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Distribution of saposin proteins (sphingolipid activator proteins) in lysosomal storage and other diseases

(Tay–Sachs disease/Sandhoff disease/fucosidosis/Gaucher disease/Niemann–Pick disease)

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ABSTRACT **Saposins (A, B, C, and D) are small glycoproteins required for the hydrolysis of sphingolipids by specific lysosomal hydrolases. Concentrations of these saposins in brain, liver, and spleen from normal humans as well as patients with lysosomal storage disease were determined. A quantitative HPLC method was used for saposin A, C, and D and a stimulation assay was used for saposin B. In normal tissues, saposin D was the most abundant of the four saposins. Massive accumulations of saposins, especially saposin A (about 80-fold increase over normal), were found in brain of patients with Tay–Sachs disease or infantile Sandhoff disease. In spleen of adult patients with Gaucher disease, saposin A and D accumulations (60- and 17-fold, respectively, over normal) were higher than that of saposin C (about 16-fold over normal). Similar massive accumulations of saposins A and D were found in liver of patients with fucosidosis (about 70- and 20-fold, respectively, over normal). Saposin D was the primary saposin stored in the liver of a patient with Niemann–Pick disease (about 30-fold over normal). Moderate increases of saposins B and D were found in a patient with GM1 gangliosidosis. Normal or near normal levels of all saposins were found in patients with Krabbe disease, metachromatic leukodystrophy, Fabry disease, adrenoleukodystrophy, I-cell disease, mucopolysaccharidosis types 2 and 3B, or Jansky–Bielschowsky disease. The implications of the storage of saposins in these diseases are discussed.**

Sphingolipid activator proteins are small glycoproteins required for the maximal hydrolysis of sphingolipids by specific lysosomal hydrolases. Sphingolipid activator protein 1 discovered in 1964 (1) (herein termed saposin B) activates the hydrolysis of cerebroside sulfate, GM1 ganglioside, and globotriaosylceramide by arylsulfatase A, acid β-galactosidase, and α-galactosidase, respectively (2–5), and also appears to promote hydrolysis of a variety of other lipids including glycerolipids (6). A second activator protein, discovered in 1971 (7), sphingolipid activator protein 2 (herein termed saposin C) activates the hydrolysis of glucosylceramide and galactosylceramide by β-glucosylceramidase (EC 3.2.1.45) and galactosylceramide β-galactosidase (EC 3.2.1.46), respectively (8). Saposin C acts differently than saposin B since it interacts with the above enzymes increasing their maximal velocity and decreasing their Michaelis constant (9–11), whereas saposin B binds lipid substrates solubilizing them for hydrolysis. A third activator protein termed sphingolipid activator protein 3, also known as GM2 activator, is a specific activator for the hydrolysis of ganglioside GM2 by GM2 β-*N*-acetylgalactosaminidase (12, 13). Sphingolipid activator protein 3 is genetically distinct and unrelated to the saposins studied here.

The two remaining saposin proteins, saposins A and D, were discovered (16) in 1989 after the cDNA encoding them was analyzed. Studies of the proteolytic processing of saposins B and C showed that both are initially biosynthesized as a large molecular mass precursor (70 kDa) that, after proteolytic processing, generates the 12-kDa saposin proteins (14, 15). This laboratory cloned a cDNA for saposins B and C and showed that a common precursor generates both polypeptides (16). O'Brien *et al.* (16) and Collard *et al.* (17) proposed that two additional activator proteins similar in structure to saposins B and C are generated by proteolysis of the same precursor, which we call prosaposin.

Fürst *et al.* (18) obtained protein sequencing data on a polypeptide that they isolated. After comparison with nucleotide sequences (16), they came to the conclusion that a common precursor gives rise to three activator proteins, saposins B and C and the third protein that they isolated and called component C located within the carboxyl-terminal portion of the precursor; they did not identify saposin A.

