motif. Saposin A contains an additional glycosylation site and a single tryptophan. The oligosaccharides on saposins are not required for in vitro activation functions. Saposins A and C were produced in E.coli to contain single tryptophans at various locations to serve as intrinsic fluorescence reporters, i.e., as topological probes, for interaction with phospholipid membranes. Maximum emission shifts, aqueous and solid quenching, and resonance energy transfer were quantified by fluorescence spectroscopy. Amphipathic helices at the amino- and carboxyl-termini of saposins A and C were shown to insert into the lipid bilayer to about 5-carbon bond lengths. In comparison, the middle region of saposins A or C were either embedded in the bilayer or solvent exposed, respectively. Conformational changes of saposin C induced by phosphatidylserine interaction suggested the reorientation of functional helical domains. Differential interaction models are proposed for the membrane-bound saposins A and C. By site-directed mutagenesis of saposin A and C, their membrane topological structures were correlated with their activation effects on acid β-glucosidase. These findings show that proper orientation of middle segment of saposin C to the outside of membrane surface is critical for its specific and multivalent interaction with acid β-glucosidase. Such membrane interactions and orientations of the saposins determine the proximity of their activation and/or binding sites to lysosomal hydrolases or lipid substrates.
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INTRODUCTION

Saposins, a family of small (~80 amino acids) heat stable glycoproteins, are essential for the in vivo hydrolytic activity of several lysosomal enzymes in the catabolic pathway of glycosphingolipids (1-3). Four members of saposins, A, B, C, and D, are proteolytically derived from a single precursor protein, termed prosaposin (4-8). The primary sequences of saposins are highly homologous with ~60% amino acid similarity. In addition, each of the saposins has six conserved cysteines that form three intradomain disulfide bridges whose placements are identical (9). In a native state, five consensus N-glycosylation sequences in prosaposin are occupied by oligosaccharide chains (10-12). Each of saposins B, C, and D has one such sequence, while saposin A has two. Non-glycosylated saposins retain their respective activation effects using in vitro assays (12-15).

A multiple α-helical bundle motif, characterized by a three conserved disulfide structure and several amphipathic peptides, is found in the saposins, and also in saposin-like proteins and domains, i.e., NK-lysin, surfactant-associated protein B (SP-B), acid sphingomyelinase (ASM), acyloxyacyl hydrolase (AOAH), plant aspartic proteases and pore-forming peptides (amoebapores), [reviewed in (16)]. This common backbone structure was characterized by the NMR structure of NK-lysin (Fig. 1A), as well as by the crystal structure of prophytepsin (a plant aspartic protease) (17,18). The ‘saposin fold’ is a common fold in a single globular structure for all the saposins and saposin-like proteins and domains.
Despite the shared saposin fold structure in solution, saposins and saposin-like proteins have diverse *in vivo* biological functions. Since all these proteins bind to or interact with lipid membranes, one can speculate that the specific biological functions of saposins and saposin-like proteins are the results of the differential interactions with the biological membrane environments. For instance, SP-B and NK-lysin are saposin-like proteins with completely different biological functions. SP-B plays a crucial role in the rapid adsorption of lipids at the air/water interface (19). NK-lysin is a tumor-lysin and antibacterial polypeptide (20). A postulated model for SP-B action on lipid membranes included a small alteration of the saposin fold in lipid-bound SP-B, while membrane-associated NK-lysin maintains the saposin fold without any structural changes. In the SP-B-lipid binding model, portions of the hydrophobic helical stretches are buried in the bilayer in an orientation parallel to the lipid acyl chains. Other parts of helices are positioned parallel to the membrane surface (21). The insertion of  $\alpha$-helical peptides and the electrostatic interaction between charged residues of the protein and polar head groups of lipid stabilize SP-B at lipid membrane surface. Lipid-bound NK-lysin model has half of the molecule embedded into the membrane and the more hydrophilic negatively-charged half remains solvent-exposed (22). Most of the positively-charged residues located in an equatorial belt around the saposin fold of the molecule interact with the negatively charged head groups of lipid. Apparently, the different interaction modes of SP-B and NK-lysin with lipid membrane are due to the different charge arrangements and amphipathic properties of helices in these two molecules.

Prosaposin is a multifunctional precursor preprotein. A complete deficiency of prosaposin with a mutation in the initiation codon caused the storage of multiple glycosphingolipid substrates
resembling a combined lysosomal hydrolase deficiency (23). Patients lacking the individual saposins B and C showed a variant form of metachromatic leukodystrophy (24-26) and Gaucher disease (27,28), respectively. These define the primary physiological functions of saposin B and C in lysosomes. The structural characteristics of these saposins are of great importance to their diverse mechanisms of activation on their respective cognate glycosphingolipid hydrolases. Mechanistic and kinetic studies of lysosomal enzyme activation by saposins B and C have shown different modes of action. In general, activation by saposin B is via solubilizing and presenting glycosphingolipid substrates to several lysosomal enzymes (2). Saposin C promotes acid β-glucosidase activity by inducing in the enzyme conformational change at acidic pH (29-31). The enzyme activity is thought to be optimized by saposin C via membrane perturbation (32). However, the binding of saposin C to phospholipid bilayers is poorly characterized. In vitro and ex vivo saposins A and D functions are to enhance the degradation of galactosylceramide and ceramide/sphingomyelin, respectively (12,33-35). To date, the precise physiological functions of saposins A and D remain unknown.

