Vol. 181, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1991 Pages 286-292

ISOLATION AND CHARACTERIZATION OF PROSAPOSIN FROM HUMAN MILK

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Received October 14, 1991

Prosaposin is the precursor protein for saposins, which are small lysosomal proteins required for the hydrolysis of sphingolipids by specific lysosomal hydrolases. Prosaposin, in addition to generating the saposins in the lysosomes, also exists as an unprocessed ~70-kDa protein in many tissues and secretory fluids. In this study, we isolated prosaposin from human milk. Milk was fractioned by ammonium sulfate precipitation, then chromatographed with DEAE-Sephacel and G-3000 SW gel permeation-HPLC. A fraction containing prosaposin was finally purified with the anti-saposin C IgG attached affinity column. The protein staining of the purified preparation on SDS-PAGE and the Western blotting showed a single band. The sequence of the initial 10 amino acids from N-terminus of the purified protein was identical to the sequence of prosaposin deduced from cDNA. Although prosaposin itself showed ß-glucosidase activator activity at a slight degree, the activity increased much after trypsin treatment. Western blotting of the trypsin-treated sample confirmed the formation of small saposin-like bands from prosaposin by the action of trypsin.

Saposins are small heat-stable glycoproteins that are required for the hydrolysis of sphingolipids by specific lysosomal hydrolases [1]. The existence of prosaposin, a precursor protein for four saposins has been predicted from the analysis of the cDNA for saposin C [2-4]. Four saposin domains appear to be formed from prosaposin by a specific peptidase in the lysosomes. At the junctional boundaries of each saposin domain there occur basic amino acid residues that are potential proteolytic cleavage sites. The patterns of processing of prosaposin are quite different in different tissues in rat [5]. The precursor form is the major form in brain and muscle but cleavage products dominate in other tissues. Recently we demonstrated that prosaposin is secreted from platelets upon stimulation by thrombin and exists abundantly in various secretory fluids [6]. Collard et al. showed that the nucleotide sequence of the cDNA for rat sulfated glycoprotein 1 (SGP-1), one of the major secretory proteins from Sertoli cells in the testis, coincides in sequence with human prosaposin [7]. SGP-1 appears to have important effects on sperm cell differentiation, since it is secreted into seminiferous tubules from Sertoli cells during certain stages of spermatogenesis.

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0006-291X/91 $1.50

MATERIALS AND METHODS

Materials —Human milk was kindly provided by a woman who experienced a normal delivery. Human placenta was also generously provided by a woman who experienced a normal delivery. Gaucher spleen, an autopsy sample from a patient with type I Gaucher disease, was kindly provided by Dr. S. Tsuji of Niigata University and stored at -80°C before use. DEAESephacel and Sephadex G75 were from Pharmacia. Acrylamide, sodium dodecyl sulfate, and peroxidase conjugated anti-rabbit IgG were from Wako Chemical. Immobilon transfer membranes (polyvinylidene fluoride membranes) were from Millipore. 4-Methylumbelliferyl compounds were from Sigma. Affi-Gel Hz was from Bio-Rad.

Human saposins A, B, C, and D —These were isolated from human Gaucher spleen by the method for Gunia pig saposin C isolation of Sano et al. [8] or for human saposins of Morimoto et al. [9] with slight modifications. Gaucher spleen (100 g) was homogenized in boiling water. The hot water extract was fractionated by ammonium sulfate precipitation. A fraction bound to DEAE-Sephacel and eluted with 0.5 M NaCl was purified with a column of Sephadex G-75. The fraction containing saposin C activity was further purified with a DEAE-5PW (7.5 x 75 mm, Tosoh) high performance ion-exchange column. Fraction DE-I eluted with 0.07-0.13 M NaCl, fraction DE-II eluted with 0.13-0.21 M NaCl, and fraction DE-III eluted with 0.21-0.32 M NaCl. Each fraction was purified further with a C_4 reverse phase high performance chromatography column. Finally, 2.3 mg of saposin D was obtained from DE-I, 3.5 mg of saposin B and 2.8 mg of saposin A from DE-II, and 2.8 mg of saposin C from DE-III. The identity of each saposin was confirmed by amino acid analysis and N-terminal protein sequencing.

Monospecific anti-bovine saposin C was prepared as previously described [5].

SDS-PAGE and immunoblots —SDS-PAGE was performed by the method of Laemli with 15% acrylamide [10]. The samples were electroblotted to Immobilon membranes after electrophoresis. Blocking was performed with crude casein from bovine milk (provided by the Meiji Central Research Institute of Meiji Milk Products Co., Ltd.), and then saposin related peptides were detected indirectly with anti-bovine saposin C IgG as previously described [5].

