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Pulmonary surfactant protein B: a structural model and a functional analogue

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Abstract

Surfactant proteins B and C (SP-B and SP-C), together with phospholipids, are important constituents of pulmonary surfactant and of preparations used for treatment of respiratory distress syndrome (RDS). SP-B belongs to the saposin family of homologous proteins, which include other lipid-interacting proteins, like the membranolytic NK-lysin. SP-B, in contrast to other saposins, is hydrophobic and a disulfide-linked dimer, and its mechanism of action is not known. A model of the three-dimensional structure of one SP-B subunit was generated from the structure of monomeric NK-lysin determined by nuclear magnetic resonance, and the SP-B dimer was formed by joining two subunits via the intersubunit disulfide bond Cys48–Cys48′. After energy minimization, intersubunit hydrogen bonds/ion pairs were formed between the strictly conserved residues Glu51 and Arg52, which creates a central non-polar region located in between two clusters of positively charged residues. The structural features support a function of SP-B in cross-linking of lipid membranes. Mixtures of phospholipids, an SP-C analogue and polymyxin B (which cross-links lipid vesicles but is structurally unrelated to SP-B) exhibit in vitro surface activity which is indistinguishable from that of analogous mixtures containing SP-B instead of polymyxin B. This suggests an avenue for identification of SP-B analogues that can be used in synthetic surfactants for treatment of RDS.

Keywords: Surfactant protein B; Saposin; Membrane protein; Molecular modelling; Structure prediction; Polymyxin B

1. Introduction

Pulmonary surfactant contains about 80 weight % phospholipids, 10% neutral lipids and 10% proteins. Four surfactant proteins (SPs) have been characterized, namely SP-A, SP-B, SP-C and SP-D [1]. SP-B, which constitutes approximately 0.5–1 weight % of pulmonary surfactant, is the only protein for which there is firm evidence for an essential function in respiration. Human hereditary deficiency of SP-B causes severe respiratory distress soon after birth [2] and SP-B null mice likewise exhibit fatal respiratory dysfunction [3]. In contrast, SP-A knock-out mice do not show signs of respiratory malfunction, but are more susceptible to bacterial infections than wild-type mice [4], and SP-D knock-out mice exhibit disturbed surfactant homeostasis but no apparent

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; PG, phosphatidylglycerol; RDS, respiratory distress syndrome; SP, surfactant protein

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respiratory dysfunction [5,6]. SP-B and SP-C are present in natural surfactant preparations used for treatment of respiratory distress syndrome (RDS) in premature infants. The use of animal lungs for production of natural surfactants is disadvantageous in several respects, and the identification of analogues of SP-B and SP-C would be of significant value.

SP-B is a 17.4 kDa homodimeric protein. Each 79 residue polypeptide chain contains three disulfides and the dimer is held together by a disulfide bond linking Cys48 of the two subunits [1]. The SP-B polypeptide chain exhibits about 20–25% pairwise sequence identity, including identical location of the half-cystines that form intrachain disulfides, to a group of other proteins, now referred to as the saposin family, which include sphingolipid activating proteins (saposins) A, B, C and D, amoebapores, NK-lysin and parts of lipid-metabolizing enzymes [7]. This indicates that these proteins are structural homologues. However, among the saposins, SP-B is the only protein which is a covalent dimer and which is hydrophobic. The dimeric structure of SP-B may be functionally important, e.g. by making it possible to cross-link different lipid membranes, see [1].

It can be anticipated that the size and hydrophobicity of SP-B will make it difficult to experimentally determine its three-dimensional structure in the near future. The three-dimensional structure of monomeric NK-lysin in water was recently determined by nuclear magnetic resonance (NMR) methods, but does not unambiguously reveal NK-lysin's mode of interaction with lipid membranes [8,9]. We have generated an SP-B model based upon the NK-lysin NMR structure and evaluated different modes of SP-B membrane interactions. The model structure agrees with a function of SP-B in vesicle cross-linking, and surface activity experiments show that polymyxin B, a naturally occurring acylpeptide which cross-links lipid vesicles [10,11], can work as an SP-B analogue in vitro.

