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Function of saposin C in the reconstitution of glucosylceramidase by phosphatidylserine liposomes

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The function of saposin C (Sap C), a glucosylceramidase activator protein, in the enzyme stimulation by phosphatidylserine (PS) liposomes has been investigated. Using gel filtration experiments evidence was obtained for Sap C binding to PS large unilamellar vesicles (LUV) but not to glucosylceramidase. PS LUV, which by themselves are unable to tightly bind and stimulate the enzyme, acquire the capacity to also bind the enzyme after interaction with Sap C, making it express its full activity. Our results indicate that the primary step in the Sap C mode of action resides in its association with PS membranes; in turn, this association promotes the interaction between the membranes and glucosylceramidase.

Glucosylceramidase reconstitution; Saposin C; Phosphatidylserine liposome

1. INTRODUCTION

Glucosylceramidase (EC, 3.2.1.45) is the lysosomal enzyme which hydrolyses glucosylceramide to ceramide and glucose [1,2]. It has been reported that glucosylceramidase needs the assistance of a small glycoprotein cofactor, called saposin C (Sap C) or SAP-2, to attack glucosylceramide [3–6]. Actually, Gaucher's disease, an autosomal recessive disorder characterized by glucosylceramide accumulation, can be caused either by a deficiency of glucosylceramidase or of Sap C [5,6].

Sap C, together with saposins A,B and D, is derived from a single precursor protein [7,8]. The four saposins are structurally similar to each other. Saposin B stimulates the enzymatic hydrolysis of various sphingolipids, including sulfatide, GM1 ganglioside and globotriaosylceramide; saposin A and C that of glucosylceramide and galactosylceramide and saposin D that of sphingomyelin [9,10].

Glucosylceramidase is tightly bound to the lysosomal membrane; its activity is markedly impaired by delipidation and purification [4]. The purified enzyme can be reactivated by addition of acidic phospholipids, the reconstitution being facilitated by the presence of Sap C [4,11].

In vitro studies with glucosylceramidase, Sap C and acidic phospholipids are essential to understanding the physiological conditions for the enzyme action. The mechanism of glucosylceramidase activation by the combination of an acidic phospholipid such as phosphatidylserine (PS) and Sap C has been extensively investigated. It has been proposed that PS and activator protein bind at two different sites of the enzyme for the activation [12,13]; Sap C forms a complex with glucosylceramidase but not with PS [13].

In previous investigations on the interaction between acidic phospholipids and glucosylceramidase [14–16] we have shown that the enzyme activation occurs through the association of the enzyme with PS-containing liposomes. The physical organization of the lipid surface is of critical importance for this association; actually, small PS vesicles tightly bind the enzyme, while large vesicles are unable to reconstitute glucosylceramidase [16].

In search for specific compounds that might have a role in the association of glucosylceramidase with membranes, we have investigated whether Sap C can promote the reconstitution of fully active glucosylceramidase by PS LUV. This has led us to reexamine the interactions between the activator protein, the enzyme and the liposomes.

2. MATERIALS AND METHODS

2.1. *Materials*

Sodium taurocholate (TC) (synthetic, > 98% pure), and 4-methylumbelliferyl-β-D-glucopyranoside (MUGlc) were from Sigma (St. Louis, MO, USA). Triton X-100 was from Pierce (Rockford, Illinois, USA). PS from bovine spinal cord was from Calbiochem (San Diego, CA, USA). 1,2-Dioleoyl-3-*sn*-phosphatidyl-L-[3-^{14}C]serine (54 mCi/mmol) was from Amersham International (UK). The 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. [17].

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2.3. *Saposin C preparation*

Sap C was purified from spleens of patients with Type 1 Gaucher disease. The isolation procedure was basically the same as that described for Sap C from guinea pig [18]. It consisted of hot-water extraction of spleen and consecutive column chromatographies with DEAE-cellulose, Sephadex G-75 and reverse-phase HPLC with a C_4 column (Vydac). The preparation was pure according to SDS-PAGE and Western blot analysis. The purity and identity of Sap C was confirmed by automated Edman degradation of the protein, performed with an Applied Biosystems model 476A gas-phase sequencer.

2.4. *SDS-PAGE and immunoblots*

SDS-PAGE was performed by the method of Laemmli with 15% acrylamide [19]. After electrophoresis the samples were electroblotted to PVDF (polyvinylidene difluoride) membrane (Bio-Rad, Richmond, USA) and detected with a monospecific antibody against Sap C (kind gift of Dr. D. Wenger, Jefferson University, Philadelphia) using a Vectastain ABC kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions.