We isolated saposin D from the spleen of a patient with Gaucher disease, purified it to homogeneity, determined that it had the same amino acid sequence as component C, and demonstrated that it was a specific activator of sphingomyelinase (19). Similar to saposin C, saposin D appears to exert its effect upon the enzyme and did not bind sphingomyelin. We also isolated the fourth potential saposin, called saposin A, from the spleen of a patient with Gaucher disease, purified it to homogeneity, and demonstrated that saposin A, like saposin C, is a specific activator of β-glucosylceramidase and galactosylceramide β-galactosidase (20). Saposin A, like saposins C and D, activates by binding to these enzymes not to lipid substrates. Presented in Fig. 1 is the nomenclature we proposed for this family of proteins (Fig. 1).

Inui and Wenger (21) reported that saposin B accumulated in tissues of patients with lysosomal storage diseases, especially tissue from patients with infantile GM1 gangliosidosis. The accumulation of saposin C in the spleen of patients with Gaucher disease has been known since its discovery (7). In the present report we describe the tissue distributions and concentrations of saposins A, B, C, and D in lysosomal storage and other diseases. Massive accumulations, especially of saposin A, were found in brain of patients with Tay–Sachs disease or Sandhoff disease and in liver of patients with fucosidosis and accumulations of saposin D were found in patients with Niemann–Pick disease.

MATERIALS AND METHODS

Human Tissues. Human tissues used in this study were collected over many years and stored at −20°C in this laboratory. Diagnoses were made by pathological examination, analysis of storage material, and enzyme assays for the ap-

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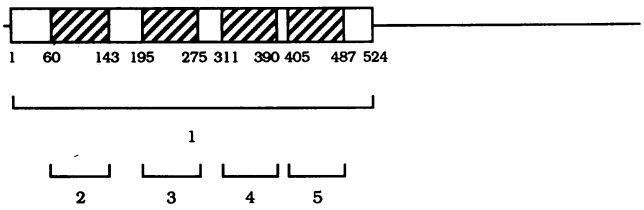

FIG. 1. Nomenclature of saposin proteins. Prosaposin, the precursor of saposins A, B, C, and D, is shown as a bar with the positions of the four saposins crosshatched. Locations of peptides are indicated by brackets. Brackets: 1, prosaposin (residues 1–524); 2, saposin A (residues 60–143); 3, saposin B (residues 195–275); 4, saposin C (residues 311–390); 5, saposin D (residues 405–487).

propriately deficient enzyme. Each disease listed is accompanied by its appropriate number as assigned by McKusick (22).

Determination of Saposin A, B, C, and D. Amounts of individual saposins were determined by a method to be described elsewhere. Individual human tissues (0.4–3.8 g) were homogenized in water and the homogenates were boiled and centrifuged. The concentrations of saposins A, C, and D in the supernatant were determined by two consecutive HPLC separations. The boiled extract was first fractionated on a C_4 reversed-phase column with an acetonitrile gradient. After the impurities were eluted, saposins A, C, and D were eluted together at high acetonitrile concentrations and this fraction was then analyzed by an anion-exchange HPLC using NaCl gradient. Saposins D, A, and C were eluted from the column in individual peaks and quantitated by measuring peak areas. Saposin B was quantified by determining its stimulative activity on pure human liver GM1 ganglioside β-galactosidase.

RESULTS

The saposin content in the brain, liver, and spleen of 5 normal individuals and 33 patients with lysosomal storage disease were analyzed. The results are presented in Table 1.