In vitro saposin A enhances acid β-glucosidase activity at µM concentration, but isolated saposin C deficiency leads to glucosylceramide storage and a "Gaucher disease-like" phenotype (36,37). Thus, saposin A is probably not an important physiologic activator of acid β-glucosidase (15,38). In a variety of cells, the physiological concentrations of saposins are estimated to be in the nmolar range (39) and saposin C provides excellence in vitro activation levels at # 200 nM. This activation was mediated via a highly specific induced conformational change and multivalent interactions. Importantly, negatively-charged phospholipid bilayers are required for
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the interactions of saposin A and/or C with acid β-glucosidase *in vitro*. Consequently, their mechanisms of interaction may be influenced by their differential interaction.

In this study, the differential interactions of saposins A and C with lipid bilayers were evaluated by intrinsic Trp fluorescence spectroscopy. Mutant saposins provided insight into the molecular basis for the conformational requirement of membrane-bound saposins to the specific activation functions.
EXPERIMENTAL PROCEDURES

Materials — The following materials were from commercial sources: acrylamide [Life Technologies, Gaithersburg, MD]; 4-methylumbelliferyl-β-D-glucopyranoside (4MU-Glc) and Ceredase® (alglucrease for injection) [Genzyme Corp., Cambridge, MA]; Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA); restriction endonucleases (New England Biolabs, Beverly, MA); Magic polymerase chain reaction prep kits (Promega, Madison, WI); C₄ reverse-phase HPLC column (Alltech Association Inc., Deerfield, IL); Egg phosphatidylcholine (EPC), brain phosphatidylserine (BPS), palmitoyl-2-stearoyl(n-doxyl)-sn-glycero-3-phosphocholine (SLPC, n = 5, 10, 16), dansyl (or 5-dimethylamino-1-naphthalenesulfonyl)-phosphatidylserine (18:1,1) (DPS) (Avanti Polar Lipids, Alabaster, AL). Anionic lipids were sodium salts. All other reagents were reagent grade or better.

Saposin Preparation — The amino acid sequences of wild-type saposins A and C are in Figure 1. The mature NH₂-terminal amino acid of the natural human saposins will be designated as 1 and used to reference the position of the amino acids in the fragments. The cDNAs for mutant proteins were generated using the QuickChange site-directed mutagenesis kit, and their sequences were verified by complete DNA sequencing.

All recombinant saposins were overexpressed in E. coli cells by using IPTG-inducing pET system (15). Expressed proteins with a His-tag were eluted from nickel columns. After dialysis, the proteins were further purified by HPLC chromatography as follows: The C4 reverse phase column was equilibrated with 0.1% trifluoroacetic acid (TFA) for 10 minutes, and then, the proteins were eluted in a linear (0-100%) gradient of 0.1% TFA in acetonitrile over 60 minutes.
The major protein peak was collected and lyophilized. The protein concentrations were determined as previously described (15).

_Vesicle Preparation_ — Unilamellar vesicles (SUV) were prepared by bath sonication (15,31). Phospholipids (0.4-50 µmole) in chloroform were dried under N₂ and then vacuum. The lipid films were suspended in 0.1 M sodium acetate, pH 4.7 and sonicated in a bath sonicator until clear. The temperature during sonication was kept at least 5°C above the Tₑ of the lipid mixtures. To prevent overheating the samples, ice was added as needed.

Since saposins cause fusion of the vesicles within 2 to 3 minutes of introduction, the selected saposins were incubated for about 20-30 minutes before collection of any fluorescence measurements. The vesicles had ~200 nm diameters (N4 + particle sizer). Using $^{31}$P-NMR spectroscopy in the presence of Mn$^{2+}$ quenching (40), lipid dispersions of egg PC in sodium acetate (pH 4.7) with vortex mixing, showed 16-28% $^{31}$P quenching by addition of MnCl₂ (5 mM). Since the $^{31}$P in PC would be distributed to inaccessible sites within multilamellar liposomes (inner leaflet and internal concentric bilayers), the quenching percentage would be expected to be significantly less than 50%. In comparison, egg PC liposomes prepared by the above sonication procedure led to a ~ 50% reduction in $^{31}$P signal upon addition of Mn$^{2+}$. These results are consistent with those expected for unilamellar vesicles.

_Saposin Activation Assays_ — The saposin activities toward acid β-glucosidase were determined fluorometrically (15,31). Assays were conducted in a detergent-free system with
phosphatidylserine liposomes. The protein-to-lipid ratios were 1:20 (mole/mole) unless otherwise indicated.

