

# An amphipathic helical motif common to tumourolytic polypeptide NK-lysin and pulmonary surfactant polypeptide SP-B

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**Abstract** The tumour-lysing and antimicrobial polypeptide NK-lysin and the pulmonary surfactant-associated polypeptide SP-B exhibit 24% residue identities (49% similarities), including six half-cystine residues in the same disulphide bonding pattern, and similar far-UV circular dichroism spectra corresponding to 45–55%  $\alpha$ -helix and 20–25%  $\beta$ -sheet structures. From this, we conclude that the conformations of NK-lysin and SP-B are similar. In contrast, the functional properties of the two proteins are dissimilar: SP-B does not exhibit antibacterial activity and NK-lysin does not significantly effect phospholipid spreading at an air/water interface. Saposins, which solubilize lipids and activate lysosomal hydrolases, the pore-forming amoebapores, and parts of acid sphingomyelinase and acyloxyacylhydrolase, also share 18–27% sequence identities with NK-lysin (and SP-B), including the six conserved half-cystine residues. The inclusion of NK-lysin extends the family of saposin-like polypeptides, all members of which appear to interact with lipids. Strictly conserved structural features with implications for helix topology and lipid interactions are observed.

**Key words:** Lipid-binding polypeptide; Secondary structure; Saposin-like module

## 1. Introduction

NK-lysin is a 78-residue polypeptide with three intrachain disulphide bridges [1]. It was recently isolated from porcine small intestine and found to possess antimicrobial and tumourolytic properties (named NK-lysin from peptide with possible Natural Killer cell origin and lytic properties). Several other antibacterial peptides, that can be grouped into families with different structural properties, have been described [2]. Many of those highlight the importance of charge and secondary structure, such as for example illustrated by PR-39 that contains 26% Arg and 49% Pro [3] and forms poly-proline helices [4]. NK-lysin is homologous to postulated peptides from human lymphocytes, suggesting immunoregulatory properties in common [1]. Likewise, several structurally different polypeptides are important in the regulation of the activity of pulmonary surfactant [5,6]. One of these, SP-B, is hydrophobic but has a fairly conventional 79-residue amino acid sequence with three intrachain disulphides [7–9] and an interchain disulphide

linking two polypeptides into a homodimer [8–10]. SP-B is considered important for alveolar stability at low lung volumes by contributing to the reduction of the alveolar surface tension [5,6]. However, the precise function of SP-B is not known and several different mechanisms of action have been suggested (cf. [11–13]). Sequence-wise, SP-B, two SP-B-like repeats in the SP-B precursor (absent in alveolar surfactant), and saposins which solubilize sphingolipids and activate lysosomal hydrolases, constitute a family of distantly related proteins [14]. In addition, a pore-forming peptide (amoebapore A) from *Entamoeba histolytica* [15], acid sphingomyelinase and acyloxyacyl hydrolase [16] belong to this 'saposin-like' family. These 80-odd residue modules exhibit about 20% pairwise residue identities including six conserved half-cystines [16].

We now notice that the disulphide pattern of NK-lysin is identical to the intrachain disulphide pattern of SP-B, and that NK-lysin belongs to the saposin-like family. Furthermore, the overall secondary structure of NK-lysin and SP-B are similar. From all this and the pattern of strictly conserved residues within the saposin family, a possible four-helix topology is proposed which is compatible with lipid-interacting properties of these proteins.

## 2. Experimental

### 2.1. Polypeptide isolation and analysis

NK-lysin was purified from porcine upper small intestine, via a concentrate of thermostable intestinal peptides [1], and SP-B was isolated from porcine pulmonary phospholipids by repeated chromatography on Sephadex LH-60 in chloroform/methanol/0.1 M HCl [17].

For SDS-PAGE, polypeptides were solubilized in 10 mM Tris-HCl, 1 mM EDTA, 2.5% SDS, pH 8.0, with or without 5.0% 2-mercaptoethanol, separated on homogeneous 20% precast Phast gels, and stained with Coomassie brilliant blue. Amino acid analysis was performed with an LKB Alpha Plus analyzer after acid hydrolysis.

