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Saposin D: A Sphingomyelinase Activator

Satoshi Morimoto*, Brian M. Martin#, Yasuo Kishimoto*[1] and John S. O'Brien*

*Department of Neurosciences, Center for Molecular Genetics, School of Medicine, University of California San Diego, La Jolla, California 92093

#National Institutes of Health, National Institute for Mental Health, Molecular Neurogenetics Unit, Building 10, Room 3D16, Bethesda, Maryland 20892

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SUMMARY: Saposin D, a newly discovered heat-stable, 10 kDa glycoprotein, was isolated from Gaucher spleen and purified to homogeneity. Chemical sequencing from its amino terminus demonstrated colinearity between its amino acid sequence and the deduced amino acid sequence of the fourth domain of prosaposin, the precursor of saposin proteins. Saposin D specifically stimulates acid sphingomyelinase but has no significant effect on the other hydrolases tested.

INTRODUCTION: The lysosomal hydrolysis of sphingolipids is catalyzed by the sequential action of acid hydrolases. Several enzymatic hydrolytic steps are stimulated by small heat-stable proteins which interact with either the substrate or the enzyme or both. Two of the heat-stable proteins have been structurally defined. The first protein, named saposin B in this report and previously designated an activator of cerebroside sulfatase (1), G_{M1} activator (2), sphingolipid activator protein 1 (3), and dispersin (4), stimulates the hydrolysis of galactocerebroside sulfate (sulfatide) by arylsulfatase A (1,5,6), G_{M1} ganglioside by ß-galactosidase (2,7) and globotriaosylceramide ($Gb0se_3Cer$) by α-galactosidase A (8). Immunological and genetic evidence has demonstrated that saposin B activates all three reactions (3,8). In a recent study, Li *et al.* (9) showed that this protein has an even broader substrate specificity than reported previously, promoting the hydrolysis of the above three glycosphingolipids, as well as G_{M2} ganglioside, sulfate esters of lactosylceramide and asialo G_{M2}, globoside, three neolacto-type glycolipids and two glycerylglycolipids. From these results, Li *et al.* (9) concluded that the hydrolysis of these lipids is

[1] To Whom Correspondence Should Be Addressed.

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promoted by a nonspecific detergent action of saposin B and called it "nonspecific activator protein". The primary structure of saposin B was determined by sequencing of a cDNA encoding it (10). The physiological significance of saposin B is underscored by the discovery of its absence in a variant form of metachromatic leucodystrophy (8).

The second protein, called saposin C in this paper and previously termed factor P (11), glucocerebrosidase activator (12), coglucosidase (13), sphingolipid activator protein 2 (3), and A_1 activator (14), stimulates the hydrolysis of glucosylceramide by glucosylceramidase (11-14), that of galactocerebroside by galactosylceramide ß-galactosidase (15) and that of sphingomyelin by sphingomyelinase (15). Unlike saposin B, saposin C appears to interact with enzymes to exert its stimulatory effect (16). The primary structure of saposin C was determined by chemical sequencing of its amino acids (17, 18) and by deducing its sequence from nucleotide sequencing of a cDNA encoding prosaposin, its precursor (19). The physiological significance of saposin C was demonstrated by discovery of its deficiency in a variant form of Gaucher's disease (20).

Recently, this laboratory has elucidated the complete nucleotide sequence of a cDNA coding for prosaposin, the precursor protein of both saposin B and C (19). Prosaposin was found to contain 4 saposin domains, two of which are saposin B and C; these are flanked by two additional domains we call saposin A and saposin D. Each saposin domain (A,B,C,D) is approximately 80 amino acid residues in length, has nearly identical placement of cysteine residues, glycosylation sites and helical regions and is flanked by proteolytic cleavage sites. Molecular models indicate that the proteins derived from each domain can fold to give rise to a conformationally rigid hydrophobic pocket held together by 3 disulfide bridges. We predicted that proteolytic cleavage of prosaposin at each domain boundary should give rise to all 4 saposins. We have recently isolated saposin D, the protein arising from domain 4. Saposin D appears to specifically stimulate sphingomyelinase to hydrolyze the phosphorylcholine moiety of sphingomyelin.

