Conformational and Amino Acid Residue Requirements for the Saposin C Neuritogenic Effect†

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ABSTRACT: Prosaposin is the precursor of four activator proteins, termed saposins A, B, C, and D, that are required for much of glycosphingolipid hydrolysis. The intact precursor also has neurite outgrowth activity ex vivo and in vivo that is localized to amino acid residues 22−31 of saposin C. Across species, this saposin C region has a high degree of identity and similarity with amino acids in the analogous region of saposin A. Wild-type and mutant saposins C and A from human and mouse were expressed in *E. coli*. Pure proteins, synthetic peptide analogues, conformation-specific antibodies, and CD spectroscopy were used to evaluate the basis of the ex vivo neuritogenic effect. Wild-type saposin A had no neuritogenic activity whereas reduced and alkylated saposin A did. Introduction of the conserved saposin A Tyr 30 (Y30) into saposin C at the analogous position 31, a conserved Ala(A)/Gly(G)31, diminished neuritogenic activity by 50−60%. Nondenatured saposin A with an introduced A30 acquired substantial neuritogenic activity. Polyclonal antibodies directed against the NH_2-terminus of saposin C cross-reacted well with reduced and alkylated saposins C and A, wild-type saposin C, and saposin A [Y30A], poorly with saposin C [A31Y], and not at all with wild-type saposin A. CD spectra of wild-type and mutant saposins C and A, the corresponding neuritogenic region of saposin C, and the analogous region of saposin A showed that more "saposin C-like" molecules had neuritogenic properties. Those with more "saposin A-like" spectra did not. These studies show that the neuritogenic activity of saposin C requires specific placement of amino acids, and that Y30 of saposin A significantly alters local conformation in this critical region and suppresses neuritogenic activity.

Prosaposin is a multifunctional protein encoded by a single locus on chromosome 10 in humans and mice. The protein is ubiquitously expressed, and has temporal and spatial transcriptional regulation (*1*). Prosaposin is synthesized as a single transcript that encodes, in tandem, four ∼80 amino acid polypeptides, termed saposins A, B, C, and D, that are needed for maximal activity of several lysosomal glycosphingolipid hydrolases (*2*−*4*) (Figure 1). Following precursor synthesis, these "activator molecules," or saposins, are proteolytically processed to mature functional proteins (*2*, *5*). Although the precise nature and subcellular location of the proteolytic process are not fully known, much, but not all, of the intersaposin cleavage and maturation occurs in the lysosome (*2*, *5*). Cathepsin D may be important to this lysosomal processing (*6*). Saposins B, C, and D each have one occupied N-linked glycosylation site, and saposin A has two (*7*−*10*). Prosaposin is trafficked to the lysosome via mannose 6-phosphate-dependent and -independent pathways (*5*). Although important for trafficking, glycosylation is not required for the function of the saposins (*8*, *11*).

Saposin C is important for optimal glucosylceramide cleavage by acid β-glucosidase within lysosomes (*12*, *13*). The genetic deficiency of saposin C leads to a "Gaucher-like" disease resembling the neuronopathic variants of acid β-glucosidase deficiency (*14*, *15*). Saposins B and D enhance the intralysosomal cleavage of sulfatide by arylsulfatase A and ceramide by ceramidase (*16*, *17*). Inherited deficiencies of saposin B lead to metachromatic leukodystrophy-like phenotypes that resemble arylsulfatase A deficiency (*18*, *19*). The cognate enzyme of saposin A is not known. The saposins also have been implicated in glycosphingolipid binding and intervesicle transfer (*10*). Saposin B partially extracts specific glycosphingolipids from membranes and presents them to arylsulfatase A in a cleavable form (*3*). The interaction of acid β-glucosidase, negatively charged phospholipids, and saposin C is required for maximal glucosylceramide cleavage rates (*20*, *21*).

In addition to being a saposin precursor, intact prosaposin has ex vivo and in vivo neuritogenic and nerve regeneration effects (*20*, *22*−*25*). In cultured neuroblastoma cells, and other neuronal-like cells of cholinergic origin, prosaposin and saposin C have potent neurite outgrowth effects (*20*, *23*,

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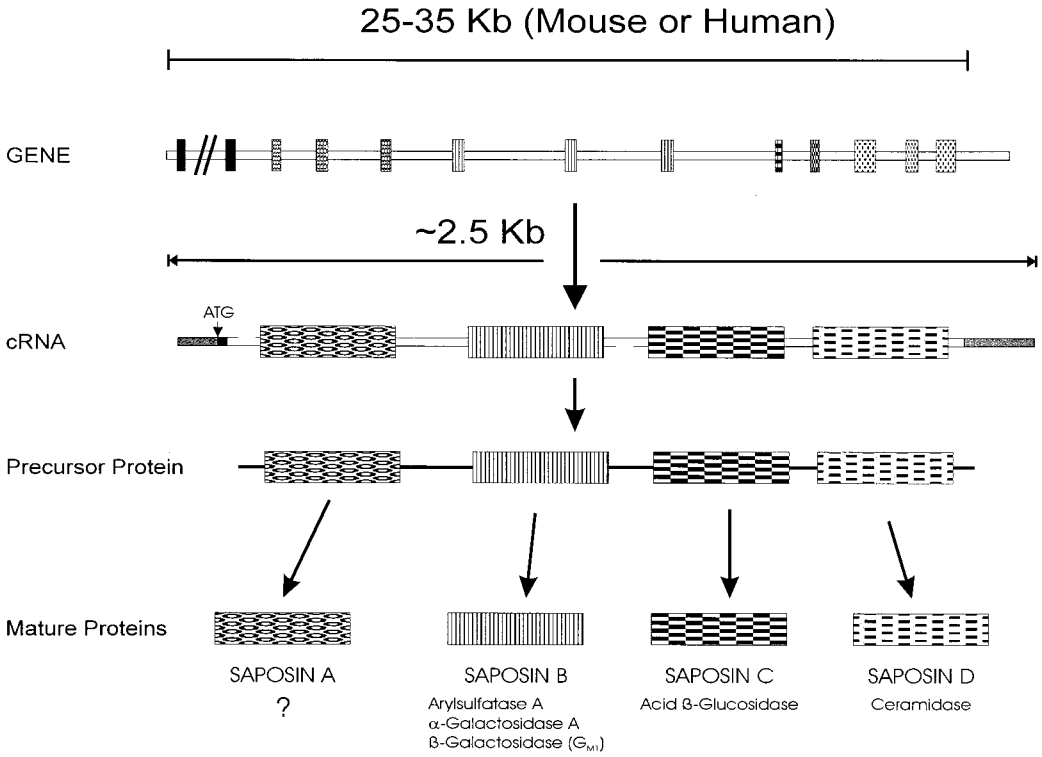