2. Materials and methods

2.1. Model building

A three-dimensional model of porcine SP-B was obtained by adopting its amino acid sequence into the known fold of NK-lysin (PDB code 1NKL; [8]) using the program ICM (version 2.7, Molsoft LLC, Metuchen, NJ, USA, 1998). SP-B residues 5–22, 23–40, 42–79 were aligned against NK-lysin residues 1–18, 23–40, 41–78, respectively, and disulfide bonds Cys8–Cys77, Cys11–Cys71 and Cys35–Cys46 were introduced. First, the tethers imposed between the corresponding residues of the two proteins were minimized. The structure was then optimized using the biased Monte-Carlo technique [12] with an initial series of 198 000 steps at 300 K. The loop region between Ala20 and Gly25 of SP-B, which is not present in NK-lysin, was then further minimized with 20 000 steps at 1500 K. Finally, the Monte-Carlo procedure was repeated for the whole structure with 201 000 steps at 1500 K and subsequently for the region Ala20–Gly25 with 200 000 steps at 1500 K.

To build the SP-B dimer, a disulfide bond was introduced between the Cys48 residues of each subunit. The dimer was then subjected to a series of 1 000 000 Monte-Carlo energy minimizations at 700 K with the program ICM. During this calculation, the polypeptide backbone was kept fixed, while all bonds of the side-chains were free. These calculations were repeated six times, each time starting with a different relative orientation of the two subunits, in order not to bias the result by the initial conformation. Finally, the dimeric structure with lowest calculated energy was used in the further analyses.

2.2. Surface activity measurements

Peptide lipid mixtures were obtained by combining, in chloroform/methanol, dipalmitoylphosphatidylcholine (DPPC), phosphatidylglycerol (PG), 3% (w/w) of a synthetic analogue of SP-C (SP-C(LKS), [13]) and, in some samples, 2% (w/w) of porcine SP-B [14]. The organic solvents were evaporated and the peptide lipid film was hydrated in 10 mmol/l HEPES buffer (pH 6.9) containing 140 mmol/l NaCl and 2 mmol/l $CaCl_2$ at a lipid concentration of 10 mg/ml. In the samples where SP-B was substituted with polymyxin B, 1% (w/w) of polymyxin B sulfate (Sigma) was added to the HEPES buffer. The peptide mass proportions are similar to those of SP-B and SP-C in pulmonary surfactant [1]. Surface tension of

the mixtures was determined in a pulsating bubble surfactometer [15] at minimum (γ_{min}) and maximum (γ_{max}) bubble radius. After 2 min equilibration at maximum bubble radius, the recordings were obtained at 37°C and approximately 50% surface compression at a rate of 40 cycles per min.

3. Results and discussion

3.1. Generation of a structural model of SP-B

The amino acid sequence of porcine SP-B was modelled into the fold of porcine NK-lysin known from NMR spectroscopy [8], according to the alignment of the SP-B and NK-lysin amino acid sequences used for prediction of secondary structure elements [7]. The results show that the NK-lysin fold can accommodate the SP-B amino acid sequence without major structural rearrangements, which supports the prediction from sequence alignments that these proteins are structurally homologous [7]. Further support for the validity of the SP-B model comes from the fact that the variable positions in the SP-B amino acid sequence, as deduced from alignment of known species variants (Fig. 1), are predominantly located at the surface of the molecule, while the non-polar residues that are conserved in the saposins [7,8] constitute the core of the protein.