### 2.2. Circular dichroism (CD) spectroscopy

SP-B or NK-lysin (about 30  $\mu$ g) was dissolved in 150  $\mu$ l 20 mM dodecylphosphocholine (DPC)/50 mM sodium phosphate buffer, pH 6.0, and diluted with 50 mM sodium phosphate buffer to 10 mM DPC. DPC was synthesized from dodecanol,  $\text{PCl}_3$  and choline acetate [18]. NK-lysin was also analyzed in the absence of lipid, and was then dissolved in 300  $\mu$ l of the sodium phosphate buffer. All samples were centrifuged for removal of non-solubilized material before analysis. After adjustment to pH 8.0, the NK-lysin and SP-B preparations were reduced by incubation for 2.5 h at 37°C in the presence of 2 mM DTT. The thermostability of the peptides was evaluated by increasing the temperature (20–90°C, 1.7°C/min) under monitoring of the ellipticity at 222 nm. CD spectra were recorded with a Jasco J-720 spectropolarimeter at 22 °C, with a scan speed of 20 nm/min (184–260 nm), a response time of 2 s, and a resolution of 10 data points/nm. Peptide concentrations were determined by amino acid analysis after spectroscopy and the mean residue molar ellipticity was expressed in  $\text{kdeg} \times \text{cm}^2 \times \text{dmol}^{-1}$ . The  $\alpha$ -helical content was calculated from the molar ellipticities at 208 and 222 nm [19] and the content of secondary

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**Abbreviations:** CD, circular dichroism; DPC, dodecylphosphocholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DTT, dithiothreitol; NK, natural killer; PA, palmitic acid; PG, phosphatidylglycerol; SP, surfactant protein.

structure elements was also estimated, using a Jasco program, by comparison of the spectra with those of proteins of known contents of secondary structure elements.

### 2.3. Measurement of adsorption properties

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), egg phosphatidylglycerol (PG), and palmitic acid (PA), (68:22:9, by weight) [20], were dissolved in chloroform/methanol (98:2, v/v) and added to the dried polypeptide (lipid/polypeptide ratio of 20:1, w/w). After sonication, the solvent was evaporated under a stream of nitrogen and the residue suspended in 150 mM NaCl by ultrasonication (50 W, 48 kHz), giving a final lipid concentration of 10 mg/ml. Surface properties were determined at 37°C with a Wilhelmy balance containing 20 ml of 150 mM NaCl. The surfactant preparations (0.1 ml) were added onto the hypophase and the surface pressure was monitored during 1 min. Values given represent three separate recordings.

### 2.4. Analysis of antibacterial activity

Antibacterial activity was determined by an inhibition zone assay in thin agarose plates seeded with *E. coli* strain D21 or *Bacillus megaterium* strain Bm11 [21]. 3 µl samples (1–4 µg/µl) were applied to wells in the agarose plates and incubated overnight. NK-lysin was added in aqueous solution and SP-B was dissolved in either 20–60% aqueous trifluoroethanol (v/v) or 10 mM DPC/50 mM sodium phosphate buffer. Up to 60% aqueous trifluoroethanol did not exhibit significant antibacterial activity, while DPC micelles alone showed low antibacterial activity.

## 3. Results and discussion

### 3.1. NK-lysin is homologous to SP-B and belongs to the saposin-like protein family

The amino acid sequence of NK-lysin is clearly related to that of SP-B. The two porcine polypeptides have 24% identical (49% similar) residues, six half-cystine residues at identical positions, and identical disulphide bridges (Fig. 1). Hence, NK-lysin is a member of the saposin family to which SP-B has already previously been assigned [14]. NK-lysin, SP-B, *Entamoeba histolytica* amoebapore A, human saposins A, B, C, D, the small subunit of human acyloxyacyl hydrolase (positions 7–80), and human acid sphingomyelinase (positions 89–165) are aligned in Fig. 1. These polypeptides exhibit 18–27% pairwise residue identities. NKG5 and 519, two proteins postulated from cDNA sequences from activated human T- and natural killer cells [22,23], show high amino acid sequence similarities to NK-lysin [1] but are not included in the comparisons now since they have not been isolated and their functions are unknown. For the same reasons the two SP-B-like repeats in the proSP-B

sequence [14] are also excluded. As seen in Fig. 1 the six half-cystine residues, at positions 8, 11, 35, 46, 71 and 77 (numbering according to the SP-B sequence), are strictly conserved and the disulphide bridges formed between these half-cystines are identical in NK-lysin [1] and SP-B [8,9], i.e. the half-cystines are linked in the order 1–6, 2–5, and 3–4. The disulphide pattern of the other members has to our knowledge not been established.