FIGURE 1: Schematic of the prosaposin gene and its transformation into mature saposins. The different rectangles represent the corresponding nucleotides in genomic DNA, mRNA, and protein for the various saposins. The completely filled exons in the gene correspond to encoded amino acid sequence that does not occur in the mature saposins, i.e., pre- and preprosaposin forms only. The enzymes listed below the mature saposins represent known in vitro and in vivo interactions.

25). These proteins also have protective effects against apoptosis in cultured granular cells from the mouse cerebellum (*26*). In vivo administration of prosaposin into ventricular fluid promotes preservation of hippocampal neurons subjected to anoxic injury (*25*). In addition, nerve regeneration rates are enhanced by application of prosaposin or saposin C between cut ends of the sciatic nerve of guinea pigs (*24*). These *ex vivo* and in vivo effects are specific to the saposin C region of prosaposin, and saposins B and D have no such effects. The neuritogenic and nerve regeneration effects are mediated by a 10−14 amino acid sequence from the NH_2-terminal half of saposin C (*20*, *23*, *25*).

Although the neuritogenic and nerve regeneration effects have been localized to a small region of saposin C, neither the specific amino acid nor the conformational requirements have been clearly delineated for this effect. In particular, the high amino acid identity between the neuritogenic region of saposin C and the corresponding region of saposin A suggests that subtle differences in amino acid sequence account for the neurite outgrowth effects of saposin C. Here genetic conservation and site-directed mutagenesis were used in combination with circular dichroism (CD)[1] spectroscopy and specific antibodies to elucidate amino acid and conformational requirements for saposin C's neuritogenic effects.

MATERIALS AND METHODS

Materials. The following were from commercial sources: Sequenase and Sequenase kit (U.S. Biochemical Corp., Cleveland, OH); QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA); restriction endonucleases (New England Biolabs, Beverly, MA); oligonucleotide synthesis and PhastSystem reagents (Pharmacia Bioteck Inc., Piscataway, NJ); pET21a(+) DNA vector, *E. coli* host strain [BL21(DE3)], and His·Bind resin (Novagen, Madison, WI); Magic polymerase chain reaction prep kits (Promega, Madison, WI); 4-vinylpyridine and 2,2,2-trifluoroethanol (Sigma Chemical Co., St. Louis, MO); guanidinium chloride (Fisher Scientific, Fairlawn, NJ); C4 reverse-phase HPLC column (Alltech Associates Inc., Deerfield, IL); PVDF membranes (Micron Separation Inc., Westborough, MA); alkaline phosphatase-conjugated goat anti-rabbit IgG and AP conjugate Substrate Kit (Bio-Rad Laboratories, Hercules, CA); synthesized peptides: human saposin C(22−31) (Peninsula Laboratories Inc., Belmont, CA) and human saposin A(21−31), saposin A(21−31)[Y30A], saposin C(22−32)-[A31Y], and mouse saposin C(22−31) (SynPep Co., Dublin, CA); NS20Y cells were from Dr. Marshall Nirenberg (National Institutes of Health, Bethesda, MA). Organic solvents were HPLC grade, and other chemicals were reagent grade or better.

Mutagenesis, Expression, and Purification of Saposins. Oligonucleotides were synthesized with Gene Assembler 4 and used without purification. The amino acid sequences of wild-type saposins are in Figure 2. The mature NH_2-terminal amino acid from the natural human saposin A or C will be designated 1 and used to identify the amino acids in a fragment. For example, saposin C(22−32) means a peptide comprised of amino acids 22−32 of the wild-type, natural saposin C sequence. A protein product or fragment containing a non-wild-type amino acid will be designated as follows:

[1] Abbreviations: CD, circular dichroism; MLR, multiple linear regression; CCA, convex constraint analysis; SELCON, self-constraint; TFE, 2,2,2-trifluoroethanol.