*Trp Fluorescence and Quenching Experiments* (31) — The intrinsic fluorescence of proteins is contributed mainly from tryptophanyl (Trp) residues. Tyr and Phe in the saposins contributed <10% of total fluorescence to the measurements. Singly substituted saposins (Trp) were created by site-directed mutagenesis. Fluorescence emission spectra were acquired by scanning (300 to 400 nm) in an SLM-Aminco Bowman Series 2 luminescence spectrometer (Urbana, IL). Excitation wavelength (λ_{EX} = 280 nm), and spectral bandwidths (4 nm) were used for the excitation and emission monochromators. Saposins (2 μM) were added to liposome suspensions at protein-to-lipid ratios of 1:20 (mole:mole). Liposomal dispersions had no fluorescence under these conditions. The associations between saposins and phosphatidylserine vesicles were determined by fluorescence quenching analysis. The aqueous quencher, acrylamide, experiments were conducted for at least 15 min. For estimating the depth of saposin penetration into the liposomal membranes, tryptophan fluorescence quenching was evaluated in the presence of liposomes containing various mole% of spin-labeled phosphatidylcholine (SLPC). The individual quenching conditions are described in the figure legends.

*Fluorescence Resonance Energy Transfer (FRET)* — FRET spectroscopy was conducted with vesicles containing PS with a dansyl-labeled head group (DPS). Donor-reporter pair fluorescence formed in saposin-DPS vesicles was analyzed by FRET. Fluorescence emission spectra were acquired from 300 to 550 nm with λ_{EX} = 280 nm. The protein:lipid ratios were 1:10 (mole:mole). The inner filter effects, if any, were corrected as follows:
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\[ F_{corr} = F_{obs} \text{ antilog } \frac{(OD_{ex} + OD_{em})}{2} \]

\( F_{corr} \) and \( F_{obs} \) are the corrected and observed fluorescence, respectively. \( OD_{ex} \) and \( OD_{em} \) are optical densities of the samples at both the excitation and emission wavelengths.

**Circular Dichroism (CD) Measurements** — The secondary structures of saposins with or without phospholipid vesicles were estimated from the CD spectra and recorded at room temperature from a Jasco J-710 spectropolarimeter (Jasco Inc., Easton, MD) using a 0.1 cm quartz cell. The reaction mixtures were in 0.005 M sodium citrate/0.01 M sodium phosphate, pH 4.7, to reduce noise at wavelength below 200 nm. Data acquisition and deconvolution calculations were done as described (31,41). The deconvolution programs were from Jasco with reference to an average of seven X-ray structures of known proteins, i.e., myoglobin, lysozyme, papain, ribonuclease, hemoglobin, \( \alpha \)-chymotrypsin A, and cytochrome \( c \). The calculations provided estimations of secondary structure of saposins as \( \alpha \)-helix (% \( \alpha \)), \( \beta \)-strand (% \( \beta \)), \( \beta \)-turn (% \( T \)), and other structures (% \( R \)). The calculations of precise secondary structures may require more reference protein sets or other deconvolution methods. These experiments were conducted to predict relative changes in CD spectra, and the results do not necessarily reflect the exact secondary structures of saposins. The CD spectra of saposins in the absence or presence of phospholipids were acquired under the same conditions. No significant CD spectra were detected from liposome samples under our experimental conditions.
Membrane Interactions of Saposins — To determine the membrane interactions of saposins, fluorescence emission shifts, fluorescence quenching, and FRET were conducted with phospholipid-containing unilamellar vesicles. Individual tryptophans (W) were created site-directed mutagenesis and were termed saposin (XW) to indicate the position (X) of the tryptophanyl residue in mature amino acid sequence of each saposins. For example, wild-type saposin A contains a Trp at residue 37, i.e., saposin A (37W). This Trp was substituted with Phe in saposin A having differently substituted Trp residues. The amino acid sequences of saposins A and C are in Figure 1B. Compared to normal saposin A, saposin C (S37W) was generated by substitution of a Trp for serine(s) at residue 37. Saposins A (0W) or C (0W) had Trps preceding the first NH$_2$-terminal amino acid in the saposin A or C sequences, respectively. Saposins A (81W) or C (81W) had Trps following the last native COOH-terminal amino acid. All Trp-substituted saposins retained their native activation properties.

