

Saposin B binds and transfers phospholipids

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Abstract Saposin B (Sap B) is a member of a family of four small glycoproteins, Sap A, B, C, and D. Like the other three saposins, Sap B plays a physiological role in the lysosomal degradation of sphingolipids (SLs). Although the interaction of Sap B with SLs has been investigated extensively, that with the main membrane lipid components, namely phospholipids and cholesterol (Chol), is scarcely known. Using large unilamellar vesicles (LUVs) as membrane models, we have now found that Sap B simultaneously extracts from the lipid surface neutral [phosphatidylcholine (PC)] and anionic [phosphatidylinositol (PI)] phospholipids, fewer SLs [ganglioside GM1 (GM1) or cerebroside sulfate (CS)], and no Chol. More PI than SL (GM1 or CS) was solubilized from LUVs containing equal amounts of PI and SLs. An increase in PI level had a poor effect on the Sap B-induced solubilization of GM1 or CS but strongly inhibited that of PC. Sap B was able not only to bind, but also to transfer phospholipids between lipid surfaces. Both the phospholipid binding and transfer activities were optimal at low pH values. These results represent the first biochemical analysis of the Sap B interaction with phospholipids. The capacity of Sap B to bind and transfer phospholipids occurs under conditions mimicking the interior of the late endosomal/lysosomal compartment and thus might have physiological relevance.—Ciaffoni, F., M. Tatti, A. Boe, R. Salvioli, A. Fluharty, S. Sonnino, and A. M. Vaccaro. **Saposin B binds and transfers phospholipids.** *J. Lipid Res.* 2006. 47: 1045–1053.

Supplementary key words phospholipid binding • phospholipid transfer • Saposin-membrane interaction

Saposin B (Sap B) is a member of a family of four small glycoproteins, Sap A, B, C, and D, generated in late endosomes/lysosomes from a single precursor, prosaposin (1, 2). Because of their ability to modulate the lysosomal enzymatic degradation of several sphingolipids (SLs), the saposins play an important role in the pathogenesis of sphingolipidoses, a group of lysosomal storage disorders characterized by SL accumulation (1, 2). Mutations af-

fecting the coding region of Sap B cause a variant form of metachromatic leukodystrophy with lysosomal storage of cerebroside sulfate (CS) (3, 4).

The structural and functional properties of Sap B have been the focus of several studies. Sap B, like the other three saposins, consists of ~80 amino acids, including six cysteines (2), forming three disulfide bridges (5). In solution, Sap B is present as a dimer, which gives rise to higher order aggregates on increasing Sap B concentration (6, 7). Crystallization has confirmed the dimer structure of Sap B, which consists of two clasped V-shaped monomers (8).

Because the lack of Sap B leads to the storage of SLs, especially CS (3, 4), the interaction of the saposin with SLs has been investigated extensively. It was shown that Sap B is able to solubilize in vitro several SLs such as CS, ganglioside GM1 (GM1), and globotriaosylceramide, whereas direct interaction with SL hydrolases has never been observed (9–12). It is thus assumed that the physiological function of Sap B is mainly related to its capacity to favor the degradative action of water-soluble SL hydrolases by binding and solubilizing SLs from lysosomal membranes. The resulting Sap B-SL complexes are more accessible substrates for the enzymes.

It should be noted that several experiments concerning the in vitro interaction between Sap B and SLs have been performed with micellar dispersions. The crucial point to understand about the actual physiological function(s) of Sap B from lipid binding experiments is that the saposin behaves in vitro as it would in vivo. To elucidate in more detail the lipid binding properties of Sap B, we have reexamined its interaction with lipids by inserting SLs into models of biological membranes such as large unilamellar vesicles (LUVs) composed of phospholipids and chole-

Abbreviations: Chol, cholesterol; CL, cardiolipin; CS, cerebroside sulfate; GM1, ganglioside GM1; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Sap B, saposin B; SL, sphingolipid; SUV, small unilamellar vesicle.

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terol (Chol), the main components of the membranes where SLs reside. Special attention has been paid to the Sap B interaction with anionic phospholipids, because they are very abundant in the late endosomal/lysosomal compartment (13) where saposins also are localized. Anionic phospholipids have been shown to be the target of two other saposins, Sap C and Sap D (14–16). Moreover, anionic phospholipids have been reported to inhibit the formation of the Sap B-CS complex, a demonstration of the capacity of Sap B to interact also with phospholipids (17, 18). The results presented here reveal that in the presence of vesicles mimicking the lipid composition of biological membranes, Sap B preferentially interacts with phospholipids, especially anionic phospholipids.

MATERIALS AND METHODS

Phosphatidylcholine (PC) from egg yolk, GM1 from porcine brain, phosphatidylinositol (PI) from bovine liver, phosphatidylethanolamine (PE) from chicken egg, and cardiolipin (CL) from bovine heart were from Avanti Polar Lipids, Inc. (Alabaster, AL). Chol was from Sigma. PC, 1,2-di[1-¹⁴C]palmitoyl (110 mCi/mmol), and Chol[7(n)-³H] (7 Ci/mmol) were from Amersham Biosciences (Buckinghamshire, UK). CS, bovine (stearoyl-sodium salt), and CS (stearoyl 1-¹⁴C) (50 mCi/mmol) were from American Radiolabeled Chemicals, Inc. (St. Louis, MO). PI, 1- α -[myo-inositol-2-³H(N)] (8.5 Ci/mmol), cholesteryl oleate (oleate-1-¹⁴C) (55 mCi/mmol), and cholesteryl oleate [cholesteryl-1,2,6,7-³H(N)] (60–100 Ci/mmol) were from Perkin-Elmer Life Sciences (Boston, MA). [³H]GM1 was prepared by labeling the ganglioside in the terminal galactose moiety (19). All other chemicals were of the purest available grade.