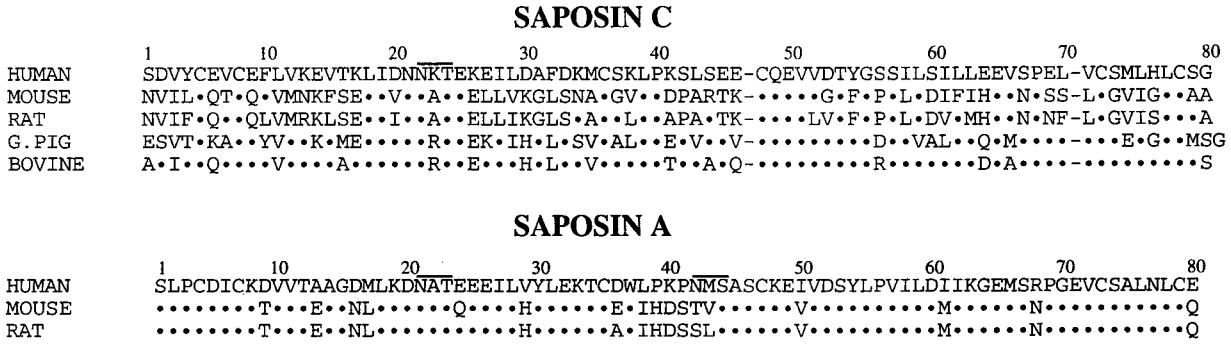

FIGURE 2: Amino acid sequences of saposins A and C and the amino acid sequence of the neuritogenic region. Human, bovine, and guinea pig saposin C amino acid sequences were from the chemically determined amino acid sequence (*43*−*45*). Mouse and rat amino acid sequences were deduced from cDNA sequences. Only the amino acid sequence of human saposin A has been chemically determined. Homology and similarity analyses were from the DNAsis program (Hitachi, Japan) using the Needleman−Wunsch algorithm. The dots indicate amino acid identity with the human sequence. The short horizontal lines designate N-glycosylation sites. Hyphens were added to achieve sequence alignment between saposins A and C.

saposin(22−32)[A31Y] means a peptide encompassing amino acids 22−32 of saposin C with the natural alanine (A) at position 31 mutated to a tyrosine (Y). The following were expressed in the pET 21a(+) system: the complete human saposins C and A (normal and mutant); the saposins C(1−41) and C(42−80); the mouse saposins C(1−41) and C(42−80). The cDNAs for mutant saposins were synthesized using the QuickChange Site-Directed Mutagenesis Kit. The fidelity of all saposin constructs was verified by complete DNA sequence analysis. For reasons that are not known, the intact cDNA encoding the mouse saposin C could not be expressed in *E. coli*, even after mutagenesis of the Shine−Delgarno sequence near the 5′ end (*20*). cDNA constructs expressed in pET 21a(+) contained sequences encoding the non-saposin pET 21a(+) amino acids MASMTGGQQMGRGS (NH_2-terminal) and KLAAALEHHHHHH (COOH-terminal) for saposins C and VNSSSVDKLAAALEHHHHHH for saposins A. These sequences do not affect saposin function (*20*, *28*). NH_2-terminal amino acid sequencing showed the absence of the initiating Met as expected. The immediate downstream amino acid from Met is Ala. In *E. coli*, the presence of this Ala signals the cleavage of the preceding Met (*29*, *30*).

Saposin expression was achieved in *E. coli* following transformation into BL21(DE3) cells that contain a isopropyl 1-thio-β-D-galactopyranoside inducible T7-polymerase gene (*28*). Transformation, incubation, induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside, cell harvest, and isolation of pure proteins were as described (*20*, *28*).

After elution from the His·Bind resin, the saposin eluates were dialyzed and lyophilized. The dried proteins were dissolved in 0.1% trifluoroacetic acid and applied to a HPLC C_4 reverse-phase column. The column was washed with 0.1% trifluoroaectic acid for 10 min, and, then, a linear (0−100%) gradient of acetonitrile was established over 60 min. Elution was monitored by UV absorbency at $\lambda = 225$ nm. The major peak was collected and lyophilized.

For some experiments, the saposins (2 mg) were reduced with DTT in 6 M guanidinium chloride and alkylated with 4-vinylpyridine (*27*). The reduced and alkylated saposins were separated from reagents by reverse-phase HPLC as above.

Neurite Outgrowth Assays. NS20Y cells were suspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/mL penicillin G, and 100 μg/mL streptomycin at 37 °C. NS20Y cells [(2−4) × 10^4/well] during the exponential growth phase were distributed into 35 mm polystyrene dishes and incubated for 24 h. Then, the medium was replaced with fresh medium containing 0.5% fetal calf serum with or without added saposins or saposin peptides. After 24 h, the cells were rinsed with phosphate-buffered saline once and fixed in phosphate-buffered saline containing 4% paraformaldehyde (*20*). The cell densities in the test and control groups were nearly equal. The numbers of cells bearing neurites were counted in at least 10 random fields (200×). Cells bearing neurites longer than 1.0× the cell diameter were scored as positive (*20*). The results are expressed as the mean and standard deviation of excess neurite activity from at least 250 cells in each of 6 independent experiments: i.e., excess neurite outgrowth (EX) = observed neurite % outgrowth in test sample − neurite outgrowth % in untreated control. The excess change in neurite outgrowth was calculated as {[EX(test 1) − EX(test 2)/EX(test 1)} × 100. The experiments were conducted by two investigators in a blinded manner. Results were unblinded after analysis. Statview (Abacus Concepts Inc., Berkeley, CA) was used for *t*-test analyses.

Western Blots. The NH_2-terminal half of human saposin C(1−41) was expressed in *E. coli* and purified as above. Polyclonal antibodies against saposin C(1−41) were raised in New Zealand white rabbits (*31*). The specificity of antiserum was determined by Western blot analysis. The antiserum recognized human saposin C and saposin C(1−41), but not saposin C(42−80). Other antibodies and Western blots were as described (*28*).