Trp fluorescence emission spectra of proteins were monitored by the change in maximum emission wavelength and intensity since the tryptophanyl environments change polarity (42). To assess the polarity shifts, the fluorescence emission spectra of Trp-saposins were obtained upon addition to suspensions of phospholipids liposomes. The fluorescence emission spectra of saposins A (0W/W37F), (37W), and (81W/W37F), or saposin C (0W), (S37W), and (81W) were obtained in the presence of brain phosphatidylserine (BPS) liposomes. Maximal emission wavelength shifts to the blue direction were noted for the Trp substituted saposins compared to those in a lipid-free background (Table I). This parameter for saposin C (S37W) did not change.
Fluorescence intensities also were increased significantly upon Trp-saposin addition to BPS or synthetic PS (18:1,1) liposomes at acidic pH (Fig. 2). These blue-shifts and intensity elevations suggested interaction of saposins with liposomal membranes during protein-lipid complexes formation. None of the Trp-saposin As or Cs showed blue-shifts with the neutral egg phosphatidylcholine (EPC) or saturated phosphatidylycerine (PS) liposomes. These results also show that the electrostatic interactions of the saposins with the charged head groups were insufficient to account for the respective spectral shifts. In the absence of phospholipids, saposin A (37W) showed the same maximal emission wavelength ($\lambda_{EM} = 351$ nm) as saposin C (S37W). This indicated that the polarity of middle regions in saposins A and C are similar in lipid-free solution.

Fluorescence quenching experiments of Trp-saposins by aqueous (polar) or hydrophobic solid (nonpolar) quenchers were conducted to determine whether saposins interacted with BPS liposomal membrane at the surface or by insertion. Aqueous fluorescence quenchers, acrylamide, were used to evaluate the accessibility of the tryptophanyl residue in saposins to the aqueous phase in the presence of BPS liposome (Fig. 3A). With the liposomes composed of BPS, the blue-shifts of the Trp-saposin fluorescence were protected effectively from quenching by acrylamide. However, saposin C (S37W), that did not have spectral shift, was largely quenched. In the absence of liposomes, all Trp-saposins were quenched to the same degree and the degree of quenching was much greater than in the presence of BPS. Together with spectral shifting experiments, the results indicated that the middle region of saposin C around 37S was exposed to the aqueous phase while the amino- and carboxyl-terminals regions were associated with membrane lipids. In comparison to saposin C (S37W), wild-type saposin A (37W) was
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quenched much less by acrylamide in the presence of BPS vesicles (Fig. 3A) (Table I). In the presence of BPS, saposins A (0W/W37F) and A (81W/W37F) displayed similar acrylamide quenching patterns. These results indicate that the NH2 and COOH termini of saposins A and C, and also, the middle region of saposin A were within the lipid environment, i.e., not aqueous accessible.

The penetration of saposins into the BPS membrane was confirmed using spin-labeled phosphatidylcholines (SLPCs). Various percentages of SLPCs (0-50%) were incorporated into BPS liposomes. The SLPCs contain doxyl groups which are located at different carbons (n) in the acyl chain: SLPC5 (n=5), SLPC10 (n=10), and SLPC16 (n=16). After addition of Trp-saposins, the protein/lipid mixtures were incubated at room temperature for 60 min, and the fluorescence intensity changes were determined. The results from the longer incubation time showed no significant differences. Substantial quenching efficiencies (30-60%) were obtained in the presence of BPS/SLPC5 liposomes for the Trp-saposins with blue shifts. For example, the Trp fluorescence of saposin C (0W), A (37W), or C (81W) was quenched 60% with BPS/SLPC5 (1:1 mole/mole) (Fig. 3B). Saposin C (S37W) did not develop spectral shifts and showed no quenching with any of the SLPCs. The quenching efficiency also was dependent upon the location of the doxyl group in the acyl chain. Less quenching effect was observed when the doxyl group was located deeper in the membrane, e.g., with BPS/SLPC10 (1:1 mole/mole) the tryptophanyl fluorescence of saposin C (0W) was quenched by only 30 % (Fig. 3C). No significant quenching was observed with BPS/SLPC16. The SLPC5 was 5 to 20-fold more effective than SLPC10 or SLPC16. These results are consistent with the NH2 and COOH ends of saposin A and B being within the liposomal membrane. The middle region of saposin A also was
embedded in the membrane to a depth of ~5 carbon bond lengths. None of the Trp-saposin Cs were quenched by EPC/SLPC liposomes (Fig. 3D).

FRET quenching was used to evaluate the proximity of the aqueous accessible region of saposin C to the membrane surface. Dansylated lipids have been used to study the interfacial interaction of proteins and lipids, because the intrinsic fluorescence energy of tryptophanyl residues in proteins can be transferred to dansyl-moiety (42). The fluorescence of dansylated macromolecule is sensitive to the polarity of the microenvironment and the nature of the conjugate molecules. Within environments of low or medium polarity, blue-shifts of the emission spectrum of dansyl-compounds are more sensitive than the quantum yield changes.

