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Saposin C induces pH-dependent destabilization and fusion of phosphatidylserine-containing vesicles

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Abstract

We have previously shown that saposin C (Sap C), a glucosylceramidase activator protein, interacts with phosphatidylserine (PS) large unilamellar vesicles (LUV), promoting the glucosylceramidase binding to the bilayer [(1993) FEBS Lett. 336, 159–162]. In the present paper the consequences of the Sap C interaction on the lipid organization of the vesicles are reported. It was found that Sap C perturbs the PS bilayer as shown by the release of an encapsulated fluorescent dye. Three different procedures, resonance energy transfer, gel filtration and electron microscopy, indicated that the activator protein is also able to make PS liposomes fuse. The effects of Sap C on PS vesicles were observed at low but not at neutral pH. The lipid composition of the bilayer also affected the Sap C-induced destabilization; in fact, the presence of PS in mixed LUV was essential for significant leakage to occur. These results demonstrate for the first time that Sap C is a protein capable of destabilizing and fusing acidic phospholipid-containing membranes in a pH-dependent fashion.

Key words: Saposin C; Membrane destabilization; Membrane fusion; Phosphatidylserine liposome

1. Introduction

Saposin C (Sap C) is a small glycoprotein required by glucosylceramidase for the hydrolysis of glucosylceramide to ceramide and glucose [1–4]. The physiological significance of Sap C has been unequivocally assessed by the identification of patients who store glucosylceramide due to a deficiency of Sap C. In these patients, affected by a variant form of Gaucher's disease, glucosylceramidase appears to be normal [3,4].

Sap C, together with saposins A,B and D, is derived by proteolytic processing from a single precursor protein, called prosaposin [5–7]. The saposins are structurally similar to one another [8,9]. Each consists of about 80 amino acids and carries one (Sap B, C, D) or two (Sap A) N-linked carbohydrate chains.

The apparent function of the saposins is that of stimulating the enzymatic hydrolysis of specific sphingolipids [8,9]. The mechanism by which saposins promote sphingolipid degradation has been better characterized for Sap B, which stimulates in vitro degradation of several sphingolipids (sulfatide, globotriaosylceramide, ganglioside GM1, etc.) by forming water soluble complexes with the substrates and thus making them accessible to the respective enzymes [10,11].

Unlike Sap B, Sap C does not appear to interact with the enzyme substrate, glucosylceramide [12]. Several authors, having extensively investigated the interactions between glucosylceramidase, Sap C and phosphatidylserine (PS), a lipid also required for the enzyme activation [2], have concluded that the mechanism of stimulation involves the binding of Sap C to the enzyme [12,13].

In contrast with these conclusions, using large unilamellar vesicles (LUV) as models of biological membranes, we have recently shown that Sap C binds to PS LUV rather than to glucosylceramidase [14]. Moreover it was found that the interaction of Sap C with the lipid surface is the prerequisite for the binding of glucosylceramidase to PS LUV and for the full reconstitution of enzymatic activity [14].

Interestingly, an immunocytochemical study of Sap C localization in lysosomes has shown the immunolabeling to be greatest around the perimeter of the matrix, thus suggesting an association between Sap C and the lysosomal membrane [15]. In view of our findings on the interaction between Sap C and components of biological membranes such as acidic phospholipids [14], a systematic study of the consequences of this interaction on the lipid organization appeared essential to understanding the role of Sap C within the lysosomes.

The aim of the present study was to delineate the nature of the Sap C effect on membranes by examining Sap C-induced changes in artificial lipid model systems, e.g. liposomes.

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Abbreviations: MUGlc, 4-methylumbelliferyl-β-D-glucopyranoside; PS, phosphatidylserine; PC, phosphatidylcholine; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; N-NBD-PE, L-α-phosphatidylethanolamine-*N*-(4-nitrobenzo-2-oxa-1,3-diazole); N-Rh-PE, L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl).

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2. Materials and methods

2.1. Materials

PS from bovine brain, L-α-phosphatidylethanolamine-*N*-(4-nitrobenzo-2-oxa-1,3-diazole) (N-NBD-PE) and L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (N-Rh-PE) were from Avanti Polar-Lipids (Alabaster, AL, USA). Phosphatidylcholine (PC) from egg yolk and calcein were from Sigma (St. Louis, MO, USA). 1,2 Dioleoyl-3-*sn*-phosphatidyl-L-[3-^{14}C]serine (54 mCi/mmol) was from Amersham International (Amersham, UK). L-α-dipalmitoyl[2-palmitoyl-9,10^{3}H(N)]-PC (50 Ci/mmol) was from NEN Research Products, DuPont de Nemours (Germany). 4-Methylumbelliferyl-β-D-glucopyranoside (MUGlc) was obtained from Koch-Light Labs (Colnbrook, UK). All other chemicals were of the purest available grade.

