Saposin D Solubilizes Anionic Phospholipid-containing Membranes*

Fiorella Ciaffoni, Rosa Salvioli, Massimo Tatti, Giuseppe Arancia‡, Pasqualina Crateri‡, and Anna Maria Vaccaro§

From the Department of Metabolism and Pathological Biochemistry and §Department of Ultrastructures, Istituto Superiore Sanità, Viale Regina Elena 299, 00161 Rome, Italy

Saposin (Sap) D is a late endosomal/lysosomal small protein, generated together with three other similar proteins, Sap A, B, and C, from the common precursor, prosaposin. Although the functions of saposins such as Sap B and C are well known (Sap B promotes the hydrolysis of sulfatides and Sap C that of glucosylceramides), neither the physiological function nor the mechanism of action of Sap D are yet fully understood. We previously found that a dramatic increase of Sap D supercellular hydrophobicity, occurring at the low pH values characteristic of the late endosomal/lysosomal environment, triggers the interaction of the saposin with anionic phospholipid-containing vesicles. We have presently found that, upon lipid binding, Sap D solubilizes the membranes, as shown by the clearance of the vesicles turbidity. The results of gel filtration, density gradient centrifugation, and negative staining electron microscopy demonstrate that this effect is due to the transformation of large vesicles to smaller particles. The solubilizing effect of Sap D is highly dependent on pH, the lipid/saposin ratio, and the presence of anionic phospholipids; small variations in each of these conditions markedly influences the activity of Sap D. The present study documents the interaction of Sap D with membranes as a complex process. Anionic phospholipids attract Sap D from the medium; when the concentration of the saposin on the lipid surface reaches a critical value, the membrane breaks down into recombinant small particles enriched in anionic phospholipids. Our results suggest that the role played by Sap D is more general than promoting sphingolipid degradation, e.g. the saposin might also be a key mediator of the solubilization of intralysosomal/late endosomal anionic phospholipid-containing membranes.

Sap D is a member of a family of four similar glycoproteins, Sap A, B, C, and D, generated from a common precursor, prosaposin, in late endosomes/lysosomes (1–4). The four saposins have been the subject of numerous structural and functional studies. They are small homologous proteins (each contains about 80 amino acids), have six cysteine residues at similar positions (1–4), and share the same disulfide structure (5, 6), similar to that of a group of saposin-like proteins such as surfactant protein B, Entamoeba histolytica pore-forming peptides, and NK-lysin (7). The maintenance of the disulfide structure is essential for the functional properties of saposins and saposin-like proteins (5, 7).

The involvement of saposins in the lysosomal degradation of sphingolipids has been unequivocally demonstrated. In fact the deficit of prosaposin and, thus, of the four saposins causes the lysosomal accumulation of several sphingolipids such as ceramide, glucosylceramide, lactosylceramide, and ganglioside GM3 (8, 9). Moreover, the description of a variant form of Gaucher’s disease due to a deficit of Sap C and of a variant form of metachromatic leukodystrophy due to a deficit of Sap B indicates that the two saposins are specifically involved in the degradation of glucosylceramides and sulfatides, respectively (10–12).

It has been reported that besides promoting sphingolipid degradation, saposins can have other functions. In fact, the four saposins as well as prosaposin have been described as serving as ganglioside binding and transport proteins (13). Moreover, prosaposin is a neurotrophic factor with neurotrophic activity localized within its Sap C domain (14).

For a long time the current hypothesis on the mechanism of action of saposins involved the concept that the activation of sphingolipid degradation was due to the interaction of saposins either with sphingolipids (e.g. Sap B with sulfatides) or with sphingolipid hydrolases (e.g. C with glucosylceramidase) (1–4). With a series of papers we have demonstrated that this was not the case at least for Sap C, whose main target is neither glucosylceramidase nor glucosylceramide but, instead, phospholipid membranes (15–18). Sap C, on interaction with anionic phospholipid-containing membranes, causes a dramatic destabilization of the lipid surface, that in turn favors the association of glucosylceramidase and the enzymatic degradation of glucosylceramide (15–18). Beside modulating the glucosylceramidase interaction with membranes, Sap C has also the capacity to aggregate and fuse anionic phospholipid vesicles at low pH values (19).

Recently, we demonstrated that Sap D, like Sap C, strongly binds to membranes and that anionic phospholipids promote and modulate its interaction with lipid surfaces (6, 16). These similarities contrast with the different physiological role played by the two saposins, namely, Sap C is involved in glucosylceramide degradation (12), whereas Sap D has been proposed as an activator of ceramide or sphingomyelin degradation (20–22). A direct interaction of Sap D with these sphingolipids seems unlikely since neither the presence of ceramide nor that of sphingomyelin increases the affinity of Sap D for lipid surfaces (6). On the other hand, it is reasonable to suppose that the high affinity of Sap D for anionic phospholipids, as reflected in its behavior in vitro (6), will in large measure dictate its behavior in vivo.