Circular Dichroism Spectra. The circular dichroism (CD) spectra were acquired at room temperature in a Jasco J-710 spectropolarimeter (Jasco Incorporated, Easton, MD). Quartz cells of 0.1 mm path length were used for measurements in the far-ultraviolet. The instrument was interfaced with a computer for data acquisition and deconvolution calculations. Three methods were used for deconvolution calculations: (a) multiple linear regression (MLR) (*32*, *33*); (b) convex constraint analysis (CCA) (*34*); and (c) the self-consistent method (SELCON) (*35*). MLR was from Jasco Co. and was used with the YANG.DAT basis database (*36*). The SOFTSEC (Softwood Co.) program was used to convert data files (ASCII files) from the JASCO spectrophotometer for use

SAPOSIN C

	10 20 30	NEURITOGENIC ACTIVITY
HUMAN	CEFLVKEVTKLIDNNKTEKEILDAF	+
HUMAN	::::::::::::**D**::::::::::	+
MOUSE	:QFVMNKFSELIV::A::ELLVKGL	+
RAT	:QLVMRKLSELII::A::ELLIKGL	+
G.PIG	:EYV::K:ME:::::R::EK:IH:L	
BOVINE	::::V::::A:::::R::E:::H:L	+
CONSENSUS C	N-TE$^{R}_{G}$-bb-$^{A}_{G}$h	

SAPOSIN C PEPTIDES

SAP(22-31)	NKTEKEILDA	+
SAP(22-32)[A31Y]	:::::::::**YF**	-
O'Brien	CEFLVKEVTKLIDNN:::::::	+
O'Brien	::::::::::::**DN**:::::::	+
RAT	.SE.II::A::EL..:.	+
MOUSE	:A::EL..K.	+

SAPOSIN A

	10 20 30	
HUMAN	CKDVVTAAGDMLKDNATEEEILVYL	-
SAP A[D20N]	::::::::::::**N**::::::::::	-
SAP A[Y30A]	::::::::::::::::::::::**A**:	+
MOUSE	::T:::E::NL::::::Q::::H::	-
RAT	::T:::E::NL::::::::::H::	-
CONSENSUS C/A	N-T$^{E\ R}_{Q\ K}$-bb-?h	

FIGURE 3: Comparison of neuritogenic active regions from saposins C and A. Sequences derived from the complete saposin C and A molecules are shown under the "saposin" headings. For these, only the "neuritogenic region" from saposin C and the corresponding region from saposin A are shown. A consensus sequence for saposin C, and A, is shown for residues 22−32 of saposin C. The saposin C peptides show the overlapping regions from various sources. The colons and periods represent identity and similarity to the respective human saposin sequence, i.e., either saposin C or saposin A, respectively. For the peptides, the comparison is with the human saposin C sequence. The saposin A sequences have been shifted to the left by one amino acid due to a deletion in the saposin C sequence. This shift also aligns the cysteine residues between saposins C and A. The boldface letters represent single amino acid substitutions that were created in the sequence. In the "neuritogenic activity" column, ND means that the sequence has not been tested. In the consensus sequences, b and h refer to branched chain and hydrophobic amino acids, respectively.

with the CCA and SELCON methods. The wavelength ranges were 190−250, 195−240, and 195−260 nm for methods a, b, and c above. All the experiments were done in 50 mM sodium phosphate buffer, pH 7.0, with a final concentration of 0.1 mg/mL for the proteins or synthetic peptides. For peptide solutions, 2,2,2-trifluoroethanol (TFE) was added to a concentration of 50%. The spectra are reported as percent of α-helix (%α), *β*-strand (%*β*), *β*-turn (%T), and random-coiled or other structures (%R) present.

Protein concentrations were determined by the Lowry method (*37*) using bovine serum albumin as the standard. The composition of the synthetic peptides was confirmed by quantitative amino acid analysis or mass spectroscopy.

RESULTS

Comparison of saposins C and A from several species showed ∼40% amino acid identity and 79% amino acid similarity across the mature sequences (Figure 2). A consensus sequence has been derived for the identified neuritogenic region of saposin C (residues 22−31) (Figure 3). Within residues 22−31, only three amino acids residues, 22, 24, and 25 were identical across all species, but neighboring branched chain amino acids were present at residues 28 and

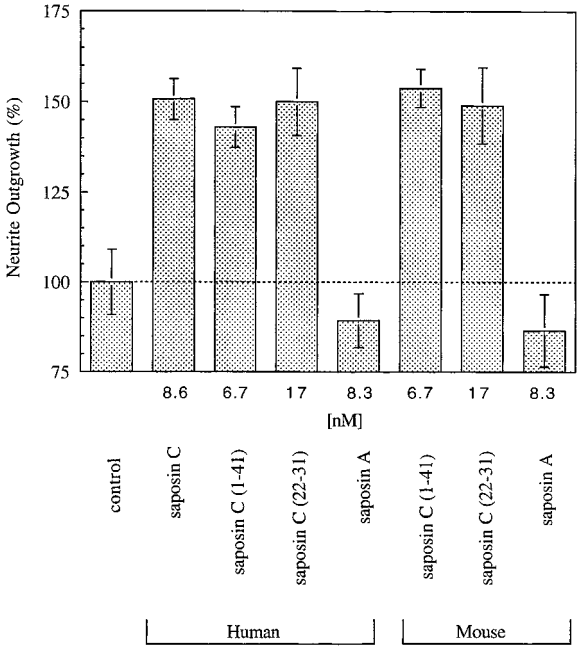

FIGURE 4: Neurite outgrowth effects of saposins on NS20Y cells. Recombinant saposin C or A and saposin C(1−41) from humans and mice were expressed in *E. coli* and purified. Human and mouse saposins C(22−31) were chemically synthesized. The concentrations are based on the calculated molecular weights from each pure protein and the protein amount as determined by the Lowry procedure (*37*). The bar heights represent the means from 6 separate experiments that included >250 cells. The error bars are one standard deviation. The control (i.e., no saposin) and the 8.3 nM saposins A from human or mouse were not statistically different ($p = 0.191$ or 0.03). The results were normalized to the control that was assigned a value of 100%.