2.2. Enzyme preparation

Glucosylceramidase was purified from human placenta following the procedure described by Murray et al. [16].

2.3. Saposin C preparation

Sap C was purified from spleens of patients with Type 1 Gaucher's disease as previously reported [14,17]. The purity of the preparation was verified by SDS-PAGE, Western blotting and N-terminal analysis [14].

2.4. Vesicle preparation

Small and large unilamellar vesicles (SUV and LUV) were prepared as previously described [18].

2.5. Stimulation of glucosylceramidase activity by PS and Sap C

The stimulatory capacity of PS liposomes and Sap C was evaluated using an assay mixture that contained, in a final volume of 0.2 ml, 10 ng of purified placental glucosylceramidase, 10 mM acetate buffer (pH 4.5–6.0), 150 mM NaCl and 1 mM EDTA (buffer A), 2.5 mM MUGlc, 2 μg of PS vesicles and 2 μg of Sap C. The assay mixtures were incubated for 30 min at 37°C. The extent of reaction was estimated fluorometrically [14]. All assays were carried out in duplicate and the results agreed within 5%.

2.6. Protein determination

The proteins were measured with the bicinchoninic acid method [19] with bovine serum albumin as a standard.

2.7. Leakage and lipid mixing assays

The leakage of liposome contents was monitored by the release of calcein trapped inside the vesicles. Calcein was previously purified on a Sephadex LH-20 column as reported [20]. LUV for leakage experiments were prepared by hydrating dried films of phospholipids in 60 mM calcein, pH 7.4 followed by 10 cycles of freeze-thawing. The resulting multilamellar vesicles were extruded 10–15 times through two 0.1 μm diameter pore polycarbonate filters (Nucleopore Corp., Pleasanton, CA). To prepare calcein-containing SUV, the freeze-thawed vesicles were sonicated until the preparation was transparent. Free calcein was separated from the dye-containing SUV and LUV by chromatography on a Sephadex G-75 column [20]. Upon addition of Sap C, leakage of calcein to the external medium was followed by the increase in fluorescence caused by calcein dilution and the consequent relief of self-quenching (excitation 470 nm, emission 520 nm). 100% leakage was established by lysing the vesicles with 0.3% (v/v) Triton X-100.

Lipid mixing was monitored by the resonance energy transfer technique [21]. Liposomes containing both N-NBD-PE and N-Rh-PE at 1 mol% each were mixed with liposomes without the fluorescence probes at a molar ratio of 1:9. The sample was excited at 475 nm while the NBD fluorescence was recorded continuously at 530 nm. The 100% lipid mixing level was set with liposomes containing 0.1% each of N-NBD-PE and N-Rh-PE at the same total lipid concentration as in the lipid mixing experiment.

Leakage and lipid mixing of liposomes were carried out at 37°C and monitored with a Fluoromax spectrofluorometer equipped with a constant temperature cell holder and stirrer (Spex Industries Inc., Edison, NJ, USA).

2.8. Chromatography on Bio-Gel A-50m columns

PS SUV were incubated with or without Sap C for 30 min at 37°C in buffer A, pH 5.0. The reaction mixture was then applied to a Bio-Gel A-50m column (10 × 200 mm; Bio-Rad, Richmond, USA) equilibrated and eluted with the same buffer. The flow rate was 0.3 ml/min. Fractions of 0.5 ml were collected. Chromatographic separations were carried out at room temperature.

2.9. Electron microscopy

Liposomes were negatively stained with a 2% phosphotungstic acid solution (pH 7.0). For a better spreading of liposomes, the Formvar carbon-coated grids were treated with 1% bacitracin [22]. Samples were observed under a Philips CM 12 transmission electron microscope.

3. Results

3.1. Sap C-induced leakage of PS-containing vesicles

Sap C is required for optimal activation of purified glucosylceramidase by PS LUV at low pH (Fig. 1 and Ref. 14). In contrast with LUV, PS SUV are able to stimulate glucosylceramidase by themselves at pH 5.5–5.8, but they become poor activators when the pH is lowered to 5.0 (Fig. 1). Again Sap C promotes the stimulating capacity of PS SUV at this lower pH (Fig. 1). It is thus evident that at pH 5.0 Sap C is required by both PS LUV and SUV for the glucosylceramidase stimulation. Liposomes of pure PC are unable to activate glucosylceramidase either in the presence or in the absence of Sap C [2,14].