Saposins in Normal Humans. Three brains, four livers, and three spleens were available from normal adults (20–50 years). Tissues were obtained at autopsy and were free of pathology by gross and microscopic examination. Brain contained 15 ± 5 μg (mean ± SD) of saposin A per g (dry tissue), 14 ± 11 μg of saposin B per g, 15 ± 5 μg of saposin C per g, and 47 ± 11 μg of saposin D per g, with a total saposin content of 92 ± 14 μg/g. Normal liver contained 25 ± 5, 21 ± 9, 30 ± 17, and 49 ± 26 μg of saposins A, B, C, and D per g, respectively, with a total saposin content of 125 ± 46 μg/g and normal spleen contained 27 ± 15, 34 ± 12, 27 ± 11, and 77 ± 11 μg of saposins A, B, C, and D, per g, respectively, with a total saposin content of 165 ± 61 μg/g. Thus, among normal tissues examined, spleen contained the highest saposin content followed by liver and brain. In each tissue, saposin D was the most abundant saposin.

Saposins in GM2 Gangliosidosis. One unexpected finding was massive accumulation of saposins in brain from patients with either Tay–Sachs disease or Sandhoff disease. The three patients with Tay–Sachs disease expired at 3, 4, and 5 years of age. In each patient, brain had the highest saposin content, 2501 to 2753 μg/g, a 27- to 30-fold increase above normal. Approximately one-half of this increase was due to saposin A, which averaged 88 times normal. When gray matter was separated from white matter about 4-fold higher concentrations of saposins were detected in the former than the latter.

A similar large increase in brain, particularly of saposins A and C, was also found in patients with Sandhoff disease. On the other hand, no such increase was seen in brain from patients with adult or juvenile type GM2 gangliosidosis. In brain extracts from a Sandhoff disease patient or a Tay–Sachs disease patient, an extra peak with retention time 15.4 min appeared after the saposin A peak on anion-exchange HPLC. Western blot of the peak after SDS/PAGE revealed that it is an unknown derivative of saposin A (data not shown).

Saposins in Gaucher Disease. Saposin C was first isolated and found to accumulate in spleen from patients with adult Gaucher disease (7). Our investigation disclosed that this saposin is not the most abundant saposin accumulating. In the four spleens examined total saposin ranged from 3267 to 3652 μg/g (dry weight), a 20- to 22-fold increase above normal; about one-half of the increase was due to saposin A, which accumulated to levels averaging 60 times normal. Saposin C was elevated 17 times normal, saposin D was 16 times normal, and saposin B was 5 times normal. Saposins did not accumulate in the brain of two patients with infantile Gaucher disease.

Saposins in GM1 Gangliosidosis. Saposin B has been shown to accumulate in the liver of patients with infantile GM1 gangliosidosis (21). Our examination of liver from two such patients showed that saposin D accumulated to nearly the same extent as saposin B (about 8 times normal). In juvenile GM1 gangliosidosis a smaller increase in saposin B was found in liver and a somewhat larger increase in saposins A and C was found in brain.

Saposins in Fucosidosis. In patients with fucosidosis, massive accumulations of saposins A and D were found in liver. In one patient saposin A accumulated to levels 102 times normal whereas saposin D was about 23 times normal. Accumulations of saposins B and C were less significant (7- to 17-fold increase). Accumulations of saposins in spleen and brain were modest.

In the brain from one fucosidosis patient, an extra peak (retention time 11.6 min) was observed after anion-exchange HPLC. Samples of this unknown peak were collected after anion-exchange HPLC for further analysis. It was found that this peak comprised two components with retention times of 56.0 and 59.6 min after hydrophobic HPLC. Neither component stimulated β-glucosidase activity but each cross-reacted with anti-saposin D antibodies on a Western dot blot. After treatment with neuraminidase from *Clostridium perfringers* (Boehringer Mannheim) both peaks shifted on anion-exchange HPLC to two peaks (8.6 and 9.0 min) identical to the peaks of saposin D (data not shown). Therefore, it was concluded that these two compounds are sialylated derivatives of saposin D. The value for saposin D presented in Table 1 includes these derivatives.

Saposins in Niemann–Pick Disease. In two patients with infantile Niemann–Pick disease, the predominant saposin accumulating was saposin D. In liver the accumulation was 31 and 24 times normal and in one spleen a 19-fold increase was found. Smaller accumulations of other saposins were found in liver and spleen, and in brain the accumulation of saposins was minimal.