Glucosylceramide ß-glucosidase and saposin C activity —Pure glucosylceramide ß-glucosidase (EC 3.2.1.45) was isolated from human placenta using two-step high-performance hydrophobic and gel permeation column chromatography according to the method of Choy [11]. Homogeneity of the purified enzyme was demonstrated by SDS-PAGE. For saposin C assay, samples were assayed for stimulatory activity toward the above enzyme by incubation with 4-methylumbelliferyl glucoside in acetate buffer, pH 4.5, with Triton X-100 as previously described [12].

Protein determination, amino acid analysis, and protein sequencing —The bicinchoninic acid method [13] was used with bovine serum albumin as standard. Amino acid compositional analysis was done with phenylisothiocyanate derivatization by the method of Tarr [14]. Manual protein sequence analyses were performed using the film method [14].

Isolation of prosaposin —All steps were carried out at 4°C. In the run described here, 500 ml of human milk were centrifuged at 10,000 *g* for 10 min. After removing the floating lipid layer, solid ammonium sulfate was added slowly, with stirring, to produce 33% saturation. After 1 h, the mixture was centrifuged. This step was repeated with the supernatant portion and 50% saturation with ammonium sulfate and the resultant pellet was dissolved in a small volume of 10 mM sodium phosphate, pH 7.0, 0.02% NaN_3 (Buffer A) and dialyzed against the same buffer. The protein content at this step was 1.7 g. The sample was applied to a column of DEAE-Sephacel (2.6 x 30 cm) previously equilibrated with Buffer A, and followed by 75 ml of Buffer A and 1,000 ml of the same buffer containing a linear gradient of 0-0.5 M NaCl. Fractions eluting in ~0.3 M NaCl, reacting strongly with anti-saposin C IgG in Western blotting, were pooled. The dissolving buffer was changed to 0.2 M potassium phosphate buffer, pH 6.9 using ultrafiltration with an Amicon YM-10 membrane and concentrated. The protein content of the preparation after the ion-exchange chromatography was 127 mg.

One tenth of this sample in 0.5 ml was injected into a loop in an HPLC system. Separation was accomplished with a gel permeation column (Tosoh TSK Gel G3000 SW, 4.6 x 600 mm). The prosaposin fractions, which eluted at around apparent

molecular weight 70 kDa (compared with the elution volume of standard proteins; ß-amylase, alcohol dehydrogenase, bovine serum albumin, and ovalbumin) were pooled. The same procedure was repeated another 9 times for the rest. The pooled prosaposin fraction was concentrated and the disolving buffer was exchanged to phosphate buffered saline (PBS) using ultrafiltration as described above. The fraction at this step contained 8.4 mg of protein. The sample was further purified with an immunoaffinity column, anti-bovine saposin C IgG immobilized to Affi-Gel Hz (6 mg of IgG/5 ml of gel). The column was washed with 100 ml of PBS, then with 100 ml of 0.5 M NaCl/PBS. Prosaposin was eluted with 50 ml of 0.1 M glycine buffer (pH 2.5).

Trypsin treatment of prosaposin—The incubation mixture contained 25 μg of prosaposin and 0.5 μg of trypsin in 26 μl of 0.1 M ammonium carbonate (pH 8.0). The reaction was performed at 37°C and stopped by the addition of 1 μl of 27 mM phenylmethylsulfonyl fluoride after 0, 2, or 4 h. Saposin C activity of each sample was assayed as described above.

RESULTS

Isolation of prosaposin from human milk—Purified protein was judged to be homogeneous by several analytical criteria, such as a single band in protein staining after SDS-PAGE and in immunoblots (Fig. 1), and a single N-terminal sequence obtained by Edman sequencing. The amino-terminal sequence obtained with our prosaposin was Gly-Pro-Val-Leu-Gly-Leu-Lys-Glu-X-Thr. This sequence was identical to the sequence starting at Gly-17 of human prosaposin deduced from cDNA [3, 4] (Fig. 2). Nakano et al. and Roman and Grabowski suggested in their cDNA studies that the first 16 amino acids satisfy the criterion for a typical signal leader sequence [3, 4]. The proposed proteolytic processing site for the signal sequence was confirmed here. Amino acid composition of the purified prosaposin matched well with that of the deduced sequence (Table 1). The estimated molecular size of prosaposin was about 74 kDa in SDS-PAGE under reduced condition and about 70 kDa by G-3000 SW gel permeation in the native state. The yield of purified prosaposin in the above procedure was 100 μg from 500 ml of human milk. We repeated the same purification procedure more than five times with fairly repeatable results, but the

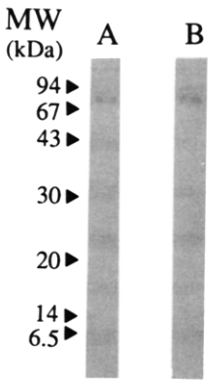

Fig. 1. SDS-PAGE and Western blot of purified prosaposin. Purified preparation (A; 500 ng; B; 100 ng) was electrophoresed, then electrotransferred to an Immobilon membrane and stained with amidoblack (A) or immunostained (B) as described in the text.

