Mapping and Analysis of the Lytic and Fusogenic Domains of Surfactant Protein B†

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Received July 8, 2004; Revised Manuscript Received November 5, 2004

ABSTRACT: Surfactant protein B (SP-B) is a hydrophobic, 79 amino acid peptide that regulates the structure and function of surfactant phospholipid membranes in the airspaces of the lung. Addition of SP-B to liposomes composed of DPPC/PG (7:3) leads to membrane binding, destabilization, and fusion, ultimately resulting in rearrangement of membrane structure. The goal of this study was to map the fusogenic and lytic domains of SP-B and assess the effects of altered fusion and lysis on surface activity. Synthetic peptides were generated to predicted helices and/or interhelical loops of SP-B and tested for fusion, lytic, and surface activities. The N-terminal half of SP-B (residues 1−37), which includes the nonhelical N-terminal amino acids in addition to helices 1 and 2, promoted rapid liposome fusion whereas shorter peptides were significantly less effective. The requirements for optimal surface tension reduction were similar to those for fusion; in contrast, helix 1 (residues 7−22) alone was sufficient for liposome lysis. The C-terminal half of SP-B (residues 43−79), which includes helices 3, 4, and 5, exhibited significantly lower levels of fusogenic, lytic, and surface tension reducing activities compared to the N-terminal region. These results indicate that SP-B fusion, lytic and surface activities map predominantly to the N-terminal half of SP-B. Amino acid substitutions in synthetic peptides corresponding to the N-terminal half of SP-B indicated that, in general, decreased fusion or lytic activities were associated with altered surface tension reducing properties of the peptide. However, the presence of fusion and lytic activities alone could not account for the surface tension reducing property of SP-B. We propose a model in which association of helix 1 with lipids leads to membrane permeabilization but not aggregation; helix 2 mediates membrane cross-linking (aggregation), which, in turn, facilitates lipid mixing, membrane fusion, and interfacial adsorption/surface tension reduction.

Pulmonary surfactant is a complex mixture of phospholipids and proteins that is synthesized, stored, and secreted by alveolar type II cells. The lipid components of surfactant, mainly dipalmitoylphosphatidylcholine (DPPC)[1] and phosphatidylglycerol (PG), reduce surface tension at the air−liquid interface in the lung and prevent alveolar collapse at end expiration. Formation and maintenance of a phospholipid-rich surface film are facilitated by the hydrophobic surfactant proteins SP-B and SP-C. SP-B deficiency results in respiratory failure, indicating that this peptide is absolutely required for lung function (*1*, *2*).

Human SP-B is synthesized as a 381 amino acid preproprotein that is processed to the 79 residue mature peptide in the distal secretory pathway of the type II cell. The mature peptide contains six cysteine residues that form three intramolecular disulfide bridges; a seventh cysteine residue forms an intermolecular disulfide bridge linking two SP-B subunits together to form homodimers. Sequence alignments reveal that the location of the cysteine residues in SP-B is a common feature among a group of proteins referred to as the saposin-like family of proteins (SAPLIP) (*3*). Members of the SAPLIP family include NK-lysin, granulysin, amoebapore, and the sphingolipid activating proteins, saposins A, B, C, and D. All SAPLIP proteins interact with lipid membranes; however, SP-B is the only membrane of this family that is always lipid associated.

SP-B interacts with the surface of the lipid bilayer via four or five amphipathic α helices (*4*). Positively charged amino acids, located predominantly in helix 1, facilitate interaction of the mature peptide with the negatively charged headgroups of phosphatidylglycerol (*5*, *6*). Addition of SP-B to liposomes composed of DPPC/PG (7:3) leads to membrane binding,

† This work was supported by National Institutes of Health Grant R37-HL56285 to T.E.W. J.P.-G. was supported by a grant from the Spanish Ministry of Science and Education (BIO2003-09056).

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[1] Abbreviations: ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; CD, circular dichroism; DPPC, dipalmitoylphosphatidylcholine; DPX, *N*,*N*′-(*p*-phenylenedimethylene)bis(pyridinium bromide); MOPSO, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; MVB, multivesicular body; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PG, phosphatidylglycerol; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; RH-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; SP-B, surfactant protein B.

10.1021/bi0485575 CCC: $30.25
Published on Web 12/24/2004

destabilization (lysis), and fusion, ultimately resulting in dramatic rearrangement of membrane structure (*7*−*9*). The fusogenic and lytic properties of SP-B are likely important for the transition of surfactant phospholipid membranes from the intracellular storage form to the functional extracellular surface film.