SP-B contains a seventh half-cystine residue (positions 48) absent in the other eight polypeptides (Fig. 1). NK-lysin, has a lysine at the corresponding position (Fig. 1), and is a monomer under both non-reducing and reducing conditions, while SP-B is a monomer only under reducing conditions, as judged by SDS/PAGE (not shown). Thus, the conclusion [8,9] that Cys<sup>48</sup> crosslinks two SP-B polypeptides into a homodimer is strongly supported by the present data.

### 3.2. NK-lysin and SP-B have similar conformations

The overall hydrophobic nature of SP-B precludes solubilization in aqueous solutions. The peptide was therefore analyzed for secondary structure in DPC micelles, which have been used in other studies of lipid-associated polypeptides (e.g. [18,24,25]). CD spectra of SP-B solubilized in aqueous trifluoroethanol are almost identical to those obtained in DPC micelles (not shown). The CD spectra of NK-lysin and SP-B are similar, and the overall secondary structure of NK-lysin is apparently not significantly altered in the presence of DPC micelles (Fig. 2). Reduction of the disulphide bonds of either NK-lysin or SP-B does not significantly affect their overall secondary structures as judged by lack of concomitant changes of their CD spectra. Furthermore, both polypeptides are apparently highly resistant to thermal denaturation, and CD spectra measured at 90°C are similar to those measured at 20°C (data not shown). However, reduction of the disulphide bonds of NK-lysin reduces its antibacterial activity (M. Andersson and G. Spyrou, unpublished). Thus, the presence of the three intrachain disulphide bridges is not necessary for maintaining the overall secondary structure, but may stabilize the tertiary structure. These findings correlate with the observation that the pore-forming activity of amoebapore is unaffected by 15 min incubation at 100°C in the absence of reducing agents, but virtually abolished by the same treatment in the presence of DTT [15].

Using the molar ellipticities at 208 and 222 nm [19], it is estimated that NK-lysin contains 52%  $\alpha$ -helix and SP-B 43%.

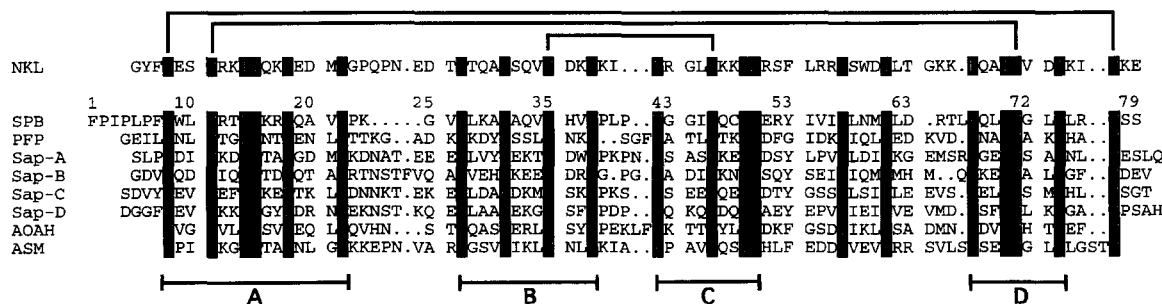


Fig. 1. The saposin-like family of proteins. Sequence alignment using the program PILEUP of saposin-like motifs of porcine NK-lysin (NKL; [1]), porcine SP-B (SPB; [7]), the pore-forming amoebapore A from *Entamoeba histolytica* (PFP; [15]), human saposins A, B, C, D (Sap-A, -B, -C, -D; [30]), positions 7–80 of the small subunit of human acyloxyacylhydrolase (AOAH; [31]) and positions 89–165 of human acid sphingomyelinase (ASM; [32]). The positions numbered correspond to the SP-B sequence. The identical disulphide bonding for both NK-lysin [1] and SP-B [8,9] is indicated at the top. Positions with strictly conserved half-cystines or hydrophobic residues (Val, Ile, Leu, Pro, Ala, Trp, Met, Phe or Tyr) are given against a black background. The proposed amphipathic  $\alpha$ -helical segments are marked A, B, C and D.