The present study was originally conceived to search for differences between the membrane binding properties of Sap C and D, which might account for their different physiological
roles. By comparing the interaction of the two saposins with vesicles, we met an unexpected property of Sap D not yet reported for any saposin, namely, the ability to disrupt anionic phospholipid-containing membranes with formation of small particles. This finding prompted us to characterize the phospholipid recombinant particles and to define the conditions that favor their formation. Our results indicate that Sap D has the potential to be a phospholipid membrane-solubilizing protein under physiological conditions.

**EXPERIMENTAL PROCEDURES**

**Materials—**Phosphatidylcholine (PC) from egg yolk and phosphatidylserine (PS) from bovine brain were from Avanti Polar Lipids, Inc. (Alabaster, AL). Phosphatidylinositol (PI) from bovine liver and cholesterol (Chol) were from Sigma. L-α-dipalmitoyl-[2-palmitoyl-9,10-3H(α)]PC (82 Ci/mmol) was from PerkinElmer Life Sciences, and 1,2-dioleoyl-3-phosphatidyl-1-[3-14C]serine (56 Ci/mmol) was from Amersham Pharmacia Biotech. All other chemicals were of the purest available grade.

**Sap C and Sap D Preparation—**Sap C and Sap D were purified from the spleens of patients with Type 1 Gaucher’s disease after a previously reported procedure (5, 6); it consisted essentially of heat treatment of a water homogenate followed by ion exchange chromatography on DEAE-Sephadex, gel filtration on Sephadex G-75, and reverse-phase high pressure liquid chromatography on a protein C4 column (Vydac). The purity of the final preparations of Sap C and Sap D was verified by N-terminal sequence analysis, SDS-polyacrylamide gel electrophoresis, and electrospray mass spectrometry (5, 6).

**Vesicle Preparation—**Multilamellar vesicles (MLVs) were prepared by mixing appropriate amounts of lipids dissolved in chloroform and evaporating the solvent under N2. PC was supplemented with [3H]PC and, in some experiments, PS with [14C]PS. The specific activities of both phospholipids were in the range of 2 × 105 dpm/mg. The dry lipids were dispersed by Vortex mixing in 2 mM l-histidine, 2 mM TES, 150 mM NaCl, 1 mM EDTA, pH 7.4. The suspension was submitted to 10 cycles of freezing and thawing and then blended by a Vortex mixer for 3–5 min. The vesicle concentration was determined by radioactivity measurements.

**Light-scattering Experiments—**The changes of vesicle turbidity were monitored by 90° light scattering. A Fluoromax-2 spectrofluorometer equipped with a constant temperature cell holder and stirrer (Spex Industries Inc., Edison, NJ) was used with excitation and emission wavelengths set at 600 nm (slit widths of 3 nm). The temperature of the cuvette holder was maintained at 37°C. All solutions were preincubated at this temperature. Lipid vesicles were added to 1 ml buffer A (10 mM acetate, 150 mM NaCl, 1 mM EDTA), pH 4.5, and monitored for 5 min. Saposin solutions were then added, and the change in light scattering was monitored as function of time.

**Gel Permeation—**Different mixtures of Sap D and labeled MLVs (see above) were concentrated by transferring them to Microcon YM-3 centrifugal filter devices (molecular mass cut-off 3 kDa) and centrifuging at 13,000 × g until all the liquid except 20 μl was passed through the filter. The presence of Sap D in each retentate was tested by SDS-polyacrylamide gel electrophoresis (see the next paragraph).

**SDS-Polyacrylamide Gel Electrophoresis—**SDS-polyacrylamide gel electrophoresis was performed with 15% acrylamide-separating gels and 4.5% stacking gels (23). After electrophoresis, Sap D was visualized by the silver-staining kit Protein (Amersham Pharmacia Biotech). SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed with 15% acrylamide-separating gels and 4.5% stacking gels (23). After electrophoresis, Sap D was visualized with the silver-staining kit Protein (Amersham Pharmacia Biotech).

**Electron Microscopy—**Different mixtures of Sap D and 20% PS containing MLVs in buffer A, pH 4.5, were incubated for 30 min at 37°C and then adjusted to pH 7.0 with 0.1 M imidazole. The samples were applied to carbon-coated copper grids (400 mesh). After 30 s on the grid, the samples were dried, negatively stained with 2% sodium phosphotungstate, and observed by transmission electron microscopy. Micrographs were taken using a Philips 208S electron microscope operating at 80 kV.