Saposin C (S37W) was added into mixed BPS/DPS liposomes for FRET analysis (Fig. 4). The emission spectrum of Trp in saposin C and dansyl-groups with BPS/DPS vesicles showed \( \lambda_{EM} \) at 351 and 503 nm (Fig. 4, lines A and B), respectively. FRET of saposin C (S37W) associated with the BPS/DPS membrane is shown in Figure 4 (line C). The decreased intensity of saposin C (S37W) was accompanied by an enhanced quantum yield of the DPS at \( \lambda = 503 \) nm. This indicated that the quenching of tryptophan fluorescence was due to FRET by DPS rather than a collision process. The Forster distance (\( R_0 \)) of the Trp-dansyl pair is ~21-24 Å (42). Therefore, the effective energy transfer of Trp-to-DPS suggested a close proximity of the middle region of saposin C to the surface of lipid membrane. Usually, two different types of protein-lipid-water forces, electrostatic and hydrophobic, have been considered for protein-lipid interactions. In hydrophobic phases the microenvironment of the dansyl-probe is less polar than in those of electrostatic type (43). This is supported by largely increasing of quantum yield and blue-shift of the emission spectrum involved in hydrophobic contact between protein and lipid. Since no
blue-shift of the DPS emission spectrum occurs from FRET, the region of saposin C at residue 37 should be in the protein-lipid-water phase of electrostatic type rather than in direct contact with the lipids.

**Effects of Phospholipids on Saposin Conformation** — Saposin A and C had different topological associations with BPS liposomal membrane. The concordant conformational changes also were studied by CD spectroscopy (Table II). The CD spectral changes in saposin C were obtained with PS (18:1,1) at pH 4.7. A relative increase in the α-helix content and a relative decrease the β-strands were observed (Table II, Fig. 5). Deconvolution of the spectra for the PS (18:1,1)/saposin A, PS (18:0,0)/saposin C or PC (18:1,1)/saposin C complexes revealed no significant changes. These results indicate that the conformational changes of saposin C upon binding to the lipid membrane were different from those with saposin A. The results also showed that the acidic (unsaturated) phospholipid-induced conformational alterations correlated with the ability of the phospholipid to enhance saposin C’s activation of acid β-glucosidase.

**Membrane Interaction and Function Correlation of Saposins** — The importance of the highly conserved Q48/E49 residues in saposin C was investigated by site-directed mutagenesis and functional analyses (44). Singly mutated saposin C (Q48N) and the doubly mutated saposin C (Q48A/E49A) have no stimulation effects acid β-glucosidase activity. To evaluate the membrane binding properties of the mutant saposin Cs, a Ser 37 to Trp was created as an intrinsic fluorescence probe on the backbone of saposins C (Q48N) and (Q48A/E49A). The resultant saposin Cs had similar defective activation functions as the S37 variants. In contrast to saposin C (S37W), the dysfunctional saposins C (S37W/Q48N) and C (S37W/Q48A/E49A) did exhibit blue-shifts of 6 and 9 nm (Table I), respectively, in the presence of BPS. This result
indicates that the Q48N and Q48A/E49A substitutions alter the interactions of saposin C with membranes and that these alterations are associated with a loss of activation function. In the absence of BPS, the $\lambda_{EM}$ of saposin C (S37W) was 6-13 nm higher than for the additionally mutated saposins (Table I). These spectral shifts of the multiply mutated saposin Cs indicate that the microenvironments surrounding the Trp had different polarities. Interestingly, CD spectra were similar to the native sequence in the absence of phospholipids (Table II). The BPS-induced relative secondary structural changes were greater with saposin C (Q48N) and C (Q48A/E49A) than with wild-type saposin C. These CD spectra changes paralleled the blue-shifts observed with the respective mutated saposin Cs in the presence of various BPS.

Our previous enzyme activation studies highlighted the importance of the COOH region and residues 47 to 60 of saposin C for (44). The residues 47 to 60 in saposin A are highly similar to the corresponding residues in saposin C (Fig. 1B, heavy line). Importantly, saposin A competes with saposin C for the binding sites on the enzyme at nmolar concentrations (15). However, nmolar concentrations of saposin A have no activation effects on the enzyme. The differential membrane interaction of saposin A and C indicated that the orientation of the corresponding domains in membrane-bound saposin A was not same as those in saposin C. Thus, the "activation" domains of saposin A may have poor accessibility to the enzyme leading to poor saposin A activation of the enzyme. To improve the orientation of activation domains, the predicted non-helical sequence KGEMSR (63-68) in saposin A was mutagenized. For these analyses, native saposin A was substituted to a more "saposin C-like" (LEEVSP) sequence to give a saposin A with the following residues, K63L, E64G, M66V. High concentrations of native saposin A ($\mu$M) are needed to attain maximal activation on acid $\beta$-glucosidase in vitro
using phospholipids assays (15). The mutant saposin As (G64E) and (K63L/G64E/M66V) produced, after a lag phase, about 70-90% of the acid β-glucosidase activation as the wild-type saposin C (Fig. 6). By fluorescence spectroscopy, the increase in enzymatic activation by the mutated saposin As correlated with significant red-shifts in emission spectra in the presence of BPS (Table I). These red-shifts (from W37) suggest that the middle regions of mutant saposin As had become exposed to more polar environments upon binding of BPS membranes. Thus, the membrane topology changes from that of normal saposin A to one that was more similar to that of saposin C and leading to development of the enzyme activation property.
DISCUSSION