Sap B preparation

Human Sap B was purified from spleens of patients with type 1 Gaucher's disease according to a previously reported procedure (5); it involved essentially the heat treatment of a water homogenate followed by ion-exchange chromatography on DEAE-Sephacel, gel filtration FPLC on a Superdex 75 HR 10/30 column (Amersham Biosciences), and reverse-phase high-pressure liquid chromatography on a protein C4 column (Vydac). Sap B was also purified from pig kidney according to a published protocol (20). The purity of the Sap B preparations was verified by N-terminal sequence analysis, SDS-PAGE, and electrospray mass spectrometry (5).

Vesicle preparation

LUVs were prepared as reported previously (15, 16). Briefly, appropriate amounts of lipids dissolved in chloroform were mixed, and the solvent was evaporated under nitrogen. The resulting lipid films were dried overnight in vacuum desiccators. The dry lipids were dispersed by vortex-mixing in buffer A (2 mM L-histidine, 2 mM N-Tris Hydroxymethyl Methyl-2-Aminoethane Sulfonic Acid, 50 mM NaCl, and 1 mM EDTA, pH 7.4) followed by 10 cycles of freeze-thawing; finally, they were passed 21 times through two stacked 0.1 μ m diameter pore polycarbonate membranes in a Liposofast-Miniextruder (Avestin, Ottawa, Canada). The vesicles were supplemented with the specified labeled lipids. PC was supplemented with [¹⁴C]PC to a specific activity of 8.5 \times 10³ dpm/nmol. PI, GM1, Chol, and CS were supplemented with

[³H]PI, [³H]GM1, [³H]Chol, and [¹⁴C]CS, respectively, to a specific activity of \sim 24 \times 10³ dpm/nmol each.

Multilamellar vesicles (MLVs) were prepared by mixing appropriate amounts of lipids dissolved in chloroform and evaporating the solvent under nitrogen. The resulting lipid films were kept overnight under vacuum. The dry lipids were dispersed by vortex-mixing in buffer A. The suspension was blended by a vortex mixer until all of the aggregates were dispersed. The vesicles were centrifuged at 40,000 *g* for 15 min at 4°C. The supernatant was discarded, and the MLV pellet, gently resuspended in buffer A, was used for phospholipid transfer experiments (see below).

Small unilamellar vesicles (SUVs) were prepared from MLVs by sonication under nitrogen in a Branson B15 sonifier at MLVs for 10 min with a microtip at a power setting of 30 W (60 s sonication, 30 s pause). The preparation was centrifuged at 100,000 *g* for 30 min, and the supernatant was used for phospholipid transfer experiments (see below).

Gel-permeation experiments

To test the Sap B-induced solubilization of lipids, LUVs (50 nmol, total lipids) with or without Sap B (2 nmol) were incubated in 100 μ l of buffer B (10 mM acetate, 50 mM NaCl, and 1 mM EDTA, pH 4.2) at 37°C for 30 min. The reaction was arrested by adjusting the pH of the solution to 7.4 with diluted NaOH, and the mixture was applied to a Sepharose CL-4B column (1 \times 30 cm) preequilibrated and eluted at room temperature with buffer A. The flow rate was 0.5 ml/min. Fractions of 0.5 ml were collected. The lipid distribution was determined by measuring the radioactivity of the fractions. When two labeled lipids were present, double isotope counting conditions were adopted. To confirm the identity of the lipids eluted from the Sepharose CL-4B column, the fractions were extracted with chloroform-methanol (2:1, v/v) and analyzed by TLC on high-performance TLC plates (Silica gel 60; Merck). Plates were developed in chloroform-methanol-water (95:5:5, v/v/v). Radioactive lipids separated on TLC plates were visualized by autoradiography.

To determine the pattern of Sap B elution from the Sepharose CL-4B column, the fractions were concentrated with a Microcon-YM-3 centrifugal filter device (molecular mass cutoff, 3 kDa) by centrifuging at 13,000 *g* until all of the liquid except 100 μ l was passed through the filter. The presence of Sap B in each retentate was tested by SDS-PAGE analysis of an aliquot of the concentrated fractions. SDS-PAGE was performed with a 10% polyacrylamide gel (21). After electrophoresis, Sap B was detected with a silver-staining kit for proteins (SilverQuest™; Invitrogen, Carlsbad, CA).

Assay of Sap B intermembrane phospholipid transfer activity

The phospholipid transfer activity of Sap B was determined by measurement of intermembrane transfer of labeled phospholipids between donor SUVs and acceptor MLVs essentially as described by DiCorleto et al. (22–24). SUVs consisting of PI/PC (5:95) were supplemented with either [³H]PI or [¹⁴C]PC to a specific activity of 24 \times 10³ or 2 \times 10³ dpm/nmol, respectively. SUVs also contained a trace of cholesteryl oleate, labeled with either ¹⁴C or ³H. MLVs consisted of PC/PE/CL (70:25:5). The phospholipid transfer incubations contained, in a total volume of 400 μ l of buffer B, pH 4.5, 0.2 nmol of Sap B (unless specified otherwise), SUVs (20 nmol, total lipids), and a large excess of MLVs (1,600 nmol, total lipids). The phospholipid transfer was arrested by adjusting the pH of the solution to 7.4 with diluted NaOH. MLVs were pelleted by centrifugation at 40,000 *g* at 4°C for 15 min. SUVs were quantitatively recovered in the superna-

tant, as judged by the recovery of labeled cholesteryl oleate, a nontransferable marker (>98%). SUVs used for PI transfer evaluation were supplemented with [^{14}C]PC and [^3H]PI and [^{14}C]cholesteryl oleate. The decrease in the $^3\text{H}/^{14}\text{C}$ ratio after incubation mea-

sured the transfer of PI from SUVs to MLVs. SUVs used for PC transfer evaluation were supplemented with [^{14}C]PC and [^3H]cholesteryl oleate. The decrease in the $^{14}\text{C}/^3\text{H}$ ratio after incubation measured the transfer of PC from SUVs to MLVs. The