A model of the SP-B dimer was generated by joining two SP-B subunits via the intersubunit Cys48–Cys48′ disulfide bond, and subjecting the dimer to energy minimization. In the calculated conformations with lowest energy, the Glu51–Arg52 dipeptide segments of the two different subunits are located in the subunit interface (Fig. 2). The location is such that they can form two intersubunit ion pairs/hydrogen bonds, i.e. one between Glu51 and Arg52′ and the other between Glu51′ and Arg52 (Fig. 3). SP-B molecules with these ion pairs/hydrogen bonds consistently showed lower energy values than models where they did not form. Furthermore, the ion pairs/hydrogen bonds were formed also when the Glu51–Arg52 dipeptide segments of each subunit were located at maximum distance in the starting conformation before the Monte-Carlo procedure. This suggests that these two intersubunit ion pairs/hydrogen bonds are formed in native SP-B. Support for this conclusion comes from the fact that the Glu51–Arg52 dipeptide segment is strictly conserved in all SP-B sequences known (Fig. 1), but is lacking in all the monomeric saposins. Notably, Glu51 is the only acidic residue that is strictly conserved in SP-B. However, it should be pointed out that the values of the energy differences between structures with and without the ion pairs/hydrogen bonds are small and that therefore the SP-B dimer can be stabilized solely from interactions between neutral residues and the intersubunit disulfide bond (see further below).

The intersubunit Glu51–Arg52′/Glu51′–Arg52 ion pairs/hydrogen bonds lock the dimer interface into a fixed position. Dimeric SP-B is an elongated molecule with approximate dimensions of 55×37 Å (Fig. 3), and a subunit interface area of about 800 Å^2. The global distribution of charged and hydrophobic residues in the SP-B model is visualized in Fig. 3. The nine positive and two negative charges per subunit are clustered in two regions located at the opposite poles of SP-B and separated by approximately 33 Å. The region between the charged clusters consists mainly of residues with non-polar side-chains, except for the Glu51–Arg52 dipeptide segment (Fig. 3). Formation of the Glu51–Arg52′/Glu51′–Arg52 hydrogen bonds/ion pairs can however neutralize these charges (see further below). The residue at position 59, which is acidic in all investigated species except pig (Fig. 1), is superficially located and lies outside the non-polar region.

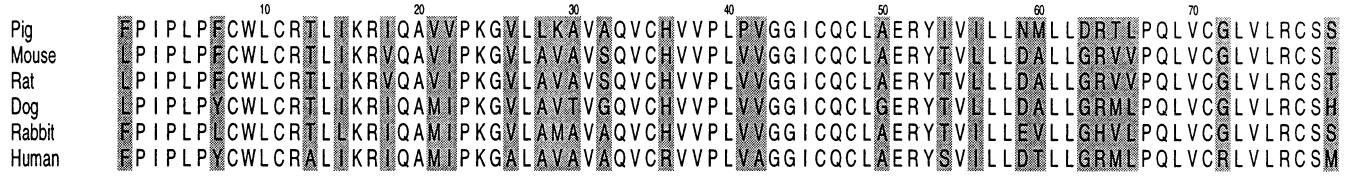

Fig. 1. Alignment of SP-B species variants. Alignment of SP-B amino acid sequences from species where the complete primary structure is established. Positions where the residues are not identical in all species are given against a gray background.