METHODS

Isolation of Saposin D. Techniques used for the isolation of saposin D were, in general, similar to those used for the isolation of coglucosidase by Sano and Radin (18). Briefly, 201 grams of spleen from a patient with adult type Gaucher's disease was homogenized with 800 ml of water and the homogenate was heated at 100°C for 10 min. The mixture was then centrifuged at 10,000 x *g* for 15 min. and the supernatant was fractionated by ammonium sulfate precipitation. The precipitates which appeared between 45 and 80% saturation, were collected and dialyzed. The dialysate was then fractionated by column chromatography on DEAE-cellulose (DE-52, Whatman). The column was eluted with a 0-0.5M NaCl linear gradient in 10 mM sodium phosphate buffer, pH 7.0. Fractions positive for stimulation of hydrolysis of 4-methylumbelliferone (4-MU) ß-D-glucoside (see below) were pooled, dialyzed and lyophilized. The residue, after dissolving in a small volume of water, was fractionated

by gel filtration using Sephadex G-75. The fractions containing stimulating activity for 4-MU glucoside hydrolysis were pooled and further fractionated by HPLC. A Varian model 5000 HPLC equipped with a Vydac protein C4 column (4.6 mm by 25 cm) was used and fractions were eluted with a linear gradient of water containing 0.1% trifluoroacetic acid and acetonitrile-water (4:1) containing 0.1% trifluoroacetic acid. Peaks were detected by measuring absorbance at 220 nm. A typical chromatogram is shown in Fig. 1. The effluent comprising the saposin D peak which eluted later than saposin B and C (shown in Fig. 1) was collected and evaporated to dryness.

Immunological Procedures: Antibodies against saposin D were prepared in a rabbit as described by Crowle (21) and purified with an affinity column which contained purified saposin D linked to Affi-Gel 10 Activated Affinity Support (Bio-Rad) as described previously (10). Rabbit antibodies against saposin B and C were provided by Dr. Arvan Fluharty and Dr. David Wenger, respectively. Dot and Western blots with these antibodies were performed as described previously (10).

Assays for Lysosomal Enzyme Activities: Sphingomyelinase activity was assayed as described by Wenger (22). [Methyl-^{14}C]sphingomyelin obtained from Dr. D. A. Wenger or from NEN/DuPont, was used as substrate. Human placental sphingomyelinase purchased from Sigma Chemical Co. or a Triton X-100 extract of a particulate fraction of human liver, as described by Radin and Berent (23) was used as an enzyme source. Galactoceramide ß-galactosidase and ß-glucosylceramidase were assayed as described by Wenger *et al.* (15). [G-^{3}H]Galactosylceramide was prepared as described previously (24). [G-^{3}H]Glucosylceramide was a gift from Dr. D. A. Wenger. Human spleen was homogenized with two volumes of water and centrifuged at 12,500 x g for 10 min and the supernatant obtained was used as an enzyme source of galactosylceramide ß-galactosidase. The same human liver preparation used for sphingomyelinase activity was used as enzyme source for ß-glucosylceramidase assay. G_{M1} ganglioside ß-galactosidase activity was assayed as described previously (25). The substrate [galactose-^{3}H] ganglioside G_{M1} which was synthesized as described (26) and purified human liver acid ß-galactosidase were provided by Dr. Yoshimi Yamamoto of this laboratory. The hydrolysis of 4-MU ß-D-glucoside or ß-D-galactoside was performed as described previously (27).

Other Analytical Methods: Peptide sequencing was accomplished using an Applied Biosystems model 470A gas-phase sequencer equipped with a model 120A on-line PTH analyzer using standard procedures supplied by the manufacturer. Protein concentration and carbohydrate content were determined by the method of Lowry *et al.* (28) and by the phenol sulfuric acid method (29).

RESULTS

Isolation and Characterization of Saposin D. Saposin D was isolated from Gaucher spleen by taking advantage of its heat-stability, acidity, small molecular size, and hydrophobicity. Since saposin B and C were also present in the DEAE column fractions containing saposin D its final purification was achieved by preparative hydrophobic HPLC. The yield of pure saposin D was 16.1 mg from 201 g of Gaucher spleen. Saposin D was eluted with a higher acetonitrile percentage than saposin B or C indicative of its greater hydrophobicity (Fig. 1). Saposin D contains a single glycosylation site (19) and was found to contain 16% carbohydrate by phenol-sulfuric acid analysis. Polyacrylamide gel electrophoresis in SDS (Fig. 2) gave a subunit molecular weight of approximately 10 kDa for saposin D similar to saposin B and C. Antibodies raised against saposin D did not cross react with saposin B or C (Fig. 2). Conversely, antibodies against saposin B or C did not cross react with saposin D