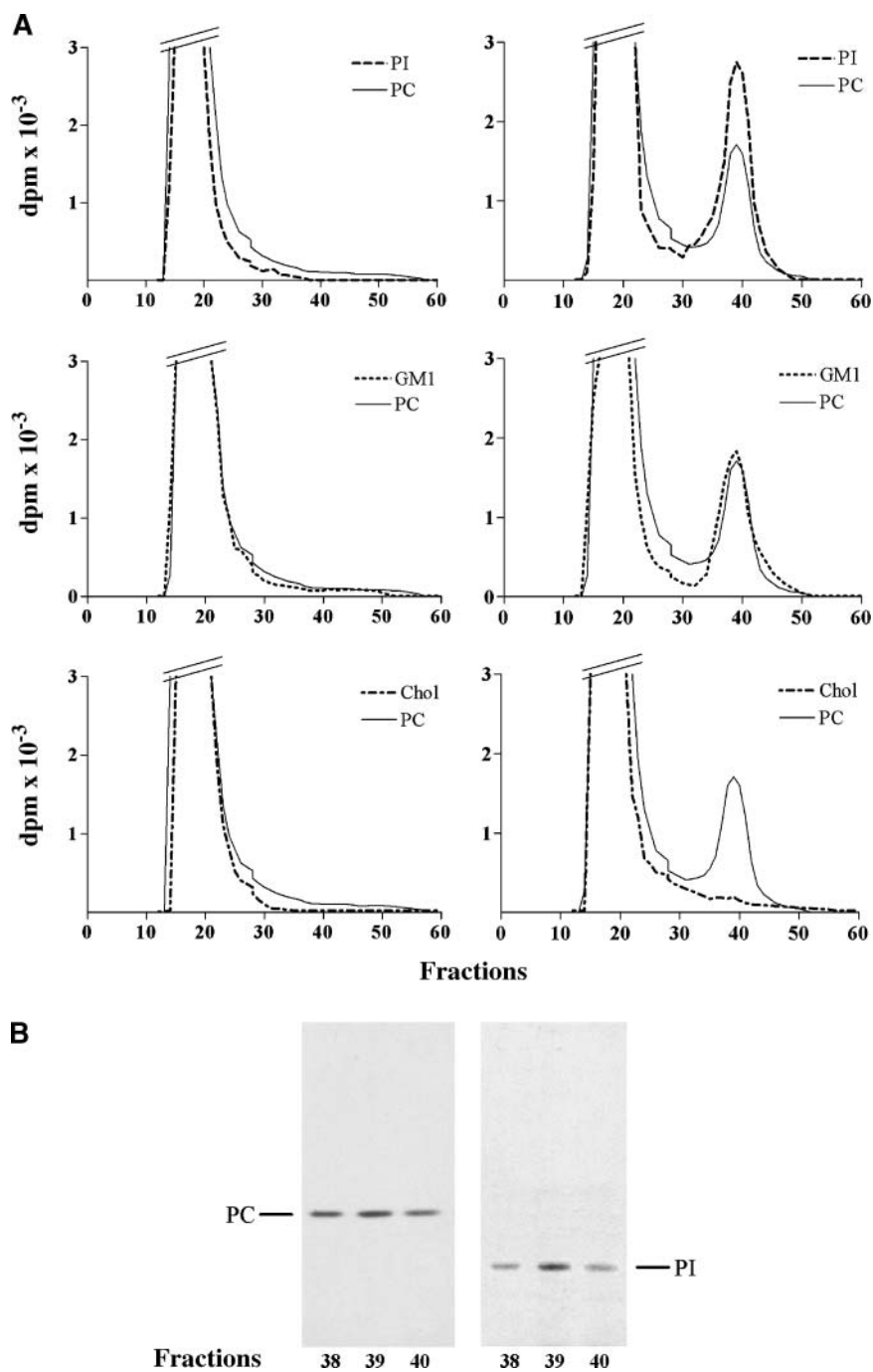


Fig. 1. Saposin B (Sap B)-induced solubilization of lipids assessed by gel permeation. A: Large unilamellar vesicles (LUVs) composed of phosphatidylinositol-phosphatidylcholine-cholesterol-ganglioside GM1 (PI/PC/Chol/GM1) (10:60:20:10; 50 nmol, total lipids) were incubated without (left panels) or with (right panels) Sap B (2 nmol) at pH 4.2, 37°C, for 30 min. The solutions were then chromatographed on a Sepharose CL-4B column, as described in Materials and Methods. The LUVs were supplemented with [^{14}C]PC and [^3H]PI (top panels), [^{14}C]PC and [^3H]GM1 (middle panels), or [^{14}C]PC and [^3H]Chol (bottom panels). The elution of lipids is given in terms of ^{14}C or ^3H dpm. B: LUVs composed of PI/PC/Chol/GM1 (10:60:20:10; 50 nmol, total lipids) were supplemented with either [^{14}C]PC or [^3H]PI, incubated with Sap B (2 nmol), and chromatographed on a Sepharose CL-4B column as described for A. Left panel, LUVs labeled with [^{14}C]PC; right panel, LUVs labeled with [^3H]PI. The specified fractions were extracted with solvents and analyzed by TLC to identify the labeled lipids as reported in Materials and Methods.

small amount of transfer (<2%) in the absence of Sap B was subtracted as background.