29 in all species, and a hydrophobic amino acid was present at residue 32: a leucine in all nonhuman sequences and a phenylalanine in the human sequence. Such a diverse representation from different species is not available for saposin A, but comparison of the sequences for saposins C and A in the neuritogenic region of saposin C revealed a high degree of similarity (Figure 3). The central amino acid in the Asn-linked oligosaccharide consensus sequence is not highly conserved, and is a neutral alanine or a positively charged lysine or arginine. The saposin C and A sequences contain significantly different amino acids at positions corresponding to 27, 30, and 31 of saposin C, and these same residues are diversely substituted across species in saposin C. Because of the high degree of similarity in the neuritogenic region, we began our analyses by testing human and mouse saposins C and A.

Saposin C(1−41) and C(22−31) from human and mouse were potent neuritogenic agents, and showed the same degree of neuritogenic activity with ∼7−17 nM (Figure 4). The maximal activity of human saposin C was at ∼8 nM (Figures 4 and 5). This equivalent potency excludes residues 23 (K→A), 26 (K→E), and 30 (D→K) as critical to the neuritogenic effect because the substitutions are highly nonconservative, particularly for charge. In comparison, intact human or mouse saposin A did not have neurite outgrowth effects (Figure 4, Table 1). Saposins A and C were reduced and alkylated to prevent refolding and disulfide formation. Using these unfolded forms of saposins A and C, neuritogenic activity was observed with the wild-type saposin A sequence to about 40% of the level achieved with

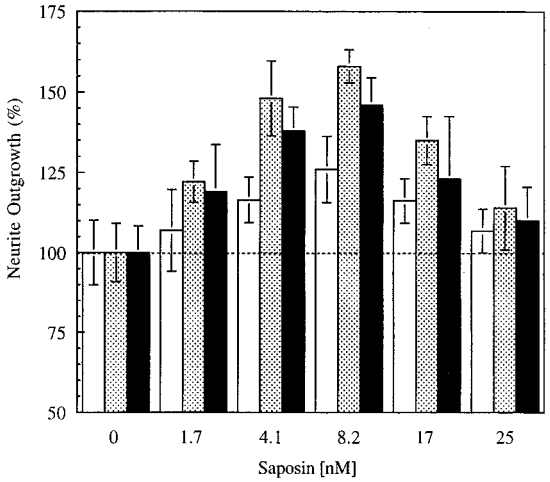

FIGURE 5: Dose−response of neurite outgrowth effect of saposins on NS20Y cells. The neuritogenic effects of saposin C (stippled) and reduced and alkylated saposin C (black) or A (white) are compared at several concentrations. The saposin concentrations, means, and standard deviations were determined as in Figure 3. For reduced or reduced and alkylated saposin C, neurite outgrowth was different from control at 1.7 ($p \sim 0.021$), 4.1 ($p \sim 0.002$), 8.2 ($p < 0.006$), and 17 ($p < 0.02$) nM. For the reduced and alkylated saposin A, the neurite outgrowth was different from the control at 4.1 ($p = 0.042$), 8.2 ($p = 0.0009$), and 17 ($p = 0.043$) nM.

Table 1: Neurite Outgrowth Effects of Various Saposins C and A on NS 20Y Cells[a]

	excess neurite outgrowth changes for saposin concn	
saposin	4.1 nM	8.2 nM
saposin C	48 ± 3.6	58 ± 1.8
reduced/alkylated saposin C	38 ± 2	46 ± 7.4
saposin C[A31Y]	21 ± 3.5	30 ± 15
saposin C[N21D]	32 ± 3.4	47 ± 10
saposin A	3 ± 0.3	−11 ± 0.9
reduced/alkylated saposin A	16 ± 0.9	26 ± 2.1
saposin A[Y30A]	24 ± 1.7	33 ± 2.5
saposin A[D20N]	−21 ± 1.4	−3 ± 0.3
saposin peptides	7.9 nM	17 nM
saposin C(22−31)	53 ± 3.2	58 ± 5.1
saposin C(22−32)[A31Y]	11 ± 0.9	26 ± 3.1
saposin A(21−31)	7 ± 0.5	9 ± 0.5
saposin A(21−31)[Y30A]	36 ± 2.9	53 ± 4.2

[a] All saposins were expressed in *E. coli* cells and purified to homogeneity. Saposin peptides were chemically synthesized. The means and standard errors are based on results from 3 separate experiments, each with over 200 cells counted. Cells bearing neurites longer than 1.0X the cell diameter were scored as positive.

similarly treated saposin C (Table 1). Moreover, the dose−response (1.7−25 nM) profiles of untreated saposin C and of the reduced and alkylated saposins C and A were similar (Figure 5). These results indicated that the secondary structures of untreated wild-type saposins C and A were subtly different and could account for their difference in neuritogenic activity in the natural conformation.