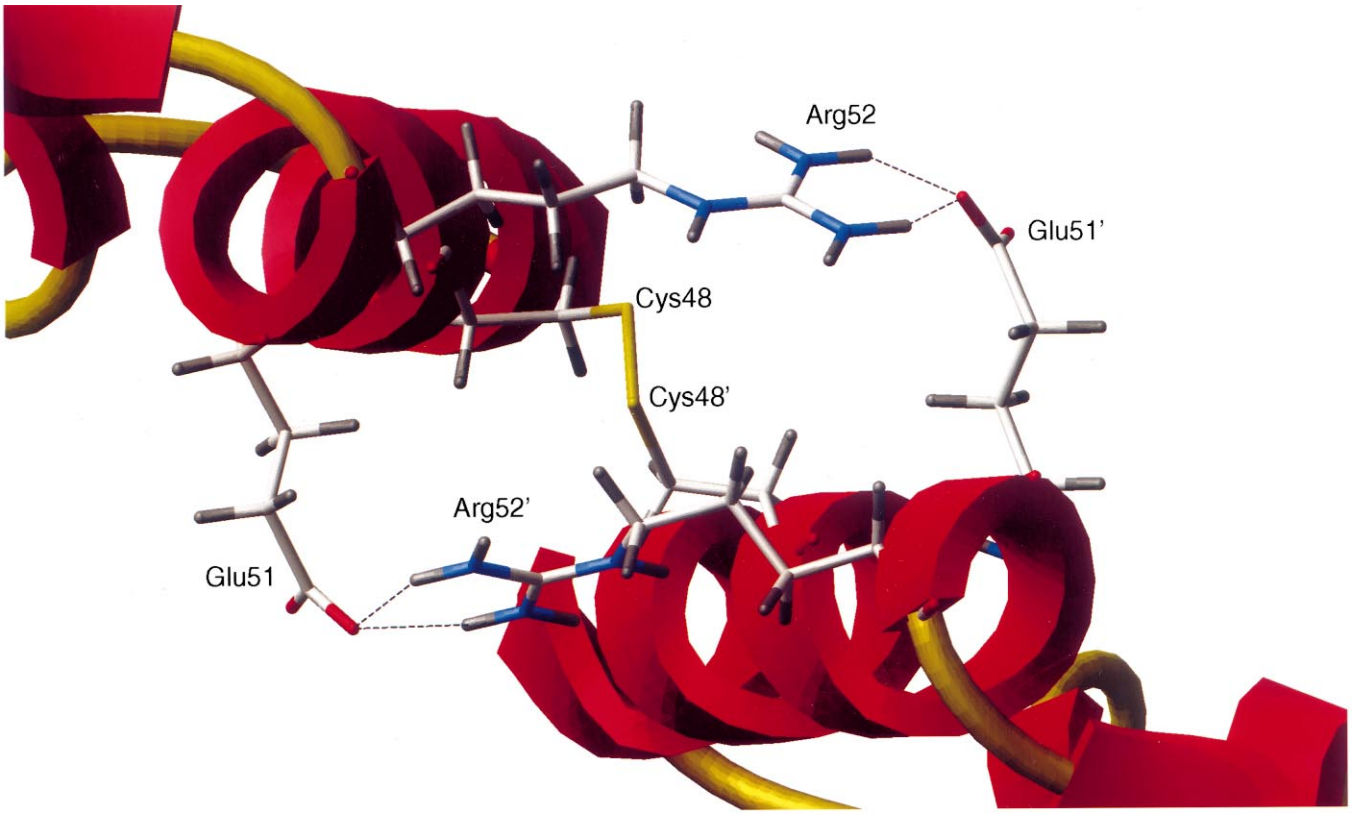

Fig. 2. Close-up of the subunit interface. Helix three of each subunit with the Cys48–Cys48′ disulfide, and the Glu51–Arg52′/Glu51′–Arg52 hydrogen bonds/ion pairs are shown. The dotted lines represent the hydrogen bonds formed between a Glu carboxyl oxygen and the Arg guanidinium group.

3.2. The SP-B model structure in lipid membranes

How SP-B interacts with lipid membranes is not understood in detail, and several different predictions of the location of the SP-B in a phospholipid environment have been put forward [1,16,17]. Clearly, the large non-polar parts of SP-B (Fig. 3) need to interact with a non-polar environment. It is widely held from several experimental studies that SP-B lacks transmembrane segments and is associated with superficial parts of lipid membranes, mainly because only limited effects on the ordering of the lipid acyl-chains have been observed, while the lipid head-groups are affected to a significant extent by SP-B [18–20]. More precisely, at low SP-B content and with gel-phase lipids, little effect on acyl-chain order can be detected, while at higher SP-B contents and with fluid phase lipids, effects on acyl-chain ordering, although still modest, can be detected [21,22]. It has been concluded that SP-B can interact with the edges of lipid bilayer discs and also induce disc formation, see [23]. The model structure is fully compatible with such an interaction; the positively charged poles of SP-B could interact with the phospholipid head-groups, and the non-polar part could interact with the acyl-chains (Fig. 4A). Likewise, SP-B situated in the superficial parts of a lipid bilayer would bury the non-polar part in the membrane acyl-chains and localize the positive clusters close to the phospholipid head-groups (Fig. 4B). SP-B located at the edges of lipid bilayer discs or in the superficial region is compatible both with current experimental data and the main features of the structural model.