RESULTS

Sap B-induced solubilization of SLs and phospholipids from LUVs

The formation of water-soluble complexes between Sap B and SLs such as GM1 and CS has been assessed previously by a variety of methods, such as gel permeation, ultracentrifugation, and electrophoresis (7, 9, 12). In those experiments, the SLs were usually presented to Sap B as micelles. To better mimic physiological conditions, we examined the interaction of Sap B with SLs inserted into LUVs composed of phospholipids (neutral and anionic) and Chol. The formation of complexes between the lipid constituents of LUVs and Sap B was assessed by gel filtration on a Sepharose CL-4B column (Fig. 1). When LUVs containing 20% Chol, 60% PC, 10% GM1, and 10% PI, one of the main anionic phospholipids of lysosomal membranes (13), were chromatographed alone, a single

peak at the excluded volume (maximum at fraction 20) was observed. After incubation with Sap B, part of the LUV components were distributed in a second peak at lower molecular masses (fractions 37–41), presumably corresponding to the Sap B-lipid complexes (Fig. 1A). Unexpectedly, the second peak contained, besides GM1, PC and PI, indicating that Sap B was able to simultaneously solubilize SLs and phospholipids (neutral and anionic). Conversely, Chol was found only in the first peak, also after incubation with Sap B. To ensure that the radioactivity in the second peak represented phospholipid solubilization and not degradation, column fractions 38, 39, and 40 (the maximum of the peak) were extracted with organic solvents and chromatographed on thin-layer plates. As shown in Fig. 1B, the radioactivity was associated exclusively with the phospholipid labeled in the experiments (PC or PI).

The distribution of Sap B in the fractions from the Sepharose CL-4B column was assessed by electrophoretic analysis (Fig. 2). In the absence of LUVs, Sap B eluted as a broad peak between fractions 38 and 44. After incubation with LUVs, the Sap B elution was shifted slightly toward higher molecular masses (fractions 37–41) and

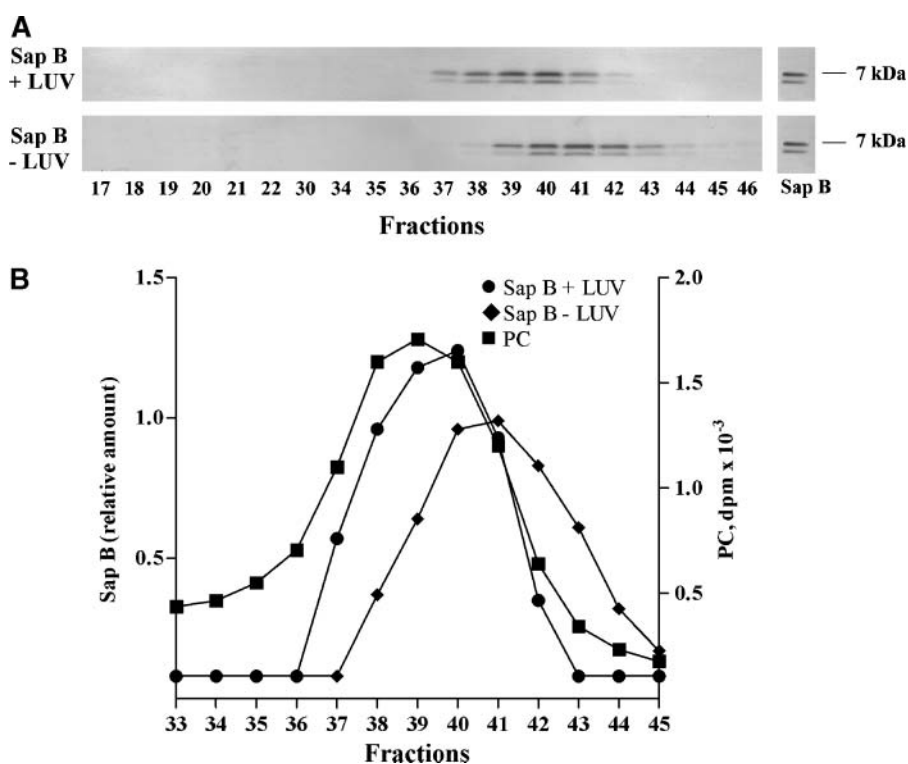


Fig. 2. Sap B elution from the Sepharose CL-4B column in the presence and absence of LUVs. A: Sap B (2 nmol) was incubated with (top panel) or without (bottom panel) LUVs composed of PI/PC/Chol/GM1 (10:60:20:10; 50 nmol, total lipids) at pH 4.2, 37°C, for 30 min. The solutions were then chromatographed on a Sepharose CL-4B column as described for Fig. 1A. LUVs were supplemented with [¹⁴C]PC. The presence of Sap B in the fractions was detected by SDS-PAGE, as described in Materials and Methods. Representative electrophoretic analyses of the fractions and of Sap B standard (0.5 μg) are presented. Molecular masses in kDa are indicated at right. B: The amount of Sap B in the fractions eluted from the Sepharose CL-4B column in the presence (closed circles) or absence (closed diamonds) of LUVs was evaluated by quantitative densitometric analysis of the Sap B bands shown in A. The relative amounts of Sap B were expressed relative to the signal obtained with 0.5 μg of Sap B, set arbitrarily as 1. The presence of lipids in the fractions was detected by PC radioactivity (closed squares).

coincided with that of the extracted lipids in the second peak (Fig. 2B). No Sap B was present in the fractions corresponding to LUVs (first peak). These results indicate that Sap B does not stably bind to the LUV surface and confirm the formation of the Sap B-lipid complexes.