To explore the possibility that these differences in the function of saposins A and C were due to single amino acid substitutions within the neuritogenic region, the homologies of amino acids in this region were compared for the most distantly related species. For saposin C, amino acid 31 (Figure 3) was an alanine in the human, bovine, and guinea pig sequences. At the homologous position in the mouse and rat was a nondisruptive substitution, glycine. The corresponding position in the saposin A sequence, amino acid 30, was preserved as a tyrosine (Y) in rodents and humans. Based on this, saposin C was expressed with a tyrosine substituted at amino acid 31, saposin C[A31Y], as was saposin A with an alanine substituted at the corresponding amino acid, saposin A[Y30A] (Table 1). This single amino acid substitution in saposin C led to a 50−60% reduction in excess neurite outgrowth activity compared to the wild-type saposin C ($p < 0.005$). At all concentrations (1.6−25 nM), the level of neurite outgrowth activity of saposin C[A31Y] was diminished when compared to the wild-type sequence (data not shown). Saposin A[Y30A] acquired neurite outgrowth activity to about 50% of that for the wild-type saposin C (Table 1). None was elicited with the natural saposin A sequence (Table 1). These results were verified by comparing the activities of the synthetic peptides, saposin C(22−31), saposin A(21−31), saposin C(22−32)[A31Y], and saposin A(21−31)[Y30A]. As shown in Table 1, the saposin C peptides had neuritogenic effects, although the wild-type sequence was more potent than saposin C(22−32)[A31Y], i.e., about 20−45% of the wild-type effects. The wild-type saposin A sequences had no neurite outgrowth activity whereas potent effects, 68−91% relative to the wild-type saposin C(22−32), were obtained with saposin A(21−31)-[Y30A]. These results demonstrate that a tyrosine at position 31 of saposin C prevents the majority of the neurite outgrowth activity. Some activity was retained in saposin C[A31Y], indicating that other saposin C peptide sequences may be important for full neurite outgrowth activity.

The N21 of saposin C is conserved in all species (Figure 2) and was thought to be important to the neuritogenic effect (*23*). In comparison, D20 of saposin A is conserved. Thus, the corresponding positions in saposins A and C are isosteric. Saposin C[N21D] and saposin A[D20N] were developed to evaluate the role of this amino acid in the neuritogenic effect. Saposin C[N21D] had 69−81% of the effect as wild-type saposin C effect ($p \sim 0.12$) (Table 1). Saposin A[D20N] did not promote neurite outgrowth at 4 or 8 nM. These experiments exclude the N21 of saposin C as being essential to its neuritogenic activity in the context of the complete saposin.

Using rabbit antibodies to the NH_2-terminal 50% of human saposin C, i.e., saposin C (1−41), Western blotting confirmed the presence of subtle conformational differences between saposin C and saposin A (Figure 6). These antibodies had specificity and reactivity for the recombinant saposins C that were reduced, or reduced and alkylated (Figure 6, lanes 6 and 4). The antibodies produced less dense bands with equivalent amounts of saposin C[A31Y] (Figure 6, lane 5) and saposin C(1−41) (Figure 6, lane 7). The greater band intensity with the reduced and alkylated saposin C indicates conformational sensitivity of the repertoire of antibodies in the antiserum. Reduced and alkylated wild-type saposin A did cross-react with these anti-saposin C(1−41) antibodies (Figure 6, lane 1) as did saposin A(Y30A) (Figure 6, lane 2). In comparison, reduced saposin A had no cross-reactivity at the 5−10 ng level (data not shown). Compared to the saposin C derivatives, reactivity was less with the above saposin A variants. These results confirm that reduction and alkylation of saposin A allowed for the exposure of NH_2-terminal saposin C-like epitopes in the saposin A NH_2-

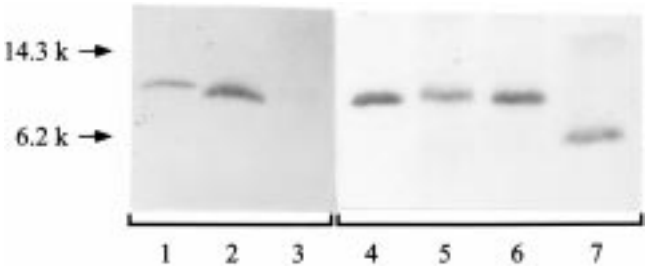

FIGURE 6: Western blot of saposins A and C. Purified recombinant proteins were electrophoresed in high-density polyacrylamide gels in the presence of 5% β-mercaptoethanol, 2.5% sodium dodecyl sulfate, 1 mM EDTA, and 10 mM Tris, pH 8.0. Rabbit antisaposin C(1−41) antiserum was use for localizing the saposins after transfer to nitrocellulose membranes. Lanes 1, 2, and 3 contained reduced and alkylated saposin A, saposin A[Y30A], and wild-type saposin A, respectively. Lanes 4, 5, 6, and 7 contained reduced and alkylated saposin C, saposin C[A31Y], wild-type saposin C, and saposin C(1−41), respectively. All lanes contained 5 ng of the specific protein.

Table 2: CD Studies of Normal and Mutant Saposin C and A Proteins and Peptides

		deconvolution calculations			
saposin[a]	program	%α	%β	%T	%R
wild-type saposin C	MLR	25.3	46.9	5.7	22.0
	CCA	14.8	50.2	34.8	
	SELCON	72.6	1.2	22.4	4.8
wild-type saposin A	MLR	29.4	42.2	2.5	26.0
	CCA	15.0	49.6	35.2	
	SELCON	74.6	0.2	19.3	8.0
reduced/alkylated saposin C	MLR	27.1	48.6	6.3	18.1
saposin C[A31Y]	MLR	21.4	51.7	7.8	19.1
saposin C[N21D]	MLR	17.5	46.0	13.1	23.4
reduced/alkylated saposin A	MLR	15.7	51.5	5.7	27.0
saposin A[Y30A]	MLR	47.3	27.3	0.0	25.4
saposin A[D20N]	MLR	27.9	43.0	9.4	19.6
saposin peptides[b]					
saposin C(22−31)	MLR	4.0	66.0	0.0	30.0
	CCA	0.1	91.4	0.0	
	SELCON	5.8	33.2	22.6	38.3
saposin C(22−32)[A31Y]	MLR	26.7	73.3	0.0	0.0
	CCA	0.1	99.5	0.0	
	SELCON	5.4	45.1	14.8	35.5
saposin A(21−31)	MLR	27.2	72.8	0.0	0.0
	CCA	0.2	99.6	0.0	
	SELCON	4.4	44.8	15.4	32.1
saposin A(21−31)[Y30A]	MLR	4.7	60.4	0.0	34.9
	CCA	0.1	79.0	0.0	
	SELCON	8.5	29.9	23.3	38.5