We have recently shown that Sap C at low pH interacts with PS vesicles [14]. Since several proteins capable of associating with vesicles cause an alteration of the lipid bilayer integrity, it was examined whether Sap C

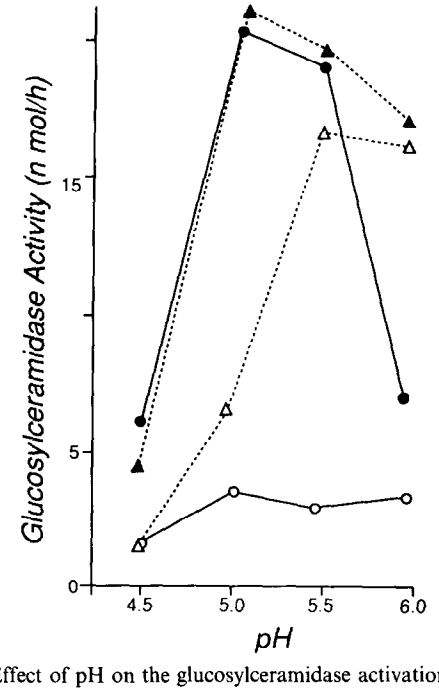

Fig. 1. Effect of pH on the glucosylceramidase activation by PS SUV and LUV in the presence and absence of Sap C. PS SUV (△,▲) or PS LUV (○,●) were added to the glucosylceramidase reaction mixture at the indicated pH values and the enzyme activity was measured in the absence (△,○) or in the presence (▲,●) of Sap C as reported in section 2.

might alter the permeability of PS membranes. When calcein-loaded PS LUV were treated with Sap C at pH 5.0, an instantaneous release of calcein occurred (Fig. 2A). The leakage depended on the ratio of Sap C to PS. Using PS LUV, an increase in the protein/lipid ratio from 1:5000 to 1:200 (the molecular mass of Sap C is about 10,000 Da) caused an increase in the percent leakage from 40 to 90% at the end of a 5 min incubation period. Fig. 2B shows that the activator protein induces leakage also from PS SUV but at protein/lipid ratios higher than those necessary for LUV.

An increase of the pH of the incubation medium altered the interaction of Sap C with the PS bilayer; at pH 5.8 the extent of Sap C-induced leakage from PS LUV was dramatically reduced (Fig. 3A).

The influence of bilayer composition on the Sap C-destabilizing effect was investigated with LUV containing different PS:PC molar ratios. As shown in Fig. 3B the presence of PS was essential for significant leakage to occur.

Given these results it can be assumed that under the conditions of optimal glucosylceramidase activation (see Fig. 1) the PS bilayers are destabilized by Sap C.

3.2. Sap C-induced fusion of PS-containing vesicles

The large Sap C-induced destabilization of the bilayer structure suggested the possibility that Sap C might induce also fusion. Resonance energy transfer between NBD-PE, as an energy donor, and Rh-PE was used to assay for liposome fusion [21]. Fig. 4 shows that at low pH Sap C, at concentrations exceeding 2 μM, induces with both PS SUV and LUV a rapid merging of membrane lipids. Initial vesicle size was not a major factor in the lipid mixing process. Mixing was not observed at neutral pH (data not shown).