The present experiments demonstrate the differential membrane interactions of saposins A and C. Native saposin C was engineered to contain specifically placed Trps as intrinsic fluorescence probes in the amino- or carboxyl- termini and middle regions. These substitutions did not alter the activation properties of these saposin Cs toward acid β-glucosidase. By fluorescence emission and quenching analyses, the saposin C amphipathic helices 1 and 5 inserted, at acidic pH, into outer-leaflet of particular negatively charged phospholipid membranes to a depth of about 5 carbon bonding lengths. A two step binding model is suggested in which saposin C has an initial interaction mediated by electrostatic effects and a second hydrophobic-interaction step that leads to insertion of the helices into lipid bilayer. This membrane binding leads to a steady-state conformational change in saposin C as assessed by CD spectroscopy. Clearly, the middle region of saposin C (S37W) is exposed to the aqueous phase as it is accessible to aqueous quenchers. Compared to saposin C, the middle region of saposin A is within the membrane environment and at a similar depth as helices 1 and 5. A model is proposed to account for the differences in saposin A and C activities vis-a-vis acid β-glucosidase (Fig. 7A and B). These models were derived from the non-glycosylated and recombinant saposins, and oligosaccharides attachment may alter some of the proposed interactions between protein and lipids. Since the region between helices 1 and 2 of saposin C is solvent exposed and contains the glycosylation site, the proposed model likely would not be greatly affected. However, the additional oligosaccharide moiety on saposin A is located in a region that might be expected to have unfavorable effects on its incorporation into the membrane to the depth proposed in the model.
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(Fig. 7B). This could make saposin A more poorly lipid-associated as observed by Vaccarro and colleagues using natural saposin A and phospholipid liposomal membranes (38).

Of the prosaposin derived saposins, saposin A shows the highest amino acid identity/similarity to saposin C. In general, the saposin fold has a structure with five amphipathic \( \alpha \)-helices folded into a single globular domain (17,18). The presentation of the fold is along a centrally located helix at amino-terminal (helix 1), against which helices 2 and 3 are packed from one side and helices 4 and 5 from the other side (Fig. 1A). This fold has been suggested to provide the primary interface for membrane interaction. CD and FTIR analyses indicate that the lipid-bound NK-lysin retains the saposin fold and that the amino- and carboxyl-termini of NK-lysin are in proximity to the middle region (22). This region localizes to the lower half of the globular saposin fold, which may be embedded into the lipid bilayer. The other half of NK-lysin has the negatively charged residues that are exposed to solvent. Our results with saposin A/BPS complexes indicate a similar structural orientation (Fig. 7A). In comparison, quenching of saposin C (S37W) by acrylamide, supports orientation of the middle region toward the solvent environment. With saposin C, the CD spectral changes upon binding show a reorganization and reorientation of the helices in saposin fold upon membrane interaction.

Electrostatic interaction between saposins and membrane is proposed to be the first step of saposin/lipid binding. The negatively charged amino acids (Glu and Asp) in saposins A and C are distributed though the whole sequence. However, the positively charged side chains of Lys and Arg are distributed differently in saposins A and C. In saposin C, all Lys residues are consolidated at 50% of amino-terminal half. These positively charged groups are important in
recognizing the negative surface of phosphatidylserine containing membrane for initial binding. To accompany the amphipathic insertion, negatively charged clusters located at 50% of carboxyl-terminal half in molecule may mainly repulse the same charged membrane surface. This repulsion triggers alteration of saposin fold and reorientation of helices in saposin C. While Lys and Arg in saposin A are evenly located in peptide sequence. Similar to NK-lysin, the rings of these residues in an equatorial belt of saposin A are likely to form a protein/lipid interface with the negatively charged surface of membrane. The non-conserved charge distribution in saposins A and C suggests the different lipid-binding properties and membrane interactions. Mutant saposins C (Q48A/E49A) and A (G64E) and were created to evaluate the effects of negatively charged side chains within the carboxyl-terminal half on the membrane interaction and activation function. Amino acids Glu$^{49}$ in saposin C and Gly$^{64}$ in saposin A are conservative residues across various of species, including human, mouse, rat and guinea pig. Reduction of one negatively charged side chain in saposin C (Q48A/E49A) leads to an inactive molecule (44). Addition of negatively charged residue in saposin A (G64E) produced a protein with increased activation effects on the enzyme. In contrast to wild-type saposins, the mutant saposins showed alteration of the lipid binding properties as determined by the pattern of changes of the fluorescence emission spectra, as well as on the CD spectra. These experiments imply the important role of highly conserved negatively charge residues at 50% of COOH-terminal half of molecule in lipid-binding processes.

The His-tag of recombinant saposins are positively charged at acidic pH. However, neither fluorescence emission shifts nor CD spectra changes were observed with Trp-free or Trp-saposin C in the presence of saturated phosphatidylserine membrane. This indicated that the (His)$_6$ tail
on carboxyl terminal of saposins was not important for the insertion of saposins into phospholipid membranes. There is a possibility that the His-tag might could have a minor impact on the saposin/membrane interactions. In our assay systems, this possible impact should be same for saposin A and C, and, thus would not affect our conclusions.