Saposins in Other Diseases. Elevation of saposin concentrations, if present, were small in brain, liver, and spleen of patients with Krabbe disease, mucopolysaccharidosis II and III, or Jansky–Bielschowsky late infantile lipidosis. No increase of saposins were found in liver and spleen of a patient with Fabry disease, in brains from two patients with adrenoleukodystrophy, in brain of a patient with Alzheimer disease in an area with numerous neuritic plaques, in brain and spleen of a patient with I-cell disease, or in brain and liver of a patient with late infantile metachromatic leukodystrophy.

DISCUSSION

Prosaposin is proteolytically processed to generate saposins A, B, C, and D by cleavage at lysine or arginine residues at the boundaries of each saposin domain generating equimolar quantities of saposins A, B, C, and D (Fig. 1). Saposin D is 2–3 times more abundant than saposins A, B, and C in most

Table 1. Saposin tissue distribution

Source	Tissue	Saposin, μg/g (dry weight) A	B	C	D	Total
	Normal tissues					
U	Brain	9	14	16	34	73
	Liver	29	23	59	92	203
	Spleen	42	51	41	116	250
M	Brain	15	0	21	61	97
	Liver	30	34	20	31	115
	Spleen	31	29	26	50	136
Y	Liver	19	9	23	47	98
A-101	Liver	22	17	18	27	84
	Spleen	7	22	14	66	109
A-14	Brain	22	28	9	47	105
	Tay–Sachs disease (27280)					
M	Brain	1395	186	444	599	2623
	Liver	85	22	49	181	337
	Spleen	276	82	154	415	927
L	Brain	1410	189	519	383	2501
	Liver	90	170	64	170	494
	Spleen	207	252	110	388	957
S	Total brain	1152	438	523	644	2758
	White matter	476	—*	186	241	903
	Gray matter	1583	—*	572	697	2851
	Liver	99	31	65	140	336
	Spleen	242	104	153	334	834
	Adult GM2 gangliosidosis (27280)					
A	Brain	115	35	91	77	318
	Liver	78	72	46	136	332
	Juvenile GM2 gangliosidosis (23071)					
L	Brain	96	28	46	86	256
	Liver	87	46	15	86	234
	Spleen	350	64	42	110	566
	Infantile Sandhoff disease (26880)					
H	Brain	1097	119	427	460	2103
	Spleen	287	119	155	347	909
R	Brain	991	55	548	385	1978
	Adult Gaucher disease (23080)					
N	Spleen	1602	203	662	1185	3652
M	Spleen	1533	235	685	1069	3522
T	Spleen	1604	103	479	1474	3660
H	Spleen	1472	124	488	1184	3267
	Infantile Gaucher disease (23090)					
A	Brain	19	34	9	21	83
Z	Brain	16	19	5	10	50
	Infantile GM1 gangliosidosis (23050)					
B	Liver	136	410	177	359	1082
W	Liver	190	287	231	428	1137
	Juvenile GM1 gangliosidosis (23060)					
J	Brain	260	79	182	158	679
	Liver	93	118	186	266	662
	Spleen	182	96	286	292	856
L	Brain	326	24	73	196	619
	Liver	186	195	257	491	1129
W	Brain	353	77	189	184	802
	Fucosidosis (type 2) (23000)					
M	Brain	89	128	32	207	456
	Liver	2754	295	217	1126	4393
	Spleen	262	728	52	162	1205
G	Liver	959	376	516	1079	2929
	Spleen	124	221	34	136	515
	Niemann–Pick disease type A (infantile) (25720)					
F	Brain	152	106	207	159	623
	Liver	228	137	531	1498	2395
S	Brain	110	40	166	143	458
	Liver	451	355	642	1174	2623
	Spleen	614	247	596	944	2401
	Infantile Krabbe disease (24520)					
J	Brain	32	17	12	71	133
	Liver	40	13	27	140	219
	Spleen	47	27	24	81	179
	Mucopolysaccharidosis II (Hunter disease) (30990)					
B	Brain	21	11	19	109	160
	Liver	88	36	82	149	356
	Spleen	144	51	79	197	472
A	Liver	142	80	70	169	462
	Spleen	123	—*	70	167	360
	Mucopolysaccharidosis III (Sanfilippo type B) (25292)					
D	Brain	141	23	52	234	450
	Liver	100	56	83	120	359
	Spleen	133	277	97	152	658
	Jansky–Bielschowsky (late infantile lipidosis) (20450)					
R	Brain	53	9	48	55	155
	Liver	20	41	135	97	293
	Spleen	124	59	166	263	613
	Fabry disease (30150)					
W	Liver	49	108	63	135	356
	Spleen	54	69	127	255	506
	Adrenoleucodystrophy (30010)					
B	Brain	16	27	18	58	118
A	Brain	14	23	19	61	117
	Alzheimer disease (10430)					
N	Brain	39	—*	35	57	131
	I-cell disease (25250)					
G	Brain	3	28	7	15	53
	Spleen	33	34	72	139	278
	Late infantile metachromatic leucodystrophy (25010)					
P	Brain	20	20	110	187	337
	Liver	111	32	60	341	544