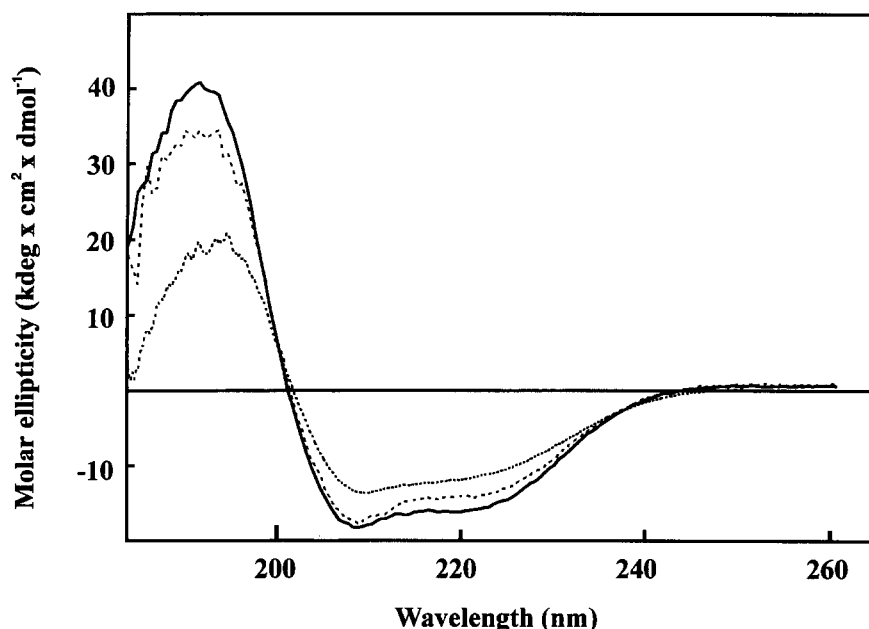


Fig. 2. CD spectra of NK-lysin and SP-B. NK-lysin in 50 mM sodium phosphate buffer, pH 6.0 (solid line), NK-lysin in 10 mM DPC/50 mM sodium phosphate buffer, pH 6.0 (dashed line) and SP-B in 10 mM DPC/50 mM sodium phosphate buffer, pH 6.0 (dotted line).

Comparison of the spectra with reference spectra yields 50%  $\alpha$ -helix for NK-lysin and 40% for SP-B (24% and 23%  $\beta$ -sheet, respectively). The values for SP-B are in excellent agreement with the overall secondary structure determined in DPPC/PG (7:3, w/w) bilayers by Fourier transform infrared spectroscopy (about 45%  $\alpha$ -helix and 20%  $\beta$ -sheet) [26]. Combined, the related amino acid sequences (Fig. 1), the close similarity in overall secondary structure (Fig. 2) and the identical disulphide bridges connecting distant parts of the polypeptide chain, strongly indicate that the three-dimensional structures of NK-lysin and SP-B are similar.

### 3.3. NK-lysin and SP-B exhibit different functions

The similar conformations of NK-lysin and SP-B raise the question whether they also exhibit overlapping functional properties. However, SP-B solubilized in DPC micelles or in 20–60% aqueous trifluoroethanol does not show detectable antibacterial activity toward either *Escherichia coli* or *Bacillus megaterium*. In contrast, NK-lysin exhibits lethal concentrations in the low  $\mu$ M range against these two bacteria [1]. On the other hand, NK-lysin has minimal effect on the spreading kinetics of a DPPC/PG/PA mixture at an air/water interface or on the surface pressure achieved after 1 min, while SP-B has a clear effect on both these parameters (Fig. 3). NK-lysin and SP-B thus have different specific functions.

### 3.4. Possible structural basis for lipid interactions of SP-B and other saposin-like proteins

All saposin-like proteins except NK-lysin are known to interact with lipids and it is not unlikely that the observed antimicrobial and tumourolytic functions of NK-lysin are also mediated via lipid interactions. Most, but not all, antibacterial polypeptides permeabilize membranes, exemplified by the  $\alpha$ -helical magainins and the disulphide-containing defensins [2]. A close inspection of the similarities between the members of the

saposin-like family (Fig. 1) reveals common structural features that may explain the lipid-interacting properties. Four stretches with intermittent repeats of hydrophobic residues (including half-cystine residues) are conserved in all sequences (positions 8–22, 27–38, 42–50 and 67–74, Fig. 1). Each of these four segments can form an amphipathic  $\alpha$ -helix (Fig. 4A). Except for positions 57–61 (Fig. 1), which might form just one helix turn, no additional such strictly conserved segments can be discerned. Hence the four amphipathic  $\alpha$ -helices (Fig. 4A) might constitute a common denominator for the polypeptide backbone fold of the saposin-like module. Prolines do not interfere with the helix predictions (only the first turn of helix C and D contain Pro and this does not interfere with the helical hydro-