Saposins A and C have highly identical/similar sequences and conserved α-helical domains. However, saposin A is not a significant physiological activator of acid β-glucosidase. Previously, our *in vitro* data showed activation of acid β-glucosidase by saposin A at µM levels whereas saposin C's effects were at nM levels (15). The present studies show a direct correlation of activation functions and orientation of the saposins A and C in the phospholipid membranes. Saposin C (S37W, Q48N) and (S37W, Q48A/E49A) were inactive proteins probably due to the loss of a turn structure in the TYG (aa 53 to 55) region between helices 3 and 4 (44). Fluorescence and CD spectral analyses also show different BPS induced membrane conformations in these mutant saposin Cs compared to the wild-type. To evaluate the relationship of these changes to activity, mutant saposin As were generated that preserved the helical domains for activation, but the non-helical sequence, KGEMSRP, between helices 4 and 5 was mutagenized selectively to be more "saposin C-like" LEEVSP. At nmolar concentrations, saposin As (G64E) and (K63L/G64E/M66V) activated acid β-glucosidase to nearly the same levels as wild-type saposin C. The apparent \( K_d \) (activation) for these mutants were decreased over 20-fold compared to wild-type saposin A, but was about twice that of wild-type saposin C. The significant red shift in \( \theta_{EM} \) max of these mutant saposin As indicates a dramatically increased polarity of the solvent surrounding the tryptophan residue (W37) in saposin A upon binding to lipid bilayer. These results indicate that helices 2 and 3 in the mutated saposin As are
more solvent exposed, and more closely resemble those in saposin C. Thus, the solvent exposure of the region between helices 2 and 3 in saposin C is directly related to its activation potential and lack of such exposure explains the poor activity of saposin A in this assay. The results showed that the membrane-association modes of saposins A and C are the determinants of their specific biological functions. An extension of this observation implies that the diverse functions of saposins or saposin-like proteins result from differential orientations of their helices with respect to lipid membranes.

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Saposins Membrane Interaction

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FIGURE LEGENDS

Figure 1. Amino acid sequences of human NK-lysin, and saposins A and C. (A) NMR model of NK-lysin with the amino acid sequence below. (B) The sequences of saposins A and C show conservation of the 6 cysteines (boldface C) and the first N-glycosylation consensus sequence (*). The second N-glycosylation site in saposin A also is occupied. The heavily underlined residues, 48-62 in saposin C, encompass the activation region for acid β-glucosidase. The region from residues 63-69 (underlined in saposin A) is a non-helical structure. The residues K63, G64, and M66 of saposin A were substituted by the corresponding amino acids L63, E64, and V66 from saposin C, respectively. The predicted α-helical regions and others are indicated: LBR: lipid binding region. NR: neuritogenic region. AR: activation region.

Figure 2. Emission spectra of saposin C (0W) in the absence or presence of BPS. The spectra were acquired at λ_{EX} = 280 nm in 0.1 M sodium citrate/ 0.2 M sodium phosphate, pH 4.7, at room temperature. The protein:lipid (P:L) ratios were 1:0, 1:1, 1:3, 1:5, 1:10, and 1:20 as indicated. Saposin concentration was 2 µM.

Figure 3. Fluorescence Quenching of Trp-saposin Cs with water-soluble (A) or lipid (B, C, D) agents. (A) Aqueous quenching by acrylamide of Trp-saposins (2 µM) fluorescence was conducted in the absence (open symbols) and presence (filled symbols) of BPS (20 µM). In Stern-Volmer plots, squares, triangles, diamonds,
and circles are saposins C (0W), C (S37W), C (81W), and A (37W), respectively. (B) (C) and (D) [inverse Stern-Volmer plots for quenching of Trp-saposins with spin-labeled phosphatidylcholine (SLPC)]. (B) the quenching of saposins C (S37W) (○), C (81W) (▼), A (37W) (■) with various mole % of 5-doxyl-SLPC in BPS liposomes. (C) Quenching of saposin C (0W) with (5-doxyl) (■), (10-doxyl) (▼), and (16-doxyl) (●) SLPC in PBS. (D) Quenching of saposins C (0W) ( ), C (S37W) (~), C (81W) (O), and A (37W) ( ), S37W with (5-doxyl)-SLPC in EPC. Protein:lipid = 1 : 20 (mole:mole), pH 4.7, room temperature.

Figure 4. **Evidence for FRET between Trp-saposin C and dansyl-PS.** The emission spectra of saposin C(S37W) in BPS (line A), BPS/DPS(1:1, mole:mole) liposomes (line B), and saposin C(S37W) and BPS/DPS mixture (line C). Experimental conditions: protein:lipid = 1 : 10 (mole:mole), Saposin concentration = 5 µM, pH 4.7, room temperature, λex = 280 nm.