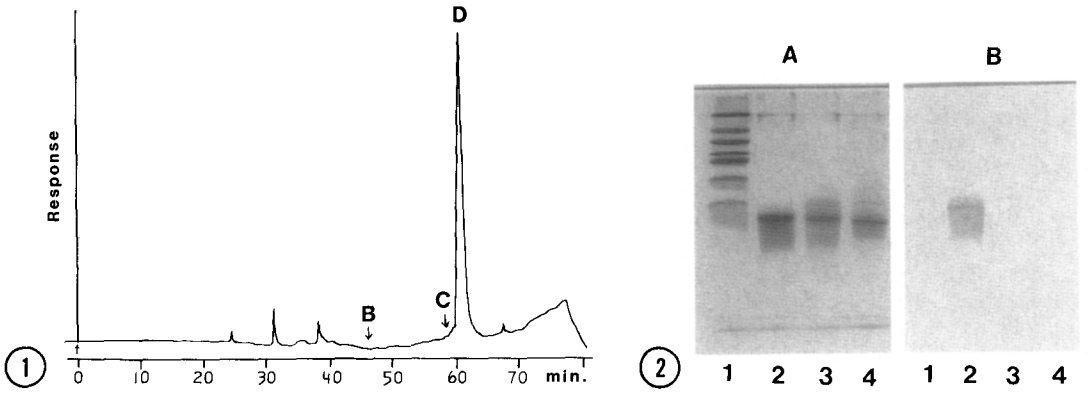

Figure 1. HPLC of Saposin D. Purified saposin D was analyzed by HPLC as described in Methods. Under the same conditions, saposin B and C appear with retention times as indicated by arrows.

Figure 2. SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting of Saposin D. The purified saposin D (8.6 μg, lane 2 and 6) was electrophoresed together with saposin C (7.6 μg, lane 3 and 7) and saposin B (13.6 μg, lane 4 and 8) on 17.5% polyacrylamide slab gels containing SDS. Lanes 1 and 5 contained a protein standard mixture (Sigma Chemical Co.). The lowest protein marker is α-lactalbumin (14,200 Da). Section A was stained with Coomassie brilliant blue and B was immunoblotted with anti-saposin D antiserum as described in Methods.

(data not shown). Chemical sequencing of the N-terminal amino acids of saposin D revealed the first 19 amino acids to be identical to the polypeptide predicted to arise from proteolysis of domain 4 of prosaposin (amino acids 392 to 474) (19).

Specificity of Sphingomyelinase Stimulation by Saposin D. In the presence of Triton X-100, sphingomyelinase activity was stimulated 100% by as little as 2 μg (less than 1 μM) of saposin D as shown in Fig. 3. Stimulation was not increased when the amount of saposin D was increased indicating near saturation at this concentration. Stimulation was not likely due to a nonspecific detergent nature of saposin D because the assay mixture contained sufficient Triton X-100 to solubilize the sphingomyelin present in the assay. In addition, saposin C, which stimulates ß-glucosylceramidase activity (16, 30) and has a structure similar to saposin D (17,18,19,31) did not possess stimulatory activity in the assay. With addition of Triton X-100, 27 μg and 54 μg of Saposin D increased the sphingomyelinase activity 3- and 5-fold, respectively. However, the activity obtained with 54 μg was only 10% of that in the presence of Triton X-100. Saposin D did not stimulate ß-glucosylceramidase activity (Fig. 3), galactosylceramide ß-galactosidase activity or G_{M1} ganglioside ß-galactosidase activity (Table 1). The hydrolysis of 4-MU ß-D-glucoside was slightly enhanced when saposin D was present (also shown in Table 1). Saposin D did not stimulate hydrolysis of the other 4-MU derivative substrates including pyrophosphate, α-galactoside, ß-galactoside, α-glucoside, α-mannoside, ß-fucoside, ß-glucuronide, and ß-N-acetylglucosamide (data not shown).