In contrast to the situations where SP-B is located at lipid disc edges or in the superficial parts of a bilayer, the lack of experimental support for a transmembrane orientation of SP-B is not possible to rationalize in a straightforward manner from the model. Naively, the model structure is possible to accommodate in a transmembrane manner in a phos-

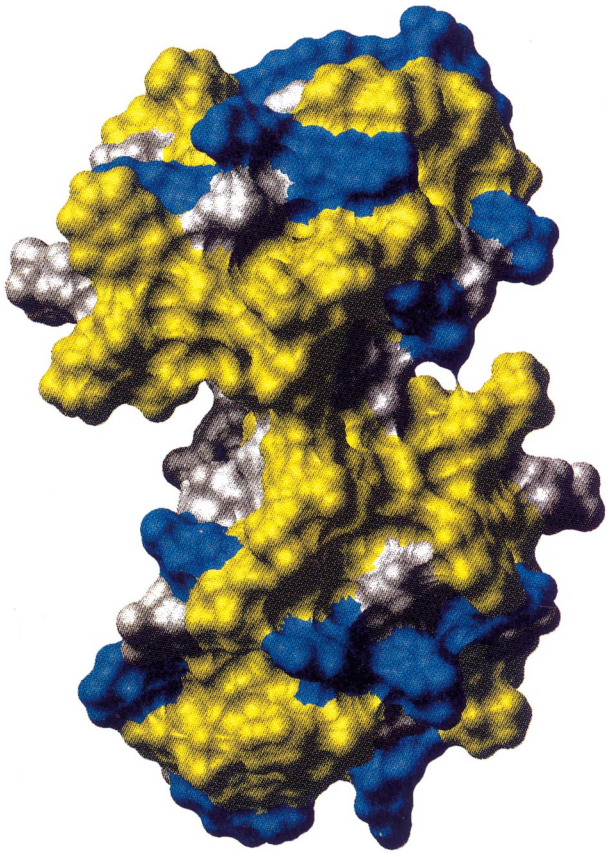

Fig. 3. Polarity of the surface of SP-B. Skin representation of the SP-B dimer where charged residues (Lys, Arg, His, Glu, Asp) are colored blue and hydrophobic residues (Cys, Met, Leu, Ile, Val, Trp, Phe) colored yellow. Other residues are in white. The figure was made with the program ICM.

pholipid bilayer. In the SP-B model, there is a distance of 33–40 Å between the clusters of positively charged residues (Fig. 3), which is in good agreement with the distance between the polar phospholipid head-group regions of a fluid DPPC (the main constituent of surfactant) membrane, about 37 Å [24]. A transmembrane orientation would locate the Glu51–Arg52 segments in the acyl-chain part (Fig. 4C). In a lipid environment, formation of the Glu51–Arg52′/Glu51′–Arg52 intersubunit ion pairs/hydrogen bonds is expected to be favored over the exposure of charged side-chains. Vandenbussche and coworkers [19] concluded from dichroic infrared spectra of SP-B in phospholipid membranes that 60–80% of the SP-B α-helices are parallel to the acyl-chains. With a transmembraneous insertion of the SP-B model, 50–60% of the helical residues would assume an orientation parallel to the acyl-chains. Furthermore, the most likely orientation of NK-lysin in lipid membranes from all available data [9] is similar to the orientation that each SP-B subunit will assume if the dimer is situated in a transmembraneous manner. From the model data, it is not possible to identify one preferred orientation of SP-B in a lipid membrane. Perhaps, the apparent versatility of the SP-B/lipid interactions (Fig. 4) reflects a dynamic behavior of the protein in a membrane environment.