Values can be converted to molar quantities using the known molecular masses of each (saposins B, C, D, 12 kDa; saposin A, 16 kDa). Numbers in parentheses are from ref. 22.
*Undetectable.

normal tissues studied, which may suggest a slower degradation rate for saposin D than for saposins A, B, and C.

In Tay–Sachs disease and Sandhoff disease a massive accumulation of GM2 ganglioside occurs in neurons secondary to β-*N*-acetylhexosaminidase deficiency (13). The accumulation of saposins especially saposin A (about 80 times normal) is in the same range as that of GM2 ganglioside, although the actual quantities of the protein are lower than the ganglioside by about an order of magnitude. It is known that in lysosomal storage diseases activities of lysosomal enzymes are generally increased (except for that of the deficient enzyme) but these increases are rarely higher than 3 times normal. In tissues where GM2 ganglioside does not accumulate such as in liver and spleen and in later onset GM2 gangliosidosis where GM2 accumulation in brain is smaller, saposin A concentrations were much lower, indicating parallel storage of ganglioside GM2 and saposin A. In attempting to understand this coincident association we determined the

effect of saposin A on *in vitro* hydrolysis of ganglioside GM2 by GM2 β-*N*-acetyl-galactosaminidase and no stimulation was observed (20). Their concomitant storage may imply a complex between ganglioside GM2 and saposin A. Since ganglioside GM2 accumulates predominantly within lysosomal storage granules called cytoplasmic membranous bodies in neurons in patients with Tay–Sachs disease (23), saposin A may be similarly localized. Previous analyses of the cytoplasmic granules stored in Tay–Sachs disease (24) have indicated that the isolated granules contained about 10% protein on a weight basis. The actual content may be higher since leaching out of protein could have occurred during isolation of the granules. It will be of interest to determine whether the protein portion of cytoplasmic membranous bodies comprises saposins, especially saposin A.

It is also necessary to determine whether saposins play any role in the neurological deterioration that occurs in Tay–Sachs disease. The massive quantities that are stored are large enough to implicate them in the neuronal pathology. From studies in man (25) and in experimental animals with GM1 and GM2 gangliosidosis (26), neurological deterioration and symptomatology are best correlated with alterations of neuronal membrane morphology including proliferation of growth-cone-associated neurites from the axon hillock (secondary neurites) and the presence of bizarre swellings (meganeurites) interposed between the soma and the initial segment of the axon. The relationship between ganglioside accumulation and these distortions of neuronal structure is unclear. Similar membranous neuronal changes have been documented in feline mannosidosis in which gangliosides do not accumulate (27). Unfortunately, we did not have access to brain tissue from feline mannosidosis to determine saposin concentrations. The relationship between saposin accumulations and aberrant neuronal structure should be explored especially since neurological deterioration is directly correlated with the morphologic changes.