2.5. *Glucosylceramidase and Sap C assays*

The standard assay mixture used to measure glucosylceramidase activity contained, in a final volume of 0.2 ml: 0.1/0.2 M citrate/phosphate buffer (pH 5.6), 2.5 mM MUGlc, 0.1% (v/v) Triton X-100 and 0.25% (w/v) TC. When the stimulatory capacity of PS and/or Sap C was evaluated, the assay mixture contained, in a final volume of 0.2 ml: 10 mM acetate buffer (pH 5.4), 150 mM NaCl, 1 mM dithioerythritol and 1 mM EDTA (buffer A), 2.5 mM MUGlc and different amounts of lipid vesicles and/or Sap C as specified in the experiments. The effect of Sap C was also checked in the presence of Triton X-100, as previously reported [12,13]. In this case the assay mixture contained, in a final volume of 0.2 ml: 50 mM acetate buffer (pH 4.5), 2.5 mM MUGlc and 0.05% (v/v) Triton X-100.

The assay mixtures were incubated for 30 min at 37°C. The extent of reaction was estimated fluorometrically. All assays were carried out in duplicate and agreed within 5%.

1 U of glucosylceramidase is defined as the amount of enzyme which hydrolyzes 1 nmol of MUGlc/h in the standard assay.

2.6. *Protein determination*

The proteins were measured with the bicinchoninic acid method [20] with bovine serum albumin as standard.

2.7. *Vesicle preparation*

PS vesicles were prepared as previously described [16]. In short, small unilamellar vesicles (SUV) were obtained by submitting the lipid suspension to sonication under nitrogen in a Branson B 15 Sonifier, while large unilamellar vesicles (LUV) were prepared by filter exclusion through two stacked 0.1 μm pore size polycarbonate filters (Nucleopore Corp., Pleasanton, CA) using a high pressure extrusion apparatus (Lipex Biomembranes Inc., Vancouver, BC).

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

To analyze the physical interaction of Sap C with lipid vesicles and glucosylceramidase, the samples were incubated for 15 min at 37°C in buffer A and then loaded on Bio-Gel A-50m columns (10 × 170 mm; Bio-Rad, Richmond, USA) preequilibrated and eluted with the same buffer. When the sample contained glucosylceramidase, the column was first eluted with 20 ml of buffer A and then with buffer containing 2% (w/v) TC. A new column was used for each experiment. The flow rate was 0.3 ml/min for all columns. Fractions of 0.4 ml were collected. Chromatographic separations were carried out at room temperature.

3. RESULTS AND DISCUSSION

3.1. *Effect of Sap C on glucosylceramidase activation by PS LUV*

We have previously shown that, at difference with PS SUV, PS LUV are unable to fully stimulate purified glucosylceramidase [16]. At a concentration of PS LUV up to 10 μg/ml less than 10% of the activity measured in an assay medium containing either detergents (standard assay, see section 2) or PS SUV was observed (Table I). Thus we have investigated the possibility of promoting enzyme activation by PS LUV with Sap C, a well-known glucosylceramidase activator [3,4]. As a matter of fact we have found that Sap C dramatically enhances the stimulating power of LUV (Fig. 1), while it has a small effect on the activation exerted by SUV, by themselves able to efficiently stimulate the enzyme (Table I). The stimulation in the Sap C-PS LUV system is similar to the optimum obtained both in the standard and the PS SUV systems (Table I). In agreement with previous reports [4,11], Sap C alone is nearly uneffective on glucosylceramidase activity (Fig. 1 and Table I). The pH optimum of glucosylceramidase in the Sap C-PS LUV system is in the range 5.0–5.4, slightly lower than that previously found when PS SUV were used as activators (pH 5.8, Ref. [16]).

Since several authors who have investigated the mechanism of glucosylceramidase activation by Sap C have included Triton X-100 in the assay [12,13], we have compared the stimulation exerted by Sap C in the presence of either PS LUV or Triton X-100. As shown in Table I the Sap C–PS LUV system is much more efficient than the Sap C–Triton X-100 system. Most probably the stimulation mediated by the detergent is related with the reported formation of a high molecular weight complex containing Sap C and Triton X-100 micelles [21].