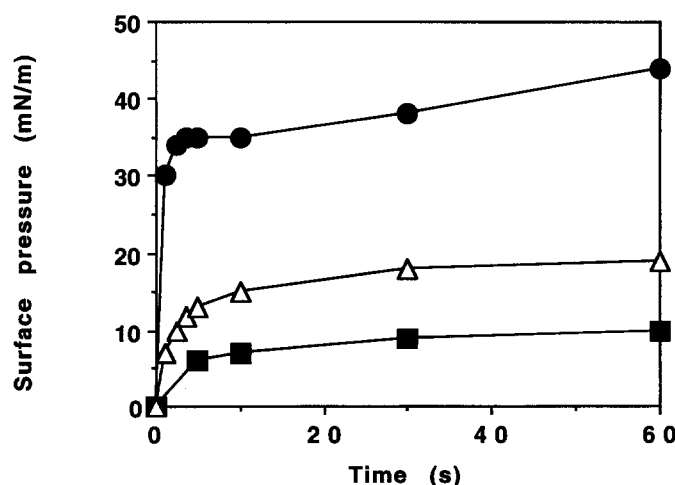


Fig. 3. Surface properties of lipids mixed with NK-lysin or SP-B. Spreading kinetics at an air/water interface of DPPC/PG/PA (68:22:9, by weight) alone (■), and in the presence of 5% (w/w) NK-lysin (△) or SP-B (●). Each value represents the mean of three separate measurements.