In infantile Sandhoff disease, storage of GM2 ganglioside in brain is quantitatively similar to that in Tay–Sachs disease (except that asialo-GM2 also accumulates) (13) and similar massive accumulations of saposin A were present. Asialo-GM2 and globoside are stored in spleen in Sandhoff disease, but ganglioside GM2 concentrations are insignificant and saposin concentrations are not large (2–3 times normal in spleen) pointing to neuronal ganglioside GM2 storage as the predominant feature accompanying saposin A accumulation. Another unexpected finding was the very large accumulation of saposins A and D in spleen of patients with adult Gaucher disease. It had been noted (7) that saposin C accumulates in the spleen of patients with Gaucher disease but our results demonstrate that saposins D and A accumulate to levels many times higher. Although these saposins do not form a complex with glucocerebroside, their accumulation in the spleen of patients with Gaucher disease indicate that saposins may undergo an uncharacterized association with either increased glucocerebroside or mutated glucosylceramide β-glucosidase, as suggested by Datta and Radin (28). They reported increase of saposin C in mouse tissues after injection of glucocerebroside or of an inactivator of glucosidase. They also reported that the uptake of β-glucosylceramidase by neuroblastoma cells was enhanced by adding saposin C (29). The role of saposins A and D in the pathophysiology of the glucocerebroside storage in Gaucher disease is worthy of study, especially since saposin A is now known to be a second activator of β-glucosylceramidase (20). Glucocerebroside storage in brain of patients with infantile Gaucher disease is much less than in liver and saposin concentrations were much lower therein in accord with the proposed association of saposins and glucocerebroside.

The accumulation of saposins A and D in liver of patients with fucosidosis was also unexpected. We have demonstrated (20) that neither saposin A nor C stimulates α-L-fucosidase activity using a synthetic α-L-fucoside substrate (4-methylumbelliferyl α-L-fucoside) but no studies were conducted using fucoglycolipids as substrates. Accumulations of saposins A and D were much smaller in the brain and since neutral fucoglycolipids are not stored in brain of patients with fucosidosis, the parallel storage of fucoglycolipid and saposins A and D may indicate a complex between these lipids and saposins. Experiments to explore the role of saposins A and D in fucoglycolipid hydrolysis are clearly warranted.

We reported (19) that saposin D was a specific sphingomyelinase activator. However, the degree of stimulation was lower than that of β-glucosylceramidase by saposin A or C (20), perhaps because our sphingomyelinase preparation was only partially purified whereas our preparations of β-glucosylceramidase were highly purified. The fact that saposin D is the major saposin accumulating in liver and spleen of patients with Niemann–Pick disease where sphingomyelin is massively stored is in keeping with the *in vitro* studies, suggesting that saposin D activates the lysosomal hydrolysis of sphingomyelin (19).

Insignificant accumulations of saposins were found in demyelinating disorders such as metachromatic leucodystrophy, Krabbe disease, and adrenoleucodystrophy even though storage of sphingolipids, such as cerebroside sulfate in metachromatic leucodystrophy, occurs in white matter. Normal quantities were found in I-cell disease, Alzheimer disease, and Jansky–Bielschowsky late infantile lipidosis. The failure to detect significant accumulations of saposins in visceral tissues from a patient with Fabry disease is surprising, especially since saposin B is reported to stimulate the hydrolysis of the galactosphingolipids stored in Fabry disease by α-galactosidase (4). Normal saposin concentrations were also found in liver from patients with Hunter disease or Sanfilippo disease type B, even though large accumulations of mucopolysaccharides occur within hepatic lysosomes.

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