**RESULTS**

**Sap D Induces Clearance of Anionic Phospholipid-containing Vesicles—**We have previously shown that, within the four saposins, Sap C and Sap D have the strongest capacity to destabilize anionic phospholipid-containing vesicles at acidic pH values (16, 24). Moreover, Sap C, under appropriate conditions, is able to aggregate and fuse PS vesicles (19). To address the question whether Sap C and D exert different actions on membranes, the two saposins were separately added to phospholipid MLVs, and the turbidity of the vesicles was followed by light-scattering measurements. Representative curves of the changes in scattered light intensity are shown in Fig. 1. The addition of Sap C to MLVs containing 20% PS caused a time-dependent increase in light-scattering intensity, confirming the Sap C capacity to promote aggregation of anionic phospholipid-containing membranes (19). In contrast, the addition of Sap D to the same MLV preparation induced clearance of the suspension. The rate of turbidity clarification decreased on lowering the membrane content of PS and was dramatically reduced when MLVs were devoid of anionic phospholipids. These results strongly suggest that Sap D is able to disrupt membranes and that anionic phospholipids play a critical role in this process.
The comparison of Fig. 2, B and C, suggests that the recombinant particles formed in the presence of PI are smaller than those formed in the presence of PS. To confirm that Sap C was unable to dissolve phospholipid membranes, a mixture of 10% PS-containing MLVs and Sap C was analyzed by gel permeation at pH 4.5. The elution pattern of MLVs was not affected by the presence of Sap C (data not shown).

When the PS percentage in the vesicles was increased from 10 to 20%, the peak of the recombinant particles formed after the addition of Sap D sharpened and shifted toward lower molecular masses (compare Fig. 2B and Fig. 3, upper left panel). This result is consistent with the light-scattering experiments showing that the addition of Sap D to 20% PS-containing MLVs induces a more rapid turbidity clarification than the addition to 10% PS-containing MLVs (see Fig. 1). Thus, the gel permeation experiments confirm that Sap D, but not Sap C, induces breakdown of anionic phospholipid-containing membranes, giving rise to smaller particles whose size and extent of formation depend on the type and percentage of the anionic phospholipid.

**Effect of pH on the Formation of Recombinant Particles**

Since we previously found that the interaction of Sap D with membranes is favored by an acidic environment (16), the pH dependence of the formation of recombinant particles was evaluated (Fig. 3). When the gel filtration analysis of the SapD vesicles mixtures was performed at pH 6.0, essentially all the 20% PS-containing vesicles eluted in one peak, with a maximum at fraction 18, corresponding to the MLVs. At lower pH values, progressively larger proportions of the vesicles appeared as a second peak (maximum at fraction 32–33), indicating that low pH values are required for the formation of the small particles.

To check whether the recombinant particles had the same phospholipid composition as the original MLVs, the vesicles were doubly labeled in PC and PS. Fig. 3 (upper left panel) shows that the small particles have a significantly lower PC/PS ratio ($r = 2.4$) than the parent vesicles ($r = 2.7$). The remaining MLVs eluting in the void volume, on the other hand, were enriched in PC ($r = 3.0$). Apparently Sap D selects PS over PC during the reaction with MLVs vesicles, promoting the pH-dependent formation of recombinant small particles richer in PS than the original membranes.

**Effect of the Lipid/Sap D Molar Ratio on the Formation of Recombinant Particles**

To ascertain at which lipid/saposin molar ratio the Sap D-induced breakdown of membranes becomes evident, a gel permeation analysis of Sap D mixed with different amounts of PS-containing MLVs was performed (Fig. 4). At a lipid/saposin molar ratio of 100:1, the presence of the saposin poorly affected the elution profile of the MLVs (Fig. 4A). At a molar ratio of 50:1, about 35% PC and 40% PS eluted as a broad shoulder after the MLVs peak (Fig. 4B). When the molar ratio was further decreased to 12.5:1, most of the MLVs was transformed into particles eluting in the second peak (a maximum at fraction 33) (Fig. 4C). Judging from the elution profiles, the small particles were generated when the lipid/saposin molar ratio was lower than 100:1.