3.3. *The cross-linking peptide polymyxin B mimics the surface activity of SP-B in vitro*

SP-B can fuse membranes, in particular in the presence of anionic phospholipids [25–28], and it has been suggested that SP-B can act by cross-linking two phospholipid bilayers, or a phospholipid bilayer and a phospholipid monolayer [1,13,29,30]. In line with this, the addition of SP-B to lipid/SP-C analogue mixtures lowers the surface tension at maximum radius in a pulsating bubble surfactometer, which was suggested to be an effect of SP-B binding

Table 1
Functional similarity between polymyxin B and SP-B

Peptide content (weight %)			Surface tension (mN/m)					
SP-C(LKS)	SP-B	Polymyxin B	7.5 s		1 min		5 min	
			γ_{min}	γ_{max}	γ_{min}	γ_{max}	γ_{min}	γ_{max}
3	–	–	15 (3)	39 (4)	16 (2)	42 (3)	13 (3)	44 (3)
3	2	–	2 (2)	26 (3)	1 (1)	29 (3)	<1	35 (1)
3	–	1	3 (2)	29 (3)	2 (2)	31 (3)	1 (1)	34 (2)

The preparations were composed of DPPC/PG (7:3, w/w) and the indicated amounts of an SP-C analogue (SP-C(LKS), SP-B or polymyxin B. The mixtures were examined at a concentration of 10 mg/ml in 10 mmol/l HEPES buffer (pH 6.9) containing 140 mmol/l NaCl and 2 mmol/l $CaCl_2$. The recordings were obtained with a pulsating bubble surfactometer at 37°C and 50% surface compression at a rate of 40 per min. The values are the mean (S.D.) of 3–20 measurements.

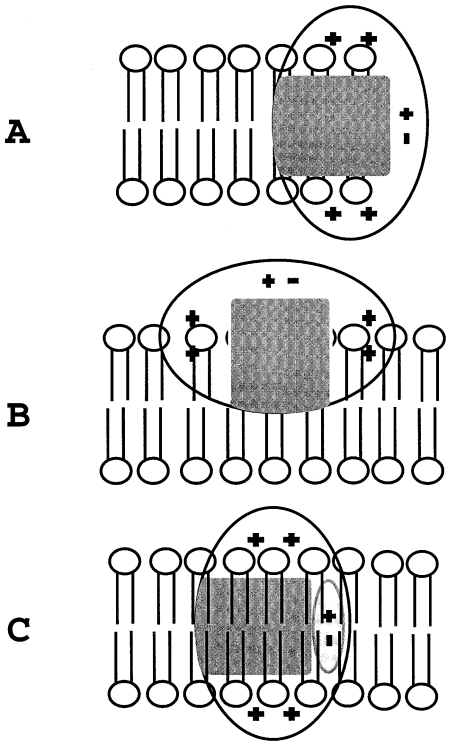

Fig. 4. Interactions between SP-B and lipid membranes. Schematic representations of different ways of locating SP-B in lipid membranes as deduced from available experimental data and the structural model of SP-B, see text for details. The SP-B dimer is represented by the oval symbol, where the positive signs at the poles symbolize the clusters of mainly basic residues, the centrally located plus and minus signs represent the Glu51–Arg52 dipeptide segments, and the central dark area symbolizes the mainly non-polar central region. (A) SP-B located at the edge of a lipid disc, (B) in the superficial region of a membrane or (C) in a transmembraneous manner. In a transmembrane orientation, the charges of the Glu51–Arg52 segment are neutralized via formation of hydrogen bonds/ion pairs, indicated by the gray area.

subphase lipid vesicles close to the air/liquid interface [13].

In order to test experimentally whether the postulated cross-linking ability of SP-B is relevant for its surface activity, we compared the effects of SP-B with those of polymyxin B, an acylated cyclic cationic nonapeptide which cross-links phospholipid vesicles by ionic interactions and promotes intervesicle lipid transfer [10,11]. Addition of 1% (w/w) of polymyxin B to vesicles composed of DPPC/PG/SP-C analogue gives practically identical reduction of γ_{min} and γ_{max} in a pulsating bubble surfactometer as the addition of 2% (w/w) SP-B (Table 1). Higher amounts of polymyxin B did not yield further reduction in surface tension. Polymyxin B has been suggested to act as a hexamer [10], and the molar ratio of SP-B and polymyxin B hexamers in the surfactant preparations studied is 0.7. This experiment suggests that the function of SP-B to a significant extent depends on cross-linking of lipid vesicles, and can be mimicked by a simpler peptide.