From the gel-permeation experiments (Figs. 1, 2), the extraction of lipids was calculated as the proportion of radioactivity eluting in the second peak together with Sap B. **Table 1** shows that the incubation of 50 nmol of LUVs (PI/PC/Chol/GM1, 10:60:20:10) with 2 nmol of Sap B (the saposin exists as a homodimer) (8) resulted in the extraction of ~13% of the 5 nmol of PI (0.65 nmol), 8% of the 5 nmol of GM1 (0.42 nmol), and 4% of the 30 nmol of PC (1.10 nmol) initially present in the vesicles. The amount of phospholipids (neutral plus anionic) complexed by Sap B was higher than that of GM1. By comparing the Sap B-induced solubilization of PI and GM1 (both lipids were present in LUVs at the same concentration), it appears that PI is extracted more efficiently than GM1.

In these initial binding experiments, Sap B purified from either Gaucher spleen or pig kidney was used. The same results were obtained with both preparations. This is in agreement with previous findings showing that Sap B isolated from pig kidney has the same activity as Sap B isolated from human tissues and is able to correct the CS hydrolytic defect in Sap B-deficient human fibroblasts (7). All later studies were thus performed with Sap B purified from pig kidney.

As shown in **Fig. 3A**, the extraction of PI, GM1, and PC increased with increasing concentrations of Sap B in the

TABLE 1. Sap B-induced solubilization of phospholipids and sphingolipids from LUVs of different composition

LUVs	Lipid Solubilization			
	PI	PC	Chol	GM1 or CS
	<i>nmol/nmol Sap B</i>			
PI/PC/Chol/GM1 (20:50:20:10)	0.50 ± 0.06	0.29 ± 0.02	0.00	0.20 ± 0.01
PI/PC/Chol/GM1 (10:60:20:10)	0.32 ± 0.03	0.55 ± 0.05	0.00	0.21 ± 0.02
PC/Chol/GM1 (70:20:10)	–	0.75 ± 0.04	0.00	0.19 ± 0.01
PI/PC/Chol/CS (20:50:20:10)	0.57 ± 0.06	0.30 ± 0.02	0.00	0.13 ± 0.02
PI/PC/Chol/CS (10:60:20:10)	0.33 ± 0.02	0.56 ± 0.06	0.00	0.11 ± 0.01
PC/Chol/CS (70:20:10)	–	0.80 ± 0.05	0.00	0.12 ± 0.01

Chol, cholesterol; CS, cerebroside sulfate; GM1, ganglioside GM1; LUV, large unilamellar vesicle; PC, phosphatidylcholine; PI, phosphatidylinositol; Sap B, saposin B. LUVs (50 nmol, total lipids) were incubated with Sap B (2 nmol) at pH 4.2, 37°C, for 30 min. The solutions were then chromatographed on a Sepharose CL-4B column as described for Fig. 1A. The LUVs were supplemented with [¹⁴C]PC, [³H]PI, [³H]GM1, [³H]Chol, or [¹⁴C]CS. The amounts of solubilized lipids were calculated from the amount of radioactivity eluting from the column together with Sap B (see Figs. 1, 2). The experiments were repeated at least three times. The values shown are means ± SD.