3.2. *Physical interaction between Sap C, PS LUV and glucosylceramidase*

In a previous paper it was shown that the low stimulation of PS LUV depended on the poor interaction between this type of vesicles and glucosylceramidase [16]. The positive effect of Sap C on the PS LUV stimulating ability suggested that this protein favoured in some way the phospholipid–enzyme interaction. Two mechanisms can be envisaged for this effect: either Sap

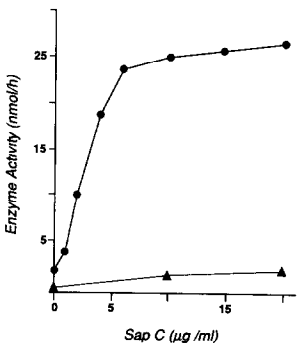

Fig. 1. Effect of Sap C on the stimulation of glucosylceramidase activity by PS LUV. Different amounts of Sap C were added to 200 μl buffer A containing 2.5 mM MUGlc and 25 U purified glucosylceramidase. Enzyme activity was measured either without further additions (▲) or in the presence of 10 μg/ml of PS LUV (●).

Table I

Comparison of the effect of Sap C on glucosylceramidase activity under various conditions

Conditions	Enzyme activity (nmol/h)	
	− Sap C	+ Sap C
1.	0.6	1.5
2. (PS LUV)	1.5	26
3. (PS SUV)	24	28
4. (Tri)	0.5	4
5. (Tri-TC)	25	25

Glucosylceramidase assays contained, in a final volume of 200 μl, 25 U of enzyme, 2.5 mM MUGlc and the following compounds. 1, buffer A; 2, buffer A and 2 μg PS LUV; 3, buffer A brought at pH 5.8 and 2 μg PS SUV; 4, acetate buffer 50 mM, pH 4.5 and 0.05% (v/v) Triton X-100; 5, citrate-phosphate buffer 0.1 M, pH 5.6, 0.1% (v/v) Triton X-100 and 0.25% (w/v) TC (standard system). Enzyme activity was measured in the absence or in the presence of 10 μg/ml of Saposin C. The different buffers were chosen so as to obtain optimal stimulation under the various conditions. Abbreviations: Tri, Triton X-100; TC, taurocholate.

C associates with PS LUV making the lipid surface more suitable for binding glucosylceramidase, or Sap C associates with the enzyme increasing the affinity of glucosylceramidase for PS LUV. Evidence for complex formation between Sap C and either PS LUV or glucosylceramidase was sought by a previously used gel permeation chromatographic technique [16]. As shown in Fig. 2A, Sap C elutes in the included volume and can activate glucosylceramidase only when PS LUV are added to the assay. On the contrary, Sap C, incubated with PS LUV before chromatography, coelutes with the phospholipid and fully stimulates the enzyme (Fig. 2B). The position of Sap C in the elution patterns was confirmed by immunoblots of the column fractions with a Sap C monospecific antibody.

Different to what happens with PS LUV, the elution of Sap C is not affected by previous incubation with glucosylceramidase. In fact, the mixture of the two proteins gives rise to well resolved peaks, one of Sap C eluting between fractions 25–40 (see Fig. 2A) and one of glucosylceramidase eluting between fractions 82–95 (see Fig. 3). It thus appears that, if a complex between glucosylceramidase and Sap C is formed at all, it is much weaker than that between Sap C and PS LUV.

Since on interaction with Sap C large PS liposomes acquire the capacity to reactivate glucosylceramidase, one can expect that likewise they become able to bind the enzyme. Actually, glucosylceramidase incubated with the PS LUV–Sap C complex coelutes with the phospholipid and the activator protein (Fig. 3). In the absence of Sap C, PS LUV and glucosylceramidase are completely separated, the enzyme being retained by the Bio-gel column, as previously reported [16].

Complex formation between saposins (A, B, C, D) and acidic phospholipids was previously excluded on the basis of experiments performed by incubating the water-soluble saposins with PS coated on Celite [13]. The lack of aqueous solubilization of PS was interpreted as lack of interaction between the protein activators and the lipid. The different experimental approaches followed by these authors and by us might explain the opposite conclusions that have been reached.

Up to now the role of Sap C in the glucosylceramidase activation has been explained as the result of the activator protein binding to the enzyme [4,10,12,13] rather than to lipids. The main line of evidence for a direct binding of Sap C to glucosylceramidase was the observation that Sepharose-linked Sap C acted as an affinity column for the enzyme [12]. It must be noted that the enzyme loaded on the affinity column was not purified and that the elution buffer contained Triton X-100, a detergent that has a strong affinity for both the activator protein [21] and glucosylceramidase. Thus, binding of glucosylceramidase to the Sepharose-linked Sap C might in fact reflect interactions of the two proteins with some lipid present in the enzyme preparation or/and with Triton X-100.