		1	5	10	15	20	25
Deduced Sequence	A)*				G S S R P V L G L K E C T		
from cDNA	B)**	M Y A L F L L A S L L G A A L A			G P V L G L K E C T		
Purified Prosaposin						G P V L G L K E X T	

A)* O'Brien et al. [2] B)**Nakano et al. [4] & Roman and Grabowski [3]

Fig. 2. N-terminal sequence of prosaposin. The deduced sequence A is from the data of O'Brien et al. [2] and the sequence B is from the data of Nakano et al. [4] and Roman and Grabowski [3].

purified protein sometimes showed an additional band of 62 kDa in SDS-PAGE. However, the N-terminal sequencing and amino acid analysis of such a batch showed the same results.

ß-Glucosidase activator activity of prosaposin before and after trypsin treatment —Prosaposin produced a slight stimulation of human glucosylceramide ß-glucosidase (Fig. 3). When 12 µg of prosaposin were added to the incubation mixture, the enzymatic hydrolysis of 4-MU ß-D-glucopyranoside was stimulated up to ~100% above the control value (Fig.3). However, the same amount of bovine serum albumin stimulated the hydrolysis 60%, and ovalbumin decreased it by 15% of the basal level. Saposin C, the ß-glucosidase activator, showed maximal stimulation of ~1100% when 12 µg were added. Although Morimoto et al. reported that saposin A was as active as saposin C in the activation of ß-glucosidase using 4MU ßDglucoside as substrate [9], in our system saposin A stimulated the hydrolysis of 4-MU ß-D-glucoside only 130% when 12 µg were added (Fig. 3). Prosaposin itself showed ß-glucosidase activator activity to a similar degree, and the activity increased much after trypsin treatment (Fig. 4). Western blotting of the trypsin-treated sample confirmed the formation of small saposin-like bands from prosaposin by the action of trypsin.

Table 1. Amino acid composition of purified prosaposin

Amino Acid	Theoretical* %	Experimental %
Asx	9.2	9.0
Glx	14.0	14.0
Ser	6.0	7.0
Gly	4.2	5.0
His	2.2	2.2
Arg	1.6	3.2
Thr	4.6	4.6
Ala	6.0	9.0
Pro	6.4	7.4
Tyr	2.2	2.2
Val	9.2	7.6
Met	3.2	2.0
Ile	5.2	4.0
Leu	10.0	10.0
Phe	1.8	2.4
Lys	8.6	6.8

* Molar percent values calculated from amino acid sequence deduced from prosaposin cDNA.

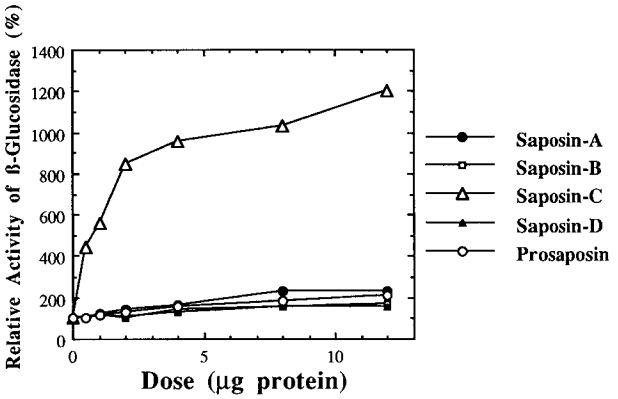

Fig. 3. Activation of glucosylceramide ß-glucosidase by saposins A, B, C, and D, and prosaposin. Various amounts of each protein were added to the incubation mixture.

DISCUSSION

There exist discrepancies in the deduced amino acid sequences in the region of the possible signal peptide and a following residue in different cDNA studies [2-4]. Nakano et al. [4] concluded that the difference appears simply because the cDNA of O'Brien et al. [2] was not full-length and lacked approximately 45 nucleotides. The N-terminal amino acid sequence of prosaposin obtained in the present study was identical to the sequence deduced by Nakano et al. [4], and Roman and Grabowski [3], starting at Gly-17. Their studies predicted the existence of a typical signal sequence in the initial 16 amino acids. Their proposed cleavage site for signal sequence was confirmed here.