[a] Acquired in 50 mM sodium phosphate, pH 7.0 [with (*a*) or without (*b*) 50% TFE], at a final concentration of 0.1 mg of saposin/mL.

terminal region. Similar studies were conducted with saposins C[N21D] and A[D20N] (data not shown). The antiserum reactivity to the respective mutant proteins did not differ from that observed with either of the respective wild-type saposins.

Secondary structural comparisons of saposin C and A mature proteins and peptides from the neuritogenic region were made by CD spectroscopy (Table 2). As estimated by the three different deconvolution programs, i.e., MLR, CCA, and SELCON, the secondary structures of wild-type saposin C were similar to those for saposin A. However, the relative changes of the α-helical and β-strand structures of reduced/alkylated and mutant saposins C and A differed with the three deconvolution methods. In Table 2, the data were deconvolved using the MLR basis sets. The mutant saposins C[A31Y] and C[N21D], and saposin A[D20N] had relatively unaltered structures whereas saposin A[Y30A] had about a 50% increase or decrease in α-helical or β-strand structure, respectively. Consequently, correlations between CD secondary structures and neuritogenic activities were not present in the intact saposins. Such correlation was present by CD spectroscopy of synthetic peptides from saposins A and C. CD analysis revealed that the synthetic peptides are mainly unstructured in water. However, upon addition of the hydrophobic membrane mimetic TFE, formation of β-sheet and α-helical structures was observed. The most pronounced changes were in 50% TFE aqueous solution. By all three deconvolution approaches, those saposin peptides with the largest β-sheet contents had poorer to absent neuritogenic activity [i.e., saposin C(22−32)[A31Y] and saposin A(21−31)]. In comparison, those saposin peptides with smaller β-sheet contents had greater neuritogenic activity [i.e., saposin C(22−31) and saposin A(21−31)[Y30A]]. These peptide results lead to the conclusion that a more "saposin C-like" predicted structure produced greater neuritogenic potency. Conversely, saposin C(22−32)[A31Y] peptide had little neuritogenic activity and a more saposin A-like predicted structure.

DISCUSSION

Prosaposin has an expanding repertoire of important intracellular and extracellular roles. Intracellularly, prosaposin is a precursor of four saposins required for the activity of several lysosomal hydrolases (*3, 38*). Extracellularly, prosaposin is found in high concentrations in seminal fluid, breast milk, bile, and cerebrospinal fluid (*39*), and appears to have differential processing in a variety of tissues (*2*). In particular cells of the brain and reproductive tract, significant amounts of prosaposin are unprocessed or partially processed for secretion extracellularly (*2, 40, 41*). The finding that prosaposin promoted ex vivo neurite outgrowth in particular neuronal cell types led to identification of the region of the precursor responsible for these effects (*20, 23*). The neurite outgrowth and the in vivo neuritogenic effects were localized to the region of amino acids 8−32 in saposin C (*20, 23, 25*). The purpose of the present investigation was to delineate some amino acid residues and/or conformational requirements for saposin C's neuritogenic effect ex vivo.

These studies are important since the corresponding amino acids 7−31 in saposin A have high homology and similarity to those in saposin C (Figures 2 and 3), but saposin A does not have neuritogenic activity. Indeed, the similarities between saposins C and A across species are so striking in this region that neuritogenic effects might have been anticipated from saposin A. We found that the wild-type human and mouse saposins A were inactive as neuritogenic agents. The only substantial amino acid difference, based upon the PAM index (*42*), was between A/G 31 of saposin C and Y30 of saposin A. The saposin A[Y30] was conserved in all mammalian species, and the saposin C[A/G 31] substitution is nonpreferential. In a previous study, the juxtaposition of N21 and N22 of saposin C was compared to the corresponding residues D20 and N21 of saposin A (*23*). Based upon these data, a synthetic peptide containing a D21 of saposin C (Figure 3) was shown not have ex vivo neuritogenic effects (*23*). In the present studies, saposin C[N21D] and saposin A[D20N] (Figure 3) were used to

evaluate this activity in the context of the intact saposins. The purified intact saposins C[N21D] and A[D20N] showed no significant alteration in their effects compared to wild-type proteins; i.e., saposins C and C[N21D] had the same neuritogenic properties, and saposins A and A[D20N] had little activity. These results exclude the importance of N21 for neuritogenic effects in the context of the intact saposin C molecule.