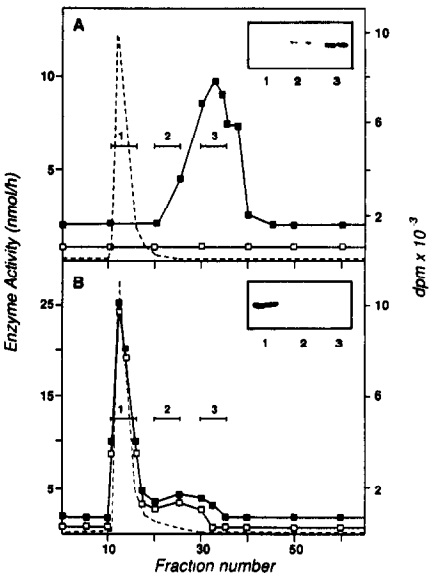

Fig. 2. Physical interaction of Sap C with PS LUV analyzed by chromatography on Bio-Gel columns. Purified Sap C (25 μg) and PS LUV (80 μg) were loaded on Bio-Gel A-50m columns either separately (A) or after mixing with each other (B). Trace amounts of labelled [^{14}C]PS was mixed with unlabelled PS prior to preparation of vesicles. The elution was performed as reported in section 2. The liposome distribution (– –) was determined by measuring the radioactivity. The glucosylceramidase activation by Sap C was tested by incubating an aliquot of the fractions (50 μl) with 150 μl buffer A containing 3.4 mM MUGlc and 25 U purified glucosylceramidase either in the absence (□) or in the presence of 10 μg/ml of PS LUV (■). The inserts show the immunoblots of pooled fractions corresponding to the bars (1, fractions 10–15; 2, fractions 20–25; 3, fractions 30–35). After SDS-PAGE analysis and electophoretic transfer of proteins, Sap C was visualized with rabbit anti-Sap C (see section 2). When Sap C (25 μg) was incubated with glucosylceramidase (up to 10,000 U) instead of PS LUV, the elution pattern of the activator protein was identical to that shown in (A).

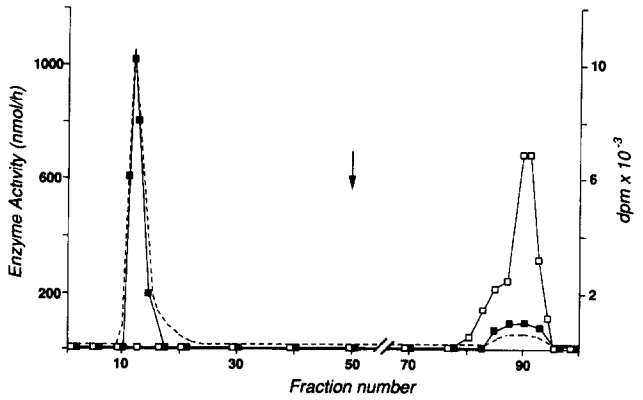

Fig. 3. Physical interaction of glucosylceramidase with PS LUV in the presence or in the absence of Sap C. Purified glucosylceramidase (3000–4000 U) was incubated with PS LUV (80 μg) in the absence (□) or in the presence (■) of 25 μg of Saposin C. The elution was performed as described in section 2. The arrow indicates when TC was added to the eluent. The distribution of PS vesicles (– –) was determined as in Fig. 2. The glucosylceramidase elution was estimated measuring its activity according to the standard assay (see section 2). When glucosylceramidase was analyzed after incubation with Sap C in the absence of PS LUV the enzyme eluted from the column only after the addition of TC to the buffer (□).

Sap C has a structure similar to that of the other three saposins (A, B, D). It was noted that there is also an extensive homology between the major pulmonary surfactant-associated protein SP-B and the four saposins [10,22]. This homology raises the possibility that the mode of their action may also be similar. Saposin B (Sap B) has been shown to bind to various lipids including sulfatides and gangliosides [9]. Similarly to Sap B, pulmonary surfactant-associated protein SP-B binds lipids interacting selectively with phosphatidylglycerol [23]. Our finding that also the activity of Sap C depends on the interaction with appropriate lipids indicates that the similar structures of SP-B, Sap B and Sap C actually correspond to similar function; the properties of all of these depend on lipid binding.

In conclusion our studies indicate that in some cases not only acidic phospholipids but also Sap C are required for the glucosylceramidase binding to membranes. We propose that the glucosylceramidase stimulation by the protein activator derives from a facilitated binding of the enzyme to a membrane surface somehow modified by its interaction with Sap C. According to this model Sap C would be the first example of a sphingolipid activator protein that acts by associating mainly with an activator lipid rather than with an enzyme or with a substrate.

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