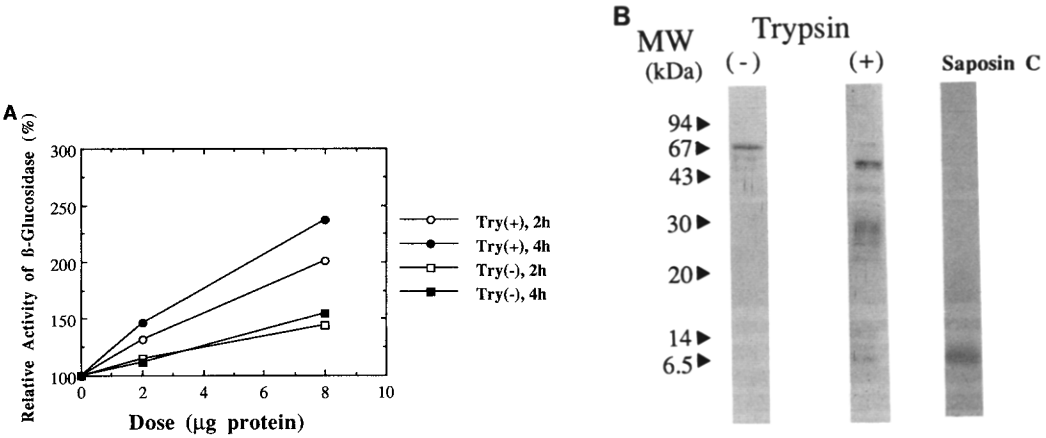

Fig. 4. Activator activity of prosaposin with or without trypsin treatment. (A) Activator activity of trypsin-treated prosaposin toward glucosylceramide ß-glucosidase. (B) Western blot of the sample treated with trypsin for 4 h.

The rat counterpart of prosaposin, SGP-1, is one of the abundant secretory proteins from Sertoli cells [7]. SGP-1 is extensively glycosylated and its 67- and 70-kDa forms produce a 57-kDa protein after treatment with endoglycosidase F. Heterogeneity in glycosylation is probably the reason why some of our prosaposin preparations showed two bands in SDS-PAGE, since the results of N-terminal sequencing and amino acid analyses of such preparations were the same as those of the preparation showing only the 74-kDa band. Native SGP-1 occurs as a noncovalent homodimer of 140 kDa [15], while native prosaposin showed apparent molecular weight of 70 kDa in HPLC gel permeation. Prosaposin secreted into milk appears to occur as a monomer.

Prosaposin showed a slight stimulation toward ß-glucosidase, but such a degree of stimulation appears to be insignificant because a control protein, bovine serum albumin showed similar stimulation.

Reduced and alkylated saposin C was easily cleaved by trypsin, but native saposin C could not be cleaved by trypsin and many other proteases (data not shown). Saposin C seems to be so compact, with its three intramolecular disulfide bonds, that it is resistant to proteolytic enzymes. Purified prosaposin, on the other hand, could be cleaved by trypsin, yielding ß-glucosidase activator activity and saposin-like bands in Western blotting. These results suggest that each of the four saposin domains in prosaposin is resistant to proteolysis; the cleavage by trypsin treatment caused cuts only in the intervening regions.

The prosaposin in milk, which is extracellularly secreted, seems to keep the same tertiary structure as prosaposins destined to be sent to cell lysosomes. What is the difference between these two types of prosaposin? The rat counterpart of prosaposin, SGP-1 is an extracellularly secreted protein which is heavily sulfated. Sulfation of carbohydrate chain or peptide backbone may function as a signal for secretion.

Although Morimoto et al. reported that saposin A was as active as saposin C in the activation of ß-glucosidase [9], in our study saposin A stimulated the hydrolysis of 4-MU ß-D-glucoside to a much smaller extent than saposin C. The identity of four saposins used in this study was confirmed by several methods, such as protein sequencing from the N-terminus and amino acid compositional analyses. Our purification method for glucosylceramide ß-glucosidase was different from the one used by Morimoto et al. [16]. Our method can produce homogeneous glucosidase but the other cannot. The discrepancy in the results may be due to the difference.

Recently, Potier et al. concluded that the 60-kDa protein which was recognized as sialidase in a sialidase/ß-galactosidase complex is derived from prosaposin [17]. Aliquots at each step of prosaposin purification did not show neuraminidase activity (data not shown), which was measured with 4-methylumbelliferyl neuraminide as substrate. More recently, Hiraiwa et al. showed that the 60-kDa protein was identical with an immunoglobulin G heavy chain protein [18].

Acknowledgments—We are most grateful to Ms. Kazumi Matsumoto for her technical assistance. This study was supported in part by a Grant-in Aid for Scientific Research on Priority Areas No. 03255212 from the Ministry of Education, Science and Culture, Japan (Y. K.) and by a Grant-in Aid for Scientific Research No. 03857146 from the Ministry of Education, Science and Culture, Japan , and by Clinical Pathology Research Foundation of Japan (A. S.). We wish to thank Dr. N. S. Radin of the University of Michigan, U. S. A., for his assistance with this manuscript.

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