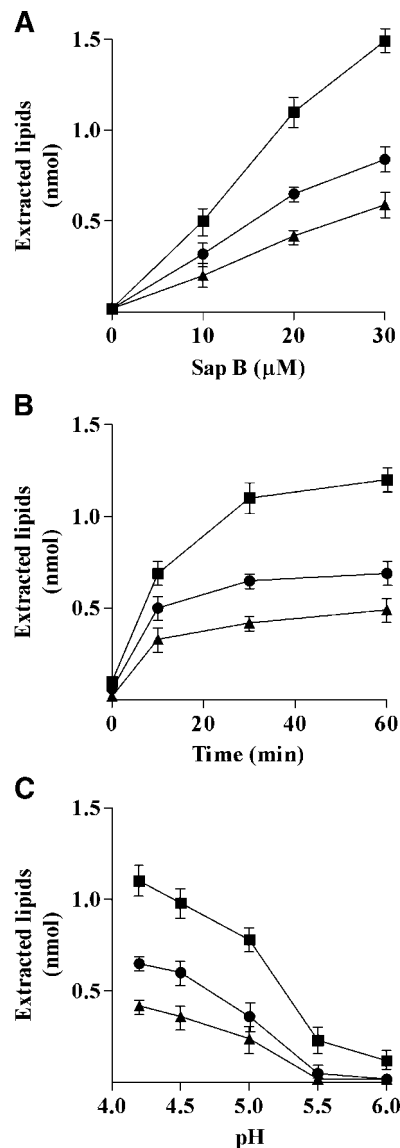


Fig. 3. Lipid extraction from LUVs as a function of the amount of Sap B, of time, and of pH. Sap B was incubated with LUVs (50 nmol, total lipids) composed of PI/PC/Chol/GM1 (10:60:20:10) and then chromatographed on a Sepharose CL-4B column as described for Fig. 1A. LUVs were supplemented with [¹⁴C]PC (closed squares), [³H]PI (closed circles), or [³H]GM1 (closed triangles), and lipid extraction was calculated as shown in Table 1. A: Increasing amounts of Sap B were incubated with LUVs in buffer B, pH 4.2, for 30 min. B: Sap B (2 nmol) was incubated with LUVs in buffer B, pH 4.2, for different periods of time. C: Sap B (2 nmol) was incubated with LUVs in buffer B, appropriately adjusted in the range from pH 4.2 to 5.5, for 30 min. For pH 6.0, the buffer was 10 mM Na-citrate, 1 mM EDTA, and 50 mM NaCl. Each point represents the mean of at least three replicates ± SD.

incubation mixture and was time-dependent, reaching a plateau level after ~30 min (Fig. 3B). Because Sap B is localized in the endolysosomal compartment, where the pH ranges between 5.5 and 4.2, the capacity of Sap B to solubilize lipids in this range of pH was assessed. Figure 3C shows that the optimal interaction with PI, PC, and GM1 occurs at the lower pH values. By increasing the pH

to >5.0 the interaction between lipids and Sap B decreased markedly.

The Sap B-induced solubilization of CS, whose lysosomal degradation is modulated by Sap B (3, 4), was analyzed by substituting CS for GM1 in the vesicles. When LUVs composed of PI/PC/Chol/CS (10:60:20:10) were incubated with Sap B, CS was solubilized (0.11 nmol of CS/nmol Sap B) together with PC and PI (0.89 nmol of total phospholipids/nmol Sap B) (Table 1). The solubilization of CS from LUVs was less efficient than that of GM1. A reduced Sap B extraction of CS compared with GM1 from micellar dispersions has been reported (9, 25).

Effect of anionic phospholipids on the Sap B-induced solubilization of SLs and neutral phospholipids

On consideration of the Sap B capacity to solubilize phospholipids together with SLs, we investigated whether the relative amount of neutral and anionic phospholipids affected the extraction of SLs. Sap B was thus incubated with LUVs, which, in addition to GM1, contained varying amounts of PC and PI. Table 1 shows that very similar amounts of GM1 were solubilized from LUVs composed of only PC and Chol or supplemented with up to 20% PI. Analogously, the addition of PI did not significantly modify the amount of CS solubilized by Sap B. Thus, the presence of anionic phospholipids on the membranes poorly affected the interaction of Sap B with SLs.

Conversely, it was noted that the increase of PI markedly decreased the Sap B-induced solubilization of PC from both GM1- and CS-containing LUVs (from 0.75–0.80 to 0.29–0.30 nmol PC/nmol Sap B in the absence or presence of 20% PI, respectively) (Table 1). To better characterize the inhibition of PC extraction by PI, the mutual effect of neutral and anionic phospholipids on their interaction with Sap B was examined in detail. LUVs composed of a constant amount of Chol (20%) and different percentages of PC and PI were incubated with Sap B, and the extraction of each phospholipid was evaluated. As shown in Fig. 4, in the absence of PI, ~1.8 nmol of PC was solubilized by 2 nmol of Sap B (a dimer). In the presence of increasing amounts of PI, the PC solubilization decreased dramatically, whereas that of PI increased concomitantly. Sap B solubilized more PI than PC when the PI concentration in the vesicles exceeded 20% of total lipids. Approximately five times more PI than PC was extracted from LUVs containing equimolar amounts of PI and PC. These results clearly indicate that the Sap B affinity for PI is much higher than that for PC and that neutral and anionic phospholipids compete for binding to Sap B. Moreover, Fig. 4 shows that the total amount of phospholipids (PC + PI) that are simultaneously extracted ranges from 1.7 to 2.3 nmol phospholipids/Sap B dimer, increasing as the PI percentage in the membranes increases.

Phospholipid transfer properties of Sap B

It has been shown previously that Sap B is able not only to bind but also to transfer GM1 and other SLs from one membrane to another (9, 25). The phospholipid binding

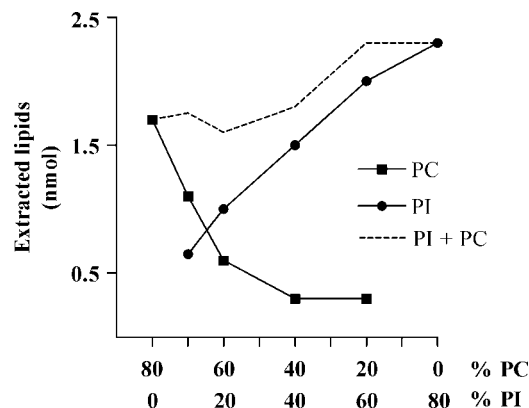


Fig. 4. Effect of the relative amounts of anionic and neutral phospholipids on their extraction by Sap B. LUVs (50 nmol, total lipids) composed of various amounts of PC and PI, as indicated, and a fixed amount of Chol (20%) were incubated with Sap B (2 nmol) in buffer B, pH 4.2, for 30 min and then chromatographed on a Sepharose CL-4B column as described for Fig. 1A. LUVs were supplemented with [^3H]PI and [^{14}C]PC. PC (closed squares) and PI (closed circles) extraction was calculated as shown in Table 1. The dashed line represents the sum of extracted phospholipids (PC + PI). Each point represents the mean of two replicate experiments.