The lytic property of SP-B may be required for intracellular maturation of the SP-C proprotein. Within type II cells, SP-B and SP-C proproteins are processed to their mature forms by proteolytic enzymes located in the late endosome/multivesicular body (MVB) (*10*, *11*). SP-B resides in the lumen of the MVB whereas the SP-C proprotein is a transmembrane protein that initially resides on the limiting membrane of the MVB (*12*). Inward vesiculation of the limiting membrane results in relocation of SP-C on internal vesicles, with the N-terminal propeptide in the lumen of the internal vesicle and the C-terminal peptide in the lumen of the MVB. Processing enzymes located in the lumen of the MVB cleave the N- and C-terminal peptides of SP-B (*13*, *14*) and the C-terminal peptide of SP-C but cannot access the N-terminus of SP-C. We proposed that the newly processed hydrophobic mature SP-B peptide associates with internal vesicles leading to membrane lysis, entry of processing enzymes, and completion of SP-C processing (*15*). This model of proSP-C processing is supported by the observation that, in the absence of SP-B, the propeptide of SP-C is not completely removed (*1*, *16*, *17*). Thus the membranolytic property of SP-B may be required for the complete processing of the SP-C proprotein in the MVB.

The fusogenic property of SP-B may be important for organization of surfactant phospholipids in the distal secretory pathway. Mature SP-B and SP-C peptides are stored with surfactant phospholipids as concentric membrane bilayers in specialized secretory granules called lamellar bodies. SP-B is transferred to the lamellar body when a late endosome/multivesicular body fuses with a lamellar body (*18*). Fusion of these two organelles also results in the transfer of the SP-C-containing internal vesicles of the MVB to the lumen of the lamellar body where they are incorporated into the surfactant membranes by a process that likely involves membrane fusion and perhaps lysis. Loss of SP-B in type II cells results in the appearance of highly disorganized lamellar bodies containing numerous MVB-derived vesicles that have not been absorbed into the surfactant membranes, indicating that vesicle/surfactant membrane fusion is an SP-B-dependent process (*1*, *18*).

Fusion and lysis may also play a role in restructuring of the surfactant phospholipid membranes in the alveolar spaces. The contents of the lamellar body are secreted into the airspaces where the surfactant membranes unravel and rearrange into a square tubular lattice structure called tubular myelin (*19*). This form of surfactant may serve as an extracellular surfactant reservoir or intermediate in the transition to the surface film at the air/liquid interface (*20*). Addition of SP-B and SP-A to liposomes composed of DPPC/PG (7:3) resulted in the formation of tubular myelin-like structures in vitro (*8*, *21*). Tubular myelin was not detected in human infants with hereditary SP-B deficiency or SP-B$^{-/-}$ mice (*1*, *22*). The formation of tubular myelin is therefore an SP-B-dependent process that likely involves lipid membrane fusion and/or lysis.

Membrane fusion and/or lysis likely plays an important role in the formation and maintenance of the alveolar surface film. At birth the initial formation of a surface film involves adsorption of newly secreted phospholipids from an extracellular surfactant pool (consisting of tubular myelin and less organized forms of surfactant, collectively referred to as large aggregate surfactant) to the air−liquid interface of the alveolus. Maintenance of a stable surface film is essential for respiration and requires insertion of phospholipids into the expanding film during inhalation followed by phospholipid packing and exclusion from the contracting surface film during exhalation. Both SP-B and SP-C facilitate the transfer of phospholipids between the large aggregate surfactant pool and the surface film; however, in mice, only SP-B deficiency results in lethal respiratory distress syndrome, indicating that this peptide plays a critical role in surface film dynamics (*1*). It is not known if the fusogenic and lytic properties of SP-B play an important role in the formation and maintenance of the surface film. As a first step toward assessing the functional importance of SP-B-mediated fusion and lysis, the fusogenic and lytic domains of human SP-B were mapped, and the requirement of these properties for surface activity was assessed.

MATERIALS AND METHODS

Materials. Dipalmitoylphosphatidylcholine, phosphatidylglycerol, 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG), and the fluorescent lipid probes *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (RH-PE) were purchased from Avanti Lipids (Birmingham, AL). Fluorescent aqueous probes 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and *N*,*N*′-(*p*-phenylenedimethylene)-bis(pyridinium bromide) (DPX) were purchased from Molecular Probes (Eugene, OR). 3-(*N*-Morpholino)-2-hydroxypropanesulfonic acid (MOPSO) and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-75 and LH-60 were purchased from Pharmacia Biotech (Piscataway, NJ). Precast 10−20% tricine gels were purchased from Invitrogen (Carlsbad, CA).

Peptide Design. Synthetic peptides were designed to the proposed helices and interhelical loops of the mature SP-B peptide (*4*, *23*) (Figure 1). Peptides were synthesized by Biosynthesis Inc. (Lewisville, TX) by F-moc chemistry and purified to >95% homogeneity by HPLC. Peptide composition was confirmed by mass spectrometry. Stock solutions (1 mg/mL) were prepared in methanol and diluted into assay buffer to achieve the peptide concentrations indicated in the figures. Appropriate solvent controls were used in each experiment.