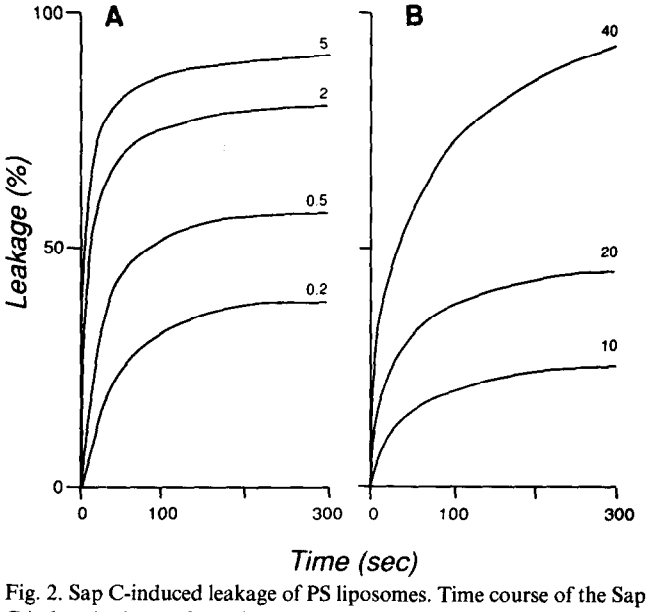

Fig. 2. Sap C-induced leakage of PS liposomes. Time course of the Sap C-induced release of calcein entrapped in PS LUV (A) or PS SUV (B). Different amounts of Sap C were injected into a stirred cuvette thermostated at 37°C, containing a 1-ml solution of liposomes (100 μM in lipid) in buffer A, pH 5.0. The final concentrations of Sap C in the mixtures, expressed as μg/ml, are indicated in the figures.

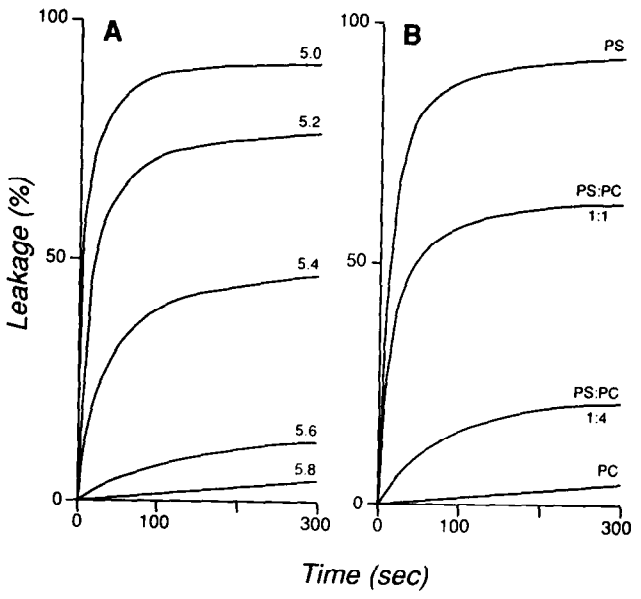

Fig. 3. Effect of pH and of bilayer composition on the Sap C-induced leakage of liposomes. Sap C-induced release of calcein entrapped in PS LUV as a function of either pH (A) or bilayer composition (B). (A) Sap C (5 μg) was injected into a stirred cuvette thermostated at 37°C, containing a 1-ml solution of PS LUV (100 μM in lipid) in buffer A, at the indicated pH values. (B) Sap C (5 μg) was injected into a stirred cuvette thermostated at 37°C, containing a 1-ml solution of LUV (100 μM in lipid) composed of only PS or PS/PC 1:1 or PS/PC 1:4 or only PC in buffer A, pH 5.

The increase of NBD fluorescence, observed upon addition of Sap C, while effectively showing a dilution of the fluorescent probe over the lipid pool does not unequivocally demonstrate the occurrence of fusion; an analogous increase would be expected to occur as a result of the fluorescent probe exchange among intact liposomes induced by the presence of Sap C. To rule out this possibility, the effect of Sap C on sonicated PS vesicles was analyzed by gel permeation chromatography. After incubation with Sap C most vesicles chromatographed in the void volume of a Biogel A-50m column in contrast with vesicles incubated in the absence of Sap C which migrated in the included fraction (Fig. 5). The increase of vesicle size shown by the gel permeation chromatography would not be expected if the increase in NBD fluorescence was due to probe exchange.

An additional proof of the Sap C-induced fusion came from the electron microscopic studies (Fig. 6). The diameter of both PS SUV and PS LUV increased more than 10-fold after the addition of Sap C at pH 5.