activity observed here suggests that Sap B might also have phospholipid transfer activity. To test this notion, we used a vesicle-to-vesicle assay system previously established to measure the transfer of PC and PI catalyzed by phospholipid transfer proteins (22–24). SUVs containing trace amounts of labeled cholesteryl oleate as a nonexchangeable marker were incubated with MLVs, and the transfer of PI or PC from one vesicle population (SUVs) to the other (MLVs) was assessed by the variations of the PI or PC/cholesteryl oleate ratios of donor SUVs. After incubation with Sap B, these ratios decreased markedly, indicating the transfer of PI and PC from SUVs to MLVs. As shown in Fig. 5A, the lipid transfer increased linearly as a function of Sap B concentration up to 0.5 μM . The transfer of the phospholipids was also dependent on the time of incubation and on the pH of the medium (Fig. 5B, C). No significant transfer was observed at neutral pH values.

DISCUSSION

In this article, we provide biochemical evidence that Sap B, a glycoprotein shown to bind and transfer SLs (7, 9–12, 25), binds and transfers phospholipids as well. In the past, the formation of Sap B-SL complexes has usually been analyzed using micellar dispersions of pure SLs. In biological membranes, SLs are present as minor components. Thus, it can be expected that the capacity of Sap B to interact with SLs *in vivo* is influenced by the physical properties of the lipid matrix in which the SLs reside. This consideration prompted us to reexamine the lipid binding properties of Sap B by inserting SLs into membrane models such as LUVs composed of phospholipids and Chol. In this system, the ability of Sap B to form complexes

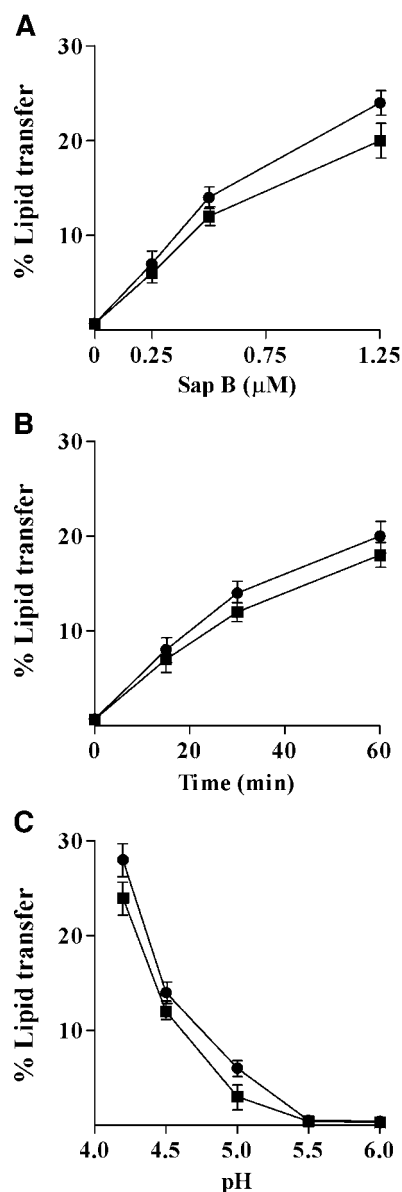


Fig. 5. Sap B transfers phospholipids within membranes in a concentration-, time-, and pH-dependent manner. Transfer of PC (closed squares) and PI (closed circles) from PC/PI (95:5) small unilamellar vesicle (SUVs) to PC/phosphatidylethanolamine/cardiophilin (70:25:5) multilamellar vesicles (MLVs) was analyzed as described in Materials and Methods. A: SUVs were incubated with MLVs in the absence and presence of increasing amounts of Sap B in buffer B, pH 4.5, for 30 min. B: SUVs were incubated with MLVs in the presence of 0.2 nmol of Sap B in buffer B, pH 4.5, for different periods of time. C: SUVs were incubated with MLVs in the presence of 0.2 nmol of Sap B in buffer B, appropriately adjusted in the range from pH 4.2 to 5.5, for 30 min. For pH 6.0, the buffer was 10 mM Na-citrate, 1 mM EDTA, and 50 mM NaCl. Each point represents the mean of at least three replicates \pm SD.

with GM1 and, to a lesser extent, with CS was confirmed, but unexpectedly, the saposin showed a better tendency to interact with phospholipids (especially anionic phospholipids) than with SLs. When the level of SLs (10% of total lipids) was close to physiological concentrations (26), Sap B extracted much more phospholipids (neutral and an-

ionic) than SLs from the lipid surface. The Sap B-induced solubilization of PI, an anionic phospholipid very abundant in endolysosomal organelles (13, 27), exceeded that of GM1 and CS, suggesting that anionic phospholipids rather than SLs are the favorite target of the saposin.

A strong competition was observed between neutral and anionic phospholipids for interaction with Sap B. Apparently, the binding of neutral and anionic phospholipids is mutually exclusive, indicating competition for the same binding site in the Sap B molecule. Sap B showed a much higher binding affinity toward PI than toward PC. Actually, PI markedly inhibited the Sap B-induced solubilization of PC, whereas PC affected the solubilization of PI to a lesser extent (Fig. 4).

It was also found that PI, while competing with PC, did not significantly affect the Sap B-induced solubilization of SLs. This observation strongly suggests that phospholipids and SLs interact with the saposin by different binding modes and, possibly, with different binding sites.