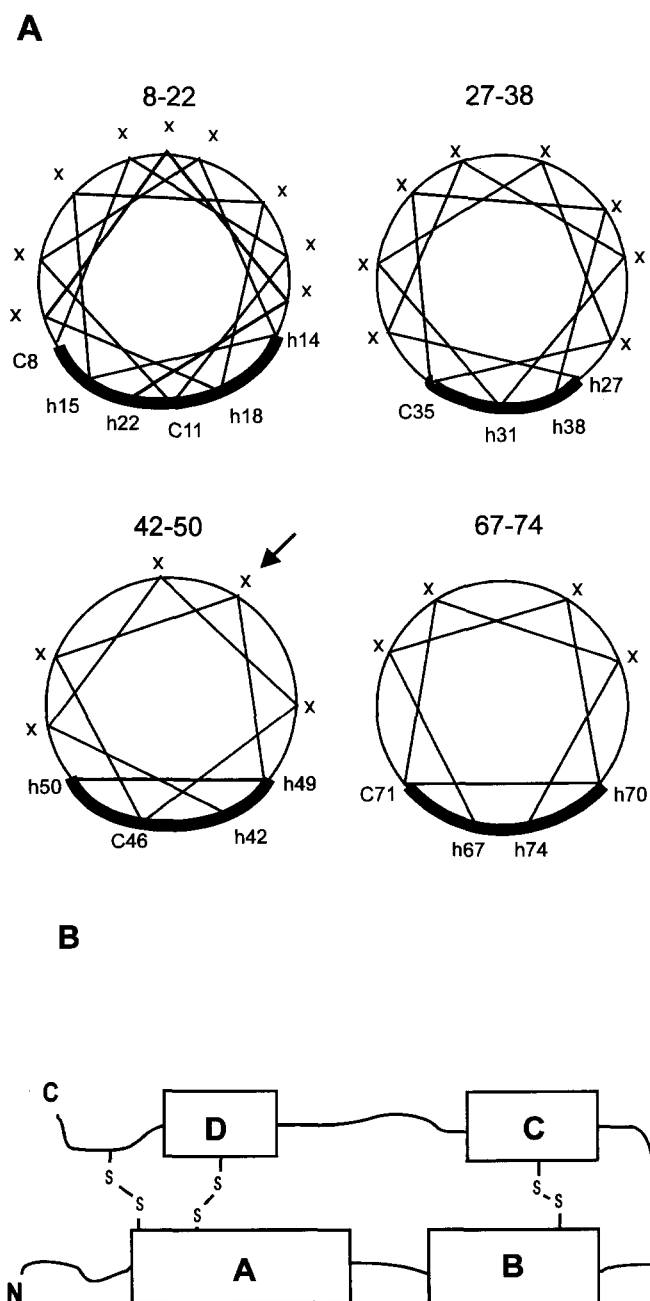


Fig. 4. Amphipathic helices and their topology in a lipid environment. (A) Helical wheel projections and (B) relative orientation of the helices. In A positions 8–22, 27–38, 42–50 and 67–74 refer to the positions of Fig. 1. Strictly conserved half-cystine and hydrophobic residues are marked (C and h, respectively) and numbered. Remaining positions are labeled X. The hydrophobic face of each helix is emphasized by a thick line. In helix C (positions 45–50), the location of Cys<sup>48</sup> in SP-B (but non-Cys in the other peptides) is marked with an arrow. (B) Schematic presentation of the saposin-like topology with the hydrophobic faces (thick lines in A) of the amphipathic helices (cylinders A–D, as also given in Fig. 1) oriented toward the paper. The distances of the segments separating the helices differ between the saposin-like polypeptides (Fig. 1) and are not drawn to scale.

gen bonding). The four proposed  $\alpha$ -helices together constitute 50–55% of the entire amino acid sequences, which is in agreement with the CD data of NK-lysin and SP-B (Fig. 2), the Fourier transform infrared data of SP-B [26] and the CD data

of saposin A, C and D (41–53%  $\alpha$ -helix) [27], but not with those of saposin B (26% helix estimated, [27]). The secondary structures of the remaining parts of the polypeptides may vary between the members of the saposin-like family, as NK-lysin and SP-B contain a significant amount of  $\beta$ -sheet structures (Fig. 2; [26]) while amoebapore is apparently all helical [15]. Recently the amino acid sequences of two amoebapore isoforms, with 47 and 57% residue identities to the sequence shown in Fig. 1, appeared [28]. Both these isoforms exhibit hydrophobic residues at the positions now found strictly conserved (Fig. 1), except at position 14 where one of the isoforms has a threonine residue [28]. This however does not interfere with the general conclusions drawn here since a conserved hydrophobic residue at the adjacent position 15 preserves the amphipathic nature of helix A also in that amoebapore isoform (cf. Fig. 4A).

Notably, the majority of the half-cystine residues in the proposed amphipathic helices are situated laterally and on the left-hand side of the hydrophobic face (when viewed along the helical axis in the orientation of Fig. 4A). Presumably, the amphipathic helices would be located in a lipid interface with the hydrophobic faces oriented toward the lipid core. This is supported by data indicating that SP-B interacts preferentially with superficial parts of DPPC/PG bilayers [26]. Considering the constraints then imposed by three intrachain disulphide bridges (Fig. 1), the relative topology of the helical segments in a lipid bilayer is concluded to be as schematically shown in Fig. 4B. Thus, the common 'saposin-like' fold in the presence of an interfacial lipid environment is proposed to consist of four amphipathic  $\alpha$ -helices where the middle two helices, and the first and last helix, respectively, are aligned from the presence of disulphide bonds in a left-handed antiparallel fashion (Fig. 4B).

SP-B differs from the other saposin-like proteins by being overall hydrophobic (38% Leu, Ile, Val) and by being a covalently linked homodimer. Half-cystine<sup>48</sup> in SP-B, which is concluded to be disulphide-crosslinked to its counterpart in another SP-B polypeptide [8,9] is located at the hydrophilic face of the postulated helix C (Fig. 4A). Consequently, it is possible that in the SP-B dimer the non-hydrophobic parts of the amphipathic helices partly are oriented 'back-to-back' and that thus the SP-B dimer may expose two hydrophobic helix surfaces, forming a possible basis for cross-linking two lipid bilayer entities. Taken together, the overall hydrophobicity and the dimeric nature of SP-B imply that this protein is constantly associated with lipids, while the other members of the family, being water-soluble and having only one hydrophobic helix face, are only intermittently lipid-associated.

Considering this proposed common helix topology for all saposin-like proteins, the basis for their different specific activities can be accounted for by additional regions and subunits in the case of acid sphingomyelinase and acylglycerol hydrolase while the different specific activities of saposins, amoebapores, NK-lysin and SP-B must reside within the module itself. Perhaps the most obvious difference between the saposin-like polypeptides is their net charge, –3 to –6 for the saposins, 0 for amoebapore A, +4 for SP-B and +6 for NK-lysin. Interestingly, in the case of amoebapore, synthetic peptides corresponding to mainly the first and third amphipathic helices exhibit some (but inferior) pore-forming activity [29] and synthetic peptides designed to be intermittently basic and hydrophobic but lacking amino acid sequence similarities to SP-B exhibit surfactant-like

activity [12]. The helix topology now proposed might aid the design and analysis of additional peptide analogues for elucidation of the structure-function relationships of saposin-like proteins.

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