4. Discussion

The ability of Sap C to activate glucosylceramidase in

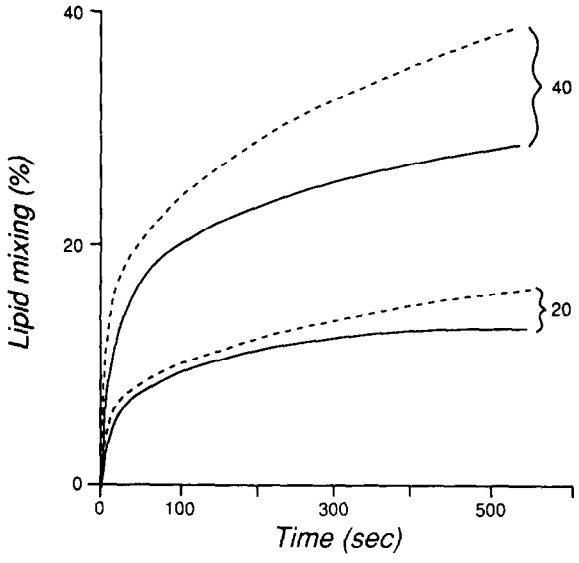

Fig. 4. Sap C-induced lipid mixing of PS liposomes. Time course of the Sap C-induced lipid mixing of PS SUV (---) or PS LUV (–). The indicated amounts of Sap C (expressed as μg/ml) were injected into a stirred cuvette thermostated at 37°C, containing a 1-ml solution of PS SUV or LUV (100 μM in lipid) in buffer A, pH 5.0. The lipid mixing was measured as reported in section 2.

the presence of PS has been known for quite a long time [1,2,23]. In contrast to previous studies that excluded a direct interaction between the activator protein and PS [13], we recently presented evidence for the spontaneous association of Sap C with PS bilayers [14]. In the present study we show for the first time that, upon addition of Sap C, structural changes of PS vesicles occur. The association with Sap C causes membrane destabilization as manifested by the leakage of the PS liposomes content. The Sap C-induced leakage is pH-dependent and is affected by the lipid composition of the bilayer. These findings reinforce our data on the strong affinity of Sap C for acidic phospholipid vesicles [14].

Sap C is effective in destabilizing both large and small PS vesicles. Although the use of LUV as models for biological membranes offers a significant advantage over SUV, it was important to assess whether Sap C also affected the lipid organization of small vesicles, since sonicated PS dispersions have represented the phospholipid preparations most commonly used to activate glucosylceramidase [2,12,13].

The commonly accepted model for the glucosylceramidase activation by Sap C and PS is based on the assumption that the enzyme binds directly Sap C [2,8,9,12] and promotes the solubilization of a limited number of PS molecules by binding the phospholipid at a site different from that of Sap C [13]. Based on our previous [14,18] and present results a new model is proposed. Sap C primarily interacts with PS-containing liposomes and destabilizes the lipid surface promoting the insertion of glucosylceramidase into the bilayer. Upon association with liposomes the enzyme might acquire its full activity in consequence of a conformational transition. According to this model the role played by Sap C in stimulating glucosylceramidase is mediated by its destabilizing effect on the membranes. This view is supported by our previous observation that organizational defects in the PS bilayer are required for the enzyme activation [18]. Actually, PS SUV, whose very small radius of curvature represents an intrinsic destabilization factor, are able to bind and stimulate glucosylceramidase also in the absence of Sap C (present paper and [18]).

We have found that Sap C also promotes the fusion of PS vesicles in a pH-dependent fashion. Fusion was evidenced with different techniques: resonance energy transfer, gel permeation and electron microscopy. The three methods gave concordant results, showing that, under appropriate conditions, Sap C transforms liposomes into much larger structures. Therefore Sap C can be considered a new member of the class of fusion proteins which, like clathrin or synexin, induce and/or enhance destabilization and fusion of artificial and biological membranes [24].