The Sap B molecular form in solution is almost exclusively dimeric (6, 7). Crystallographic analysis has confirmed the dimer structure of Sap B (8). Actually, the saposin exists as a pairwise reciprocal dimer of V-shaped, clasping monomers whose concave sides form a hydrophobic cavity. In this cavity, a phospholipid molecule copurified with the saposin was identified (8). Several phospholipid-binding proteins still contain a phospholipid-bound molecule after purification and crystallization (28, 29). The existence of an encapsulated phospholipid in purified Sap B strongly supports our results on the preferential interaction of Sap B with phospholipids. Crystallographic analysis has also revealed conformational differences between the Sap B monomers. At least three independent monomer conformations, forming distinct homodimers (symmetric and asymmetric), have been described (8). It can be envisaged that distinct Sap B dimers have different affinities for specific lipids (SLs or phospholipids) and different modes of lipid binding.

Previous data on the Sap B-induced extraction of SLs from pure micelles reported a GM1/Sap B dimer binding ratio of \sim 1 (9) and a CS/Sap B dimer binding ratio variable between 0.66 (9) and 1.3 (17). In our experimental conditions, in which the saposin has the possibility to interact with membranes containing different lipids, the total number of bound lipids is somehow higher and varies slightly according to the composition of the membrane (Table 1, Fig. 4). Actually, we found that a Sap B dimer forms complexes with \sim 2.0 nmol of lipids altogether, extracting phospholipids and SLs simultaneously. Most likely, the number of lipid molecules interacting with Sap B depends on the nature and physical organization of the lipids. The conformational variability in the dimer structure of Sap B was thought to be induced by interaction with specific lipids (8).

The lipid/Sap B dimer binding ratio of \sim 2 observed here suggests that the internal cavity of the Sap B dimer can contain more than one lipid molecule. Multiple lipid molecules have been found in the crystal structure of the GM2 activator, a lysosomal lipid binding protein (30–34).

It is interesting that the GM2 activator shares several functional, binding, and structural properties with Sap B. In fact, the GM2 activator also is involved in the lysosomal degradation of SLs (30) and can interact with both SLs and phospholipids, as suggested by several reports (35, 36). Moreover, the GM2 activator molecule contains an internal hydrophobic cavity suitable for binding lipids by two different lipid binding modes. A molecule of phosphatidylglycerol and a molecule of GM2 can satisfactorily be modeled into distinct binding sites of the spacious apolar cavity, suggesting that the GM2 activator pocket can simultaneously accommodate SLs and phospholipids (32).

This work shows that Sap B is also able to transfer phospholipids from one membrane to another. A number of transfer proteins that catalyze the transfer of phospholipids between membranes have been isolated and characterized in the tissues of several animals. In all cases, molecular masses between 20 and 30 kDa and isoelectric points between 4.7 and 5.8 have been reported (28). Moreover, a family of nonspecific lipid transfer proteins characterized by a molecular mass of ~9 kDa and a highly conserved three-dimensional structure stabilized by four disulfide bonds has been identified in plants (37, 38). The transfer proteins are specific for either the transfer of PC alone or for both PI and PC (with a marked preference for PI) or are nonspecific, being able to transfer several lipids (28). Sap B may be considered a nonspecific transfer protein that catalyzes the movement of SLs (9, 25) and of both neutral and anionic phospholipids (our results) between membrane bilayers. Sap B, unlike other phospholipid transfer proteins, can exert its activity only at low pH values, suggesting a role for Sap B in the transfer of phospholipids between internal membranes of acidic organelles, such as late endosomes/lysosomes. In other words, Sap B might regulate the level and distribution of phospholipids in endolysosomal membranes, analogously to what other phospholipid transfer proteins do in other compartments of the cell.

It has been shown previously that Sap B at low pH values (4.0–4.5) induces a small but significant destabilization of vesicles composed of Chol and neutral and anionic phospholipids (15). The interaction of Sap B with phospholipid membranes has also been confirmed using surface plasmon spectroscopy (39). The properties of Sap B observed previously and here suggest a phospholipid transfer mechanism based on a Sap B transient association and perturbation of the lipid surface followed by extraction and transport of lipids, possibly involving conformational changes of the Sap B dimer structure (8).

It was found recently that cells deficient in prosaposin and consequently in the four saposins (A–D) exhibit specific defects in CD1-mediated antigen presentation. The CD1 function can be restored by providing saposins to the cells (40–42). This finding suggests that saposins can extract lipids from endolysosomal membranes, promoting the loading and the editing of lipids on CD1 molecules. It is known that PI-related lipids are the natural major ligands of CD1d and CD1b molecules (43). The PI transfer activity of Sap B observed here suggests that this saposin

might have a specific role in exchanging PI with CD1 molecules at low pH values in the endolysosomal compartment.

The findings of this study provide the first concrete evidence that Sap B binds and transfers phospholipids, especially the anionic ones. The marked preference for anionic phospholipids is a Sap B characteristic shared with at least two other saposins, Sap C and Sap D. Apparently, anionic phospholipids play an important role in regulating the interaction of saposins with membranes. Actually, the capacity of Sap D to bind and break down membranes is favored by high levels of anionic phospholipids on the lipid surface (44). Analogously, the membrane-perturbing power of Sap C is remarkably lipid-dependent, being strongly enhanced by the presence of anionic phospholipids (15). Moreover, Sap C's ability to promote glucosylceramide enzymatic hydrolysis is regulated by its interaction with anionic phospholipids (45). The high affinity of Sap B for PI, observed here under conditions mimicking the interior environment of the late endosomal/lysosomal compartment, is thus expected to have important implications for its biological function(s). Elucidation of Sap B-lipid interactions will lead to a better understanding of the cellular processes mediated by this saposin. ■

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