Figure 5. **CD spectra of saposin C in the absence and presence of phospholipids.** (A) No phospholipids; (B) phosphatidylserine (18:0,0); (C) phosphatidylserine (18:1,1). Spectra were acquired in 0.005 M sodium citrate/0.01 M sodium phosphate, pH 4.7, at room temperature. Saposin C and phospholipid concentrations were 0.1 mg/ml and 17 µM, respectively.

Figure 6. **Activation of acid β-glucosidase by recombinant saposin C and As.** Assays were conducted in the presence of 0.4 nM purified acid β-glucosidase, 0.4 µg/ml
BPS, and various concentrations of saposins in 0.05 M sodium acetate/0.1 M sodium phosphate, pH 4.7. The fold-change refers to the ratio of final to initial activity. (■) wild-type saposin C, (●) wild-type saposin A, (▼) saposin A (G64E), and (▲) saposin A (K63L/G64E/M66V).

**Figure 7. Hypothetical Models of Membrane Interactions of Saposins A and C.**

Phospholipid bilayers contain acidic PS. Cylinders are presented the amphipathic helices in saposin A and C. (A) Membrane topological structure of saposin A: saposin fold structure of saposin A dips into the negatively-charged phospholipid membrane. (B) Membrane topological structure of saposin C: a conformational alteration of the saposin fold found in lipid-bound saposin C. The amphipathic helices at amino- and carboxyl-termini of saposin C inserted into the membrane. The middle region of saposin C is exposed to aqueous phase.
**TABLE I**

*Fluorescence Emission Maxima of Trp-saposins in the Absence and Presence of BPS*

Experimental conditions: pH 4.7, protein:lipid = 1:20 to 40 (mole:mole). The same results were observed at 22° or 37°C.

| Saposins                  | Emission Maxima (EM, nm) | EM Shift |
|---------------------------|--------------------------|----------|
|                           | -BPS | +BPS |          |
| A (0W/W37F)               | 345  | 333  | Blue     |
| A (37W)                   | 351  | 338  | Blue     |
| A (37W/G64E)              | 344  | 358  | Red      |
| A (37W/K63L/G64E/M65V)    | 339  | 350  | Red      |
| A (81W/W37F)              | 345  | 336  | Blue     |
| C (0W)                    | 339  | 333  | Blue     |
| C (S37W)                  | 351  | 351  | No       |
| C (S37W/Q48N)             | 345  | 339  | Blue     |
| C (S37W/Q48A/E49A)        | 338  | 329  | Blue     |
| C (81W)                   | 339  | 323  | Blue     |
TABLE II.

Circular Dichroism (195-250 nm) Analyses of Saposins with Various Phospholipids

Experiments conducted in 0.005 M sodium citrate/0.01 M sodium phosphate, pH 4.7, 0.1 mg of protein/ml and room temperature.

| Saposins | Phospholipids | % α  | % β  | % T  | % R  |
|----------|---------------|------|------|------|------|
| A        | None          | 44.0 | 31.9 | 0.0  | 24.1 |
| A        | PS (18:1,1)   | 39.3 | 34.9 | 0.5  | 25.4 |
| C        | None          | 29.9 | 41.7 | 0.0  | 28.4 |
| C        | PS (18:0,0)   | 30.1 | 40.4 | 1.4  | 28.1 |
| C        | PC (18:1,1)   | 30.6 | 41.0 | 0.0  | 28.4 |
| C        | PS (18:1,1)   | 49.8 | 3.9  | 14.0 | 32.4 |
| C (Q48N) | None          | 31.9 | 36.0 | 3.9  | 28.2 |
| C (Q48N) | PS (18:1,1)   | 67.5 | 0.0  | 32.5 | 0.0  |
| C (Q48A/E49A) | None       | 30.6 | 45.6 | 0.0  | 23.7 |
| C (Q48A/E49A) | PS (18:1,1) | 74.4 | 0.0  | 14.6 | 11.0 |
Figure 1. (Qi & Grabowski)

(A) NK-lysin

GYFCESCRKIIQKLEDVGPQPNEDTVQAASQVCDKLKLRLGKIMRSFLRIRSWDILTGKIPQAICVDIKICKE

(B) Saposins

S-LPCDKVTAAGMLKDNATEEIELVYLEKTCDWLPNMSASCKEIVDSYLPMILDIHKGEMSVPGESALNLCSG

SDVYCEVCEFLVEVTKLIDNNKTEILDAFDKMCSELVATSEECQEVDTYSIILSILLEEVESPLELVCMLHLC

Helix 1  Helix 2  Helix 3  Helix 4  Helix 5
Figure 2. (Qi & Grabowski)
Figure 3A (Qi & Grabowski)
Figure 3B. (Qi & Grabowski)
Figure 3C. (Qi & Grabowski)
Figure 4. (Qi & Grabowski)
Figure 5. (Qi & Grabowski)
Figure 6. (Qi & Grabowski)
Figure 7. (Qi & Grabowski)

(A) saposin A

(B) saposin C

negatively charged phospholipid bilayer
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