**Resolution of Recombinant Particles by Density Gradient Ultracentrifugation**

To further characterize the recombinant particles and to determine their density, the Sap D-induced transformation of MLVs was analyzed by density gradient centrifugation. When the 20% PS-containing vesicles were subjected to centrifugation in a KBr gradient at pH 4.5, the lipids floated at the top, whereas after incubation with Sap D at a lipid/saposin molar ratio of 25:1, a major portion of the vesicles was shifted to a peak centered at fraction 13, corresponding to the elution profile of the recombinant particles.
A density of about 1.23 (Fig. 5A). At a lipid/Sap D molar ratio of 12.5:1, an almost complete transformation of the original MLVs was observed (Fig. 5B). Vice versa, the Sap C addition to the PS-containing MLVs did not cause a significant transformation of the vesicles (Fig. 5C). The density gradient banding profiles of the recombinant particles were consistent with the elution patterns previously observed for the corresponding gel filtration samples, the peak at higher density (d = 1.23) corresponding to the peak eluting at lower molecular masses (a maximum at fractions 32–33) (compare Fig. 5A with Fig. 3, upper left panel, and Fig. 5B with Fig. 4C).

Analysis of the Sap D Binding Affinity to MLVs and Recombinant Particles—To check whether Sap D forms stable complexes with the vesicles and/or with the recombinant particles, the peaks obtained after gel permeation analysis of 20% PS-containing MLV-Sap D mixtures were analyzed by electrophoresis for the presence of the saposin. As shown in Fig. 6, upper panel, when the lipid/Sap D molar ratio of the mixture was 100:1, Sap D was found under the single MLV peak (maximum at fraction 18) eluting from the gel permeation column under this condition (see Fig. 4A). At a lipid/saposin molar ratio of 25:1, when a first MLV peak and a second peak of small recombinant particles elute from the column (see Fig. 3, upper left panel), most of the Sap D was found in the second rather than in the first peak (Fig. 6, middle panel). The same situation was observed when the lipid/saposin molar ratio was further decreased to 12.5:1, and most of the MLVs was transformed into small particles (Fig. 6, lower panel). These results indicate that Sap D remains preferentially associated with the small particles obtained after the conversion of the large vesicles.

Electron Microscopic Structure of the Recombinant Particles—The ultrastructure of the recombinant particles formed on incubation of PS-containing MLVs with Sap D was examined by negative staining electron microscopy (Fig. 7). Control MLVs are spherical in shape, with a variable diameter ranging from 400 to 1000 nm (Fig. 7A). The incubation of MLVs with Sap D at a lipid/saposin molar ratio of 25:1 gave mixtures of small spherical vesicular structures of varying sizes. The majority of the vesicles had diameters ranging from 20 to 50 nm (Fig. 7B), but vesicles with diameter larger than 200 nm were also observed (data not shown). At a lower lipid/Sap D molar ratio (12.5:1), the diameter of the vesicles further decreased (15–20 nm), and discoidal structures forming rouleaux were clearly discerned (Fig. 7C). It is thus evident that the size and morphology of the recombinant particles depend on the Sap D concentration on the membrane.

DISCUSSION

Saposins are involved in the lysosomal degradation of several sphingolipids in late endosomes/lysosomes. Most likely a crucial step in the cascade of events during the hydrolysis of these lipids is the interaction of saposins with the membranes where sphingolipids are located. We have previously demonstrated that at low pH values similar to those present in the endosomal/lysosomal environment, at least two saposins (Sap C and D) have a high affinity for phospholipid membranes, particularly in the presence of anionic phospholipids (16, 24). In the present study several independent experimental approaches have provided the first demonstration that Sap D has the capacity to break down anionic phospholipid-containing MLVs.
membranes, giving rise to small recombinant particles. The criteria for this conclusion include (a) the ability of Sap D to transform MLVs into structures that scatter less light, (b) the isolation by gel filtration or by density gradient centrifugation of stable complexes of saposin and lipid particles having a different size and density than the starting membranes, and (c) the detection by electron microscopy of small spherical and discoidal structures after incubation of MLVs with Sap D.

The membrane-disrupting activity of Sap D is expressed only when the pH of the medium is below 5.5. This observation is consistent with our previous studies showing that Sap D undergoes a hydrophobic transition at low pH values, leading to interaction with membranes (16). The change of hydrophobicity of Sap D most likely has a physiological significance because the low pH values found in late endosomes/lysosomes correspond to those required by the saposin for binding, perturbing, and solubilizing anionic phospholipid-containing membranes. Even a small pH change in the acidic organelles might markedly affect the properties of Sap D.

The formation of recombinant lipid particles induced by Sap D is dependent on the presence of negatively charged phospholipids. We observed in the past that variation of the percentage of anionic phospholipids modulates the strength of the saposin-membrane interaction (6, 24).
anionic phospholipids and the cationic sites of the saposin. Both the anionic phospholipids used in the present work, namely PS and PI, favored the SapD-induced disruption of the membrane. Apparently, the action of Sap D on the lipid surface occurs in the presence of anionic phospholipids independently of their structure.