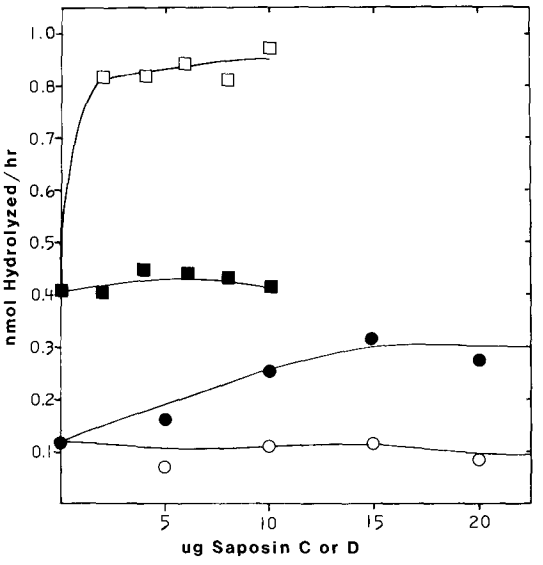

Figure 3. Effect of Saposin D on Sphingomyelinase and Glucocerebrosidase. Various amounts of saposin D were added to an assay mixture of sphingomyelinase and ß-glucosylceramidase activities and its effect was compared with that of saposin C. Sphingomyelinase activity assay mixture contained 20 nmol [methyl-^{14}C] sphingomyelin (830 cpm/nmol), 10 µg Triton X-100, and 50 µmol acetate buffer pH 5.0 in 200 µl. Crude sphingomyelinase prepared from human placenta (Sigma Chem. Co.) was used as enzyme source. Incubation was conducted at 37°C for 1 hr. ß-Glucosylceramidase assay mixture contained 5.25 nmol to [gluco-^{3}H) glucocerebroside (1358 cpm/nmol) and 20 µmol citrate-phosphate buffer pH 4.2 in 200 µl. Human liver particulate preparation solubilized by 0.1% Triton X-100 was used as enzyme source. The activities for sphingomyelinase with saposin D is shown by open squares (□) and that with saposin C is shown by closed squares (■). The activity for ß-glucosylceramidase with saposin D is expressed by open circle (O) and that with saposin C is expressed by closed circle (●). No further increase of ß-glucosylceramidase activity was observed by increasing saposins up to 35 µg each.

DISCUSSION

In accordance with our recent prediction that the proteolytic processing of prosaposin generates 4 saposin proteins of similar structure (19) we report here the isolation of a third saposin protein, saposin D, which stimulates the hydrolysis of sphingomyelin. Proof that saposin D arises from domain 4 of prosaposin was obtained by demonstrating colinearity between its first 19 chemically determined amino acids and the deduced sequence obtained from domain 4 of the prosaposin cDNA. In addition, saposin D has the correct predicted molecular weight (10 kDa) from proteolytic processing of domain 4. While this work was in progress Fürst *et al.* (31) isolated the same protein from Gaucher spleen termed component C; its amino acid sequence is identical to saposin D except for the absence of the first two N-terminal amino acids which may have been removed by peptidase action. No activating properties were reported for component C.

Saposin D has a remarkably high specificity in stimulating sphingomyelinase activity. Activation of sphingomyelinase activity by saposin C has also been reported

Table 1.

Effect of Saposin D on GM_1 Ganglioside ß-Galactosidase, Galactocerebroside ß-Galactosidase and 4-Methylumbelliferyl Glucoside ß-Glucosidase

	Saposin (µg)	nmol hydrolyzed/hr - Saposin	nmol hydrolyzed/hr + Saposin
GM_1 Ganglioside ß-galactosidase	B (42)	0	0.44
	D (45)	0	0.00
Galactosylceramidase	C (8)	0.41	0.57
	(16)	0.41	1.24
	(24)	0.41	1.48
	D (14)	0.41	0.34
	(54)	0.41	0.44
	(95)	0.41	0.44
4-Methylumbelliferyl glucoside ß-glucosidase	C (4)	3.70	9.00
	(10)	3.70	13.80
	(25)	3.70	13.80
	D (4)	3.70	5.50
	(10)	3.70	5.00
	(25)	3.70	5.00

(15); but in our hands saposin C failed to stimulate sphingomyelinase activity. Christomanou and her colleagues (20) purified two proteins, named A_1 and A_2, which stimulated sphingomyelinase activity; their A_1 protein was found by chemical sequencing to be identical to saposin C (17). It is possible that our HPLC-purified saposin C lost its sphingomyelinase activating property while retaining its ß-glucosylceramidase stimulating property. Alternatively, previous preparations of saposin C may have been contaminated with saposin D since their properties are very similar and extremely small amounts of saposin D are required for activation. Another explanation is the detergent-like property of saposin C which helped to solubilize sphingomyelin in the absence of Triton X-100.

Prior to the discovery that saposin B and C are both derived from the same precursor protein (19), these proteins were named nonsystematically. The isolation of saposin D reported here has prompted us to propose a new systematic nomenclature for the saposin proteins. As mentioned the precursor protein (prosaposin) contains 4 domains comprised of similar 80 amino acid modular units which are released upon proteolysis. We propose that those proteins which are derived from the first, second, third and fourth domains be named saposin A, B, C and D. Of the four, saposin A has yet to be isolated. Partial proteolysis of prosaposin also

yields intermediate cleavage products and these have been detected in most human tissues (19). We propose that proteins possessing two saposin domains with subunit molecular weights of 25-30 kDa be called disaposin A, B and C; containing domains 1 and 2, 2 and 3, and 3 and 4 respectively. Similarly, cleavage products containing three saposin domains with subunit molecular weights of approximately 48 and 43 kDa, be named trisaposin A and B; containing domains 1,2 and 3 and domains 2, 3, 4 respectively. In line with this proposal, we have recently isolated a glycoprotein from Gaucher spleen which appears to be disaposin A.

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