Including the work presented here, saposin C or peptides from the corresponding neuritogenic region of saposin C from various species (human, mouse, rat, and bovine species) have neuritogenic effects. The homologies of the "neuritogenic region", amino acids 8−32 for saposin C, from various species and the ability of shorter peptides to elicit neuritogenic responses in NS20Y cells indicate that the critical region can be narrowed to residues 22−31. The conserved hydrophobic amino acid 32 of saposin C is not essential since saposin C(22−31) has neuritogenic properties. Within this region, the A or G at residue 31 of saposin C is a conservative change (*42*), and the human (A31) and mouse (G31) saposins C have similarly potent neurite outgrowth effects. Position 30 of saposin C is occupied by D, K, and H in the human, rat/mouse, and bovine sources, respectively, and all have neuritogenic effects. D (pK = 3.9) and K (pK = 10.5) are acidic and basic amino acids, respectively, while H (pK = 7.0) is the strongest base at neutral pH. Also, D is a small amino acid, and K and H are more bulky. Thus, the nature of the amino acid at this position is not critical to the neuritogenic effects. Similar considerations apply to residues 23 and 27. At position 26, the K in the human and the E (pK = 4.3) in other species exclude charge and bulkiness as essential to the neuritogenic effect. Similarly, the corresponding residue in saposin A was E25. This indicates that a negative charge in this position is neither necessary nor sufficient for the neuritogenic effect. Similar considerations eliminate the cluster of residues 25−27 in saposin C or residues 24−26 in saposin A as critical to the neuritogenic effect. In the bovine saposin C, these residues are all E, i.e., very acidic. In human, the net charge for these residues is −1 due to $E^-K^+E^-$. In the mouse and rat, E^-E^-L has a charge of −2, and in guinea pig $E^-E^-K^+$ has a charge of −1. The latter shows that the position of the K in this triplet also is unimportant. In contrast, the QE^-E^- sequence in saposin A did not prohibit neuritogenic activity when Y30A was present, or when the protein was reduced and alkylated. In comparison, the substitutions A31Y or Y30A would be predicted to be highly unfavorable. The PAM index provides a frequency of 0.2% for these substitutions, indicating that these are disruptive mutations that would be suppressed in phylogenetically homologous molecules. In contrast, a D20N substitution would be sterically conservative. These considerations led us to express saposin C(A31Y) and saposin A(Y30A). These proteins had the predicted effects: (1) Saposin C[A31Y] showed a substantial decrease in neurite outgrowth activity compared to the wild-type sequence. (2) Saposin A[Y30A] showed the acquisition of neuritogenic activity to a similar level as the conversely mutagenized saposin C[A31Y]. Corresponding results were obtained using chemically synthesized peptides with the same amino acid substitutions, albeit saposin A(21−31)[Y30A] had greater potency than the corresponding intact protein, indicating the importance of secondary structure to the neuritogenic effect. Although these experiments indicated the importance of A or G 31 in saposin C, they did not explain why saposin A lacked neuritogenic effects since saposin C[A31Y] had some neuritogenic activity.

The use of reduced and alkylated saposins or synthetic peptides and antibody studies provided insight into the conformational requirements of saposin C for a neuritogenic effect. Reduction and alkylation of saposin A were required to achieve some degree of neuritogenic effect from the wild-type molecule. Presumably, this relates to the ability of saposin A to refold in solution and cloak the "neuritogenic region". This was verified by using synthetic peptides that have substantially linear structure and no disulfide formation potential. Using antibodies to saposin C(1−41), Western blot showed that the reduced and alkylated form of saposin A or saposin A[Y30A] developed reactivity to this antibody, but the reduced wild-type saposin A did not. Similarly, the antibody cross-reactivity to saposin C[A31Y], or reduced and alkylated saposin C, was decreased compared to that of intact saposin C. Also, saposin C[N21D] and saposin A[D20N] had no changes in their neuritogenic properties compared to the wild-type proteins. There was no significant change in the antibody reactivity or the CD spectrum of these mutated proteins. These results verify the conformational dependency of our antibody's reactivity and show that wild-type saposin A does not have exposed cross-reactive epitopes. These studies also show that saposin A[Y30A] assumes a conformation that allows reaction with the antibodies under reducing conditions alone. Consequently, the presence of Y30 in saposin A had significant conformational effects on the intact molecule to cloak an epitope in the Aneuritogenic region, thereby suppressing the neurite outgrowth effects of saposin A. The contextual dependency of this effect is evident from saposin C[A31Y]'s decreased, but not absent, antibody reactivity and neuritogenic effects.

These observations were supported by CD spectroscopy of saposin A[Y30A] having significantly changed the α and β structures compared to wild-type saposin A. Although wild-type saposins C and A have similar α and β structures by CD spectroscopy, a substantial relative change was observed only upon the introduction of Y30A in saposin A, but not A31Y in saposin C. CD spectroscopy of the peptides of the corresponding regions from the wild-type and mutant saposins C and A provided a more direct analyses of local conformational changes. Saposin C(22−32)[A31Y] had a CD spectrum more similar to wild-type saposin A than its parent compound, saposin C. Conversely, saposin A(21−31)[Y30A] had a CD spectrum more similar to wild-type saposin C(22−31) than to its parent compound saposin A(21−31). These results indicate that the converse substitutions in saposins C and A result in the development of a secondary structure that is more like the wild-type obverse. Importantly, the same relative structures or structural changes were obtained with each of the deconvolution methods that rely on different approaches to structural prediction. However, the calculated doubling of the helical formation in saposin A[Y30A] is rather large compared to the subtle change in conformation, as in the saposin C variants, that might be expected from single amino acid substitutions. Such large changes in conformation have precedent, albeit few *(46−49)*.

These studies verify the importance of the region spanning residues 22−31 to the neuritogenic effect of saposin C and,

thereby, prosaposin. The presence of Y30 in saposin A prevents, in great part, these effects and appears to be responsible for at least half of the neuritogenic effect of the peptide in the corresponding saposin C region. This residue alone is not completely responsible for the neuritogenic effect, since O'Brien's group synthesized peptides lacking A31 in saposin C that retained some of the neuritogenic effect (*23*). Also, our studies show that the introduction of Y31 in saposin C allows for the preservation of a low level of neuritogenic activity. Although the in vivo correlates of the ex vivo neuritogenic activity remain to be elucidated, the clear demonstration of the critical region provides targets for evaluation in such studies.

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