Another critical factor for the solubilization of the membranes is the concentration of Sap D on the lipid surface. In fact, our results show that large anionic phospholipid-containing vesicles bind the saposin up to a lipid/saposin molar ratio of about 100:1 before they break down into small recombinant complexes. It can be envisaged that electrostatic interactions provided by the anionic phospholipids attract Sap D from the medium and concentrate it on the lipid surface. When the local concentration of Sap D reaches a critical value the membrane breaks down, giving rise to small particles enriched in anionic phospholipids as compared with the original membrane. This last observation suggests that Sap D, upon association with vesicles, favors the formation of domains enriched in anionic phospholipids.

Our past investigation on the structural and membrane binding properties of saposins showed that Sap D and Sap C share several important features as follows. They have the ability to exist in both water-soluble and membrane-bound forms (16); they undergo a dramatic increase of hydrophobicity at pH 5.0–5.5, as indicated by phase partitioning experiments in Triton X-114 and fluorescence measurements (6, 16, 18, 24); they bind and perturb anionic phospholipid-containing large vesicles in a pH-dependent manner (6, 16, 24); and they have the same disulfide structure (5, 6). On the other hand, the two saposins also exhibit profound differences. In fact, Sap C, but not Sap D, is able to promote the association of glucosylceramidase with large anionic phospholipid-containing vesicles reconstituting the enzyme activity (16), a finding consistent with the notion that only Sap C is the physiological activator of glucosylceramidase. Conversely, the present work demonstrates that Sap D can break down anionic phospholipid-containing membranes, an activity not exhibited by Sap C. Thus, our findings indicate that, although both saposins have a high affinity for phospholipid vesicles, the mode of Sap D interaction with membranes is remarkably different from that of Sap C.

Sap D has been proposed to have a role in ceramide degradation, since its addition to the culture medium of fibroblasts from a patient suffering from prosaposin deficiency resulted in the reduction of ceramide storage (22). Recently it has been...
reported that not only Sap D, but also Sap A and C, reduce the ceramide storage (25). Thus, a specific physiological function of Sap D in ceramide hydrolysis is still uncertain. We have previously shown that ceramide does not increase the affinity of Sap D for the membranes (6), the real target of the saposin being anionic phospholipids. The key role played by these lipids in governing the behavior of Sap D suggests that the saposin interacts with anionic phospholipids to elicit its biological effect(s).

In the late endosomes/lysosomes, where saposins are located, the presence of internal vesicles and structures has often been observed (26–27). Recently it has been found that the network of internal vesicles in the lumen of late endosomes contains large amounts of anionic phospholipids, especially lysobisphosphatidic acid and PI, forming specialized domains where specific proteins such as the multifunctional receptor for mannose 6-phosphate-bearing ligands preferentially segregate (27). In view of our past and present results on the role of anionic phospholipids in the interaction between Sap D and membranes, it is tempting to envisage that also Sap D segregates and concentrates onto the intraendosomal/lysosomal vesicles, where it might participate to their degradation by converting them into small recombinant particles. In cells of patients affected with prosaposin deficiency, where all saposins are missing, the accumulation of intraendosomal/lysosomal multivesicular structures has been observed (9, 28). A putative role of Sap D in the degradation of anionic phospholipid-containing membranes inside acidic organelles would be consistent with this observation.

Interestingly, some of the Sap D properties characterized in the present paper are similar to those shown by exchangeable apolipoproteins, such as apoA-I, except that the Sap D properties are exhibited at low pH and the apolipoproteins properties are exhibited at neutral pH. In fact, apoA-I, under appropriate conditions, also has a marked ability to solubilize membranes, causing liposome structure to be disrupted so as to lose turbidity, especially in the presence of anionic phospholipids (29). Moreover, the Sap D-induced formation of small lipid particles resembles the recruitment of phospholipids by apolipoprotein A-I to form apolipoprotein lipid spherical and discoidal recombinant particles that can be separated from the original vesicles by gel permeation or density gradient centrifugation (30–33). Exchangeable apolipoproteins have a functional role in the remodeling of lipoproteins. The apolipoprotein-like properties exhibited by Sap D at low pH values suggest that this saposin might have a role in the remodeling of phospholipid membranes inside acidic organelles.

In conclusion, the present work has highlighted for the first time the ability of Sap D to convert membranes of appropriate composition into small recombinant particles. This finding might give critical clues to the function of this saposin, whose precise physiological and pathogenic role is still relatively unknown. Our results suggest that Sap D might have more general functions than promoting the sphingolipid degradation.