The SP-B model lends structural support to cross-linking properties of SP-B. Fig. 5 depicts how SP-B could cross-link two membranes and afford a non-polar surface for intermembrane lipid transfer. It appears possible that this arrangement can be reached both from a start situation where SP-B is in a transmembraneous orientation, and from a situation where SP-B is initially superficially located in a phospholipid bilayer (Fig. 4). Membrane cross-linking and/or fusion probably occur during several steps of the pulmonary surfactant life cycle. For example, transformations from lamellar bodies to tubular myelin and from tubular myelin to an interfacial surface active lipid layer likely involve membrane cross-linking [31].

Previously published SP-B analogues have been considered to be structurally related to the parent

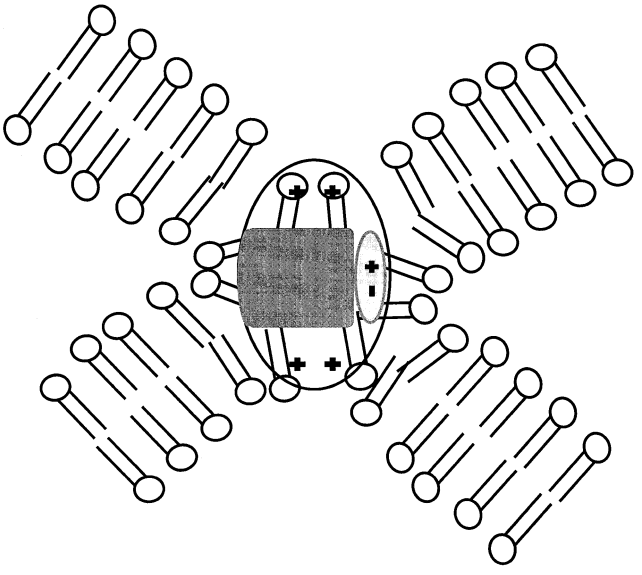

Fig. 5. Cross-linking of two vesicles mediated by SP-B. The positively charged ends of SP-B interact with the head-groups of the two vesicles and the central parts of SP-B facilitate lipid transfer by presenting a non-polar surface. The charges of the Glu51–Arg52 segment are neutralized via formation of hydrogen bonds/ion pairs, indicated by the gray area.

molecule as they represent isolated amphipathic helices of SP-B, or are simplified or permutated versions of such helices [32–34]. The present structural model shows that SP-B exhibits complex features (Fig. 3), which may be difficult to mimic by single 10–25 residue helical peptides. Some investigations have yielded significant effects of such amphipathic or model peptides on surfactant activity both in vitro and in vivo [32–34]. In contrast to this, other studies have shown that single amphipathic helices are inefficient in promoting lipid spreading and insertion at an air/water interface, and that a model peptide based on the N-terminal part of SP-B [33] does not mimic SP-B in the pulsating bubble surfactometer [13,35]. An amphipathic model surfactant peptide [34] efficiently induces lipid disc formation, but is inefficient in promoting lipid spreading [35]. SP-B promotes lipid spreading at an air/water interface [7] and lipid spreading is a central feature of surfactant activity. It therefore seems unlikely that induction of lipid disc formation is sufficient for full activity of SP analogues. To the best of our knowledge, none of the previously published SP-B analogues has been shown to cross-link membranes. The data presented here indicate that SP-B acts via membrane cross-linking and that functional SP-B analogues can be identified among compounds which lack structural similarities with SP-B but are capable of cross-linking lipid membranes.

4. Conclusions

A molecular model of a SP-B dimer shows that Glu51–Arg52 can form intersubunit hydrogen bonds/ion pairs and that the overall distribution of charged and non-polar residues is compatible with different locations of SP-B in a lipid bilayer, as well as with a function of SP-B in membrane cross-linking. The possibility that SP-B may cross-link membranes is supported by showing that polymyxin B, which is known to cross-link membranes but is structurally different from SP-B, mimics the activity of SP-B in a pulsating bubble surfactometer. Further work is needed to understand the molecular function of SP-B, and to evaluate the value of polymyxin B, or other cross-linking/fusogenic compounds, as constituents of artificial surfactants.

Acknowledgements

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