It would be of particular interest to see if Sap C, a

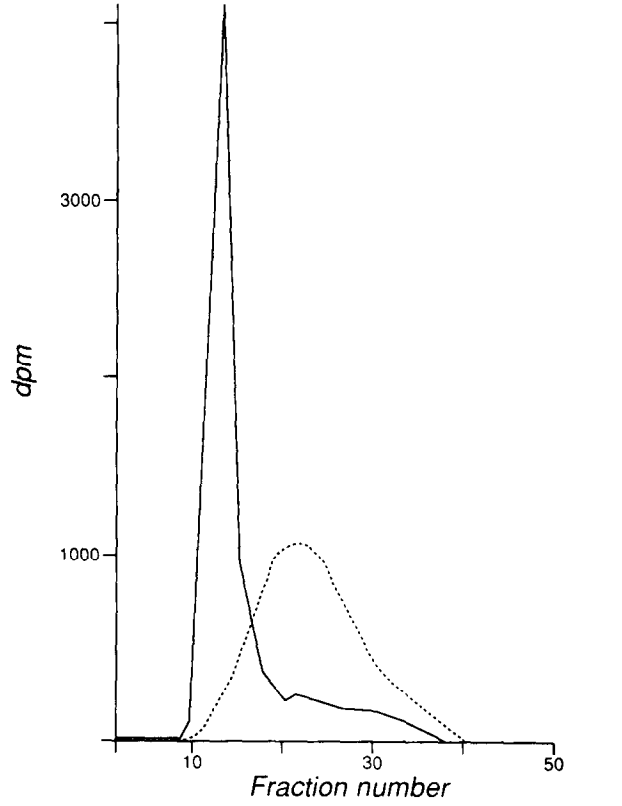

Fig. 5. Gel filtration of PS SUV incubated with or without Sap C. PS SUV (100 μg) were loaded on a Bio-Gel A-50m column after incubation without (---) or with (–) Sap C (40 μg) in buffer A, pH 5, at 37°C for 30 min. Trace amounts of labelled [^{14}C]PS was mixed with unlabelled PS prior to preparation of vesicles. The elution conditions were as reported in section 2. The liposome distribution (---, –) was determined by measuring the radioactivity.

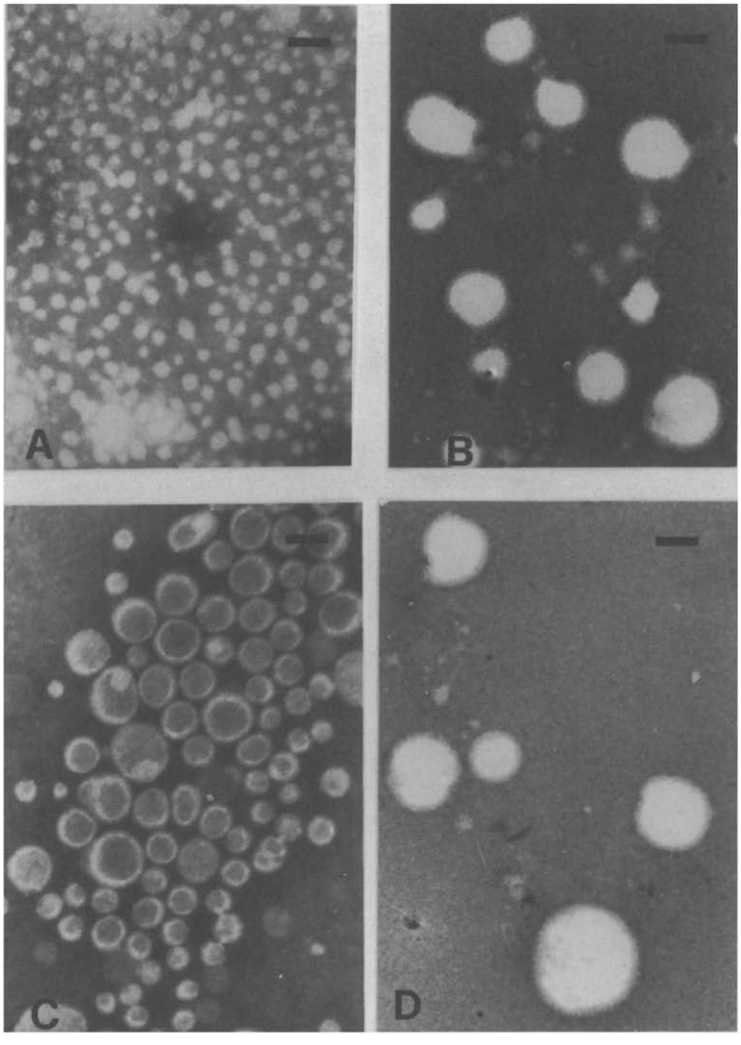

Fig. 6. Electron micrographs of liposomes. In a final volume of 0.3 ml, 100 μg of either PS SUV (A,B) or PS LUV (C,D) were incubated in buffer A, pH 5, at 37°C for 30 min in the absence (A,C) or in the presence (B,D) of 40 μg of Sap C. The negative stain of the vesicles was performed as reported in section 2. The bars denote 85 nm in A and C and 500 nm in B and D.

widely distributed protein [25], is capable of inducing fusion of natural membranes. The requirement of a low pH suggests that Sap C might exert its action only in cell compartments where an acidic environment is present such as lysosomes, where Sap C has been localized [15]. Ultrastructural findings of intralysosomal vescicular storage inclusions in skin biopsies from patients with a complete deficiency of prosaposin and thus of the four saposins (A,B,C,D) generated from it, have been reported [15]. This observation suggests that under normal conditions the accumulation of intralysosomal vesicles is prevented by the presence of one or all saposins. In consideration of its fusogenic activity, the possibility that Sap C mediates the fusion of these vesicles with the lysosomal membrane should be taken into consideration.

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