Preparation of Native Human Surfactant Protein B. Human SP-B was isolated from bronchoalveolar lavage fluid of patients with pulmonary alveolar proteinosis, as described by Shen et al. (*24*). Briefly, surfactant was isolated from bronchoalveolar lavage fluid by centrifugation and dissolved in chloroform/methanol (2:1). The organic phase was recovered, dried, dissolved in chloroform/methanol/0.1 M HCl [1:1:0.1 (v/v)], and loaded onto an LH-60 Sephadex column equilibrated in the same solvent system. Fractions eluted from the column were screened by SDS−PAGE and silver staining. SP-B-containing fractions were recovered and

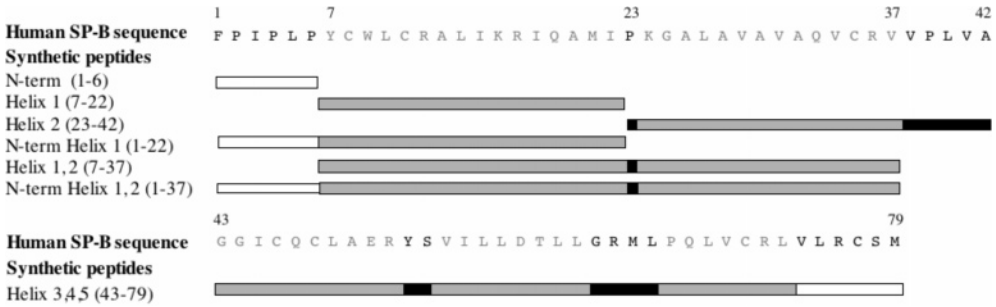

FIGURE 1: Amino acid sequences of SP-B synthetic peptides. Bars represent synthetic peptides designed to the proposed helical regions (gray) and interhelical loops (black) of the SP-B mature peptide. The numbers in parentheses represent the corresponding amino acids in human SP-B. A full-length synthetic SP-B peptide (residues 1−79) was also synthesized (not shown). It is important to note that the exact boundaries of these domains are not known.

dialyzed (SnakeSkin dialysis tubing, molecular weight cutoff of 3500; Pierce Chemical Co.) against chloroform/methanol [2:1 (v/v)] overnight at 4 °C to remove HCl, as previously described (*25*). The dialysate was dried down and stored at −80 °C.

Circular Dichroism. Far-UV circular dichroism (CD) spectra of native human or full-length synthetic SP-B and the different peptides in methanol were recorded in a Jasco 715 spectropolarimeter equipped with a xenon lamp. Methanolic solutions of all peptides and proteins were prepared at 0.2 mg/mL, and the final protein concentration of each sample was reevaluated by amino acid analysis. All of the spectra were recorded in a 0.2 mL thermostated quartz cell of 0.1 cm optical path. Ellipticity was calculated, taking 110 as the mean molecular weight per residue in SP-B.

Preparation of Phospholipid Vesicles. Phospholipids in chloroform were dried under N_2 and resuspended in 50 mM MOPSO/140 mM NaCl/0.1 mM EDTA buffer, pH 5.5 or 7.0, to a final concentration of 400 μg/mL. The phospholipid suspension was passed through a miniextruder (Avanti Lipids, Birmingham, AL) at 45 °C through two stacked 0.1 μm polycarbonate filters. A series of 10 extrusions were performed to generate a population of unilamellar liposomes with diameters of approximately 200 nm.

Fusion Assays. The increase in vesicle size associated with protein-promoted vesicle aggregation/fusion was monitored by an N4+ particle size analyzer (Coulter, Miami, FL) as we have previously described (*26*). Liposomes were added to a 2 mL cuvette (final concentration of 20 μg/mL) and mixed with individual peptides or native human SP-B (1 mg/mL stock solution in methanol) at room temperature. Light scattering produced as a result of the SP-B-induced increase in vesicle size (herein referred to as fusion) was acquired at a 90° angle, monitored for 20 min, and processed using unimodal distribution. Baseline liposome size was recorded prior to the addition of peptide.

Lipid Mixing Assays. Lipid mixing was measured as previously described (*8*). Briefly, liposomes (20 μg/mL) were prepared in the presence 0.1 M % of NBD-PE and RH-PE to incorporate the two fluorescent probes into the lipid bilayer. Liposomes containing both fluorescent probes were mixed with unlabeled liposomes (1:9) in MOPSO buffer (50 mM MOPSO, 140 mM NaCl, 0.1 M EDTA), and FRET was monitored in a RatioMaster fluorometer (Photon Technology, South Brunswick, NJ) for 200 s as synthetic peptide or native SP-B (2 μg/mL) was added. NBD excitation was at 450 nm, and emission was set to 520 nm.

Lysis Assays. Membrane leakage was measured as previously described (*8*). Liposomes were prepared in a buffer containing 12.5 mM ANTS and 45 mM DPX. Encapsulated probes were separated from free probes on a Sephadex G-75 column (1.5 cm × 20 cm). Membrane disruption causes the encapsulated ANTS probe to dilute away from the DPX quencher, resulting in an increase in fluorescence. The excitation of ANTS was set at 360 nm, and emission was recorded at 520 nm using a RatioMaster fluorometer (Photon Technology, South Brunswick, NJ) for 200 s as peptide or native human SP-B was added. Baseline fluorescence was recorded prior to the addition of peptide. Maximum fluorescence was recorded following the addition of 0.2% Triton X-100.

Measurement of Surface Activity. The surface tension reducing properties of individual peptides were assessed by captive bubble analysis (*27*). Mixtures of synthetic peptide (4%) and DPPC/POPG (7:3 by weight) were dried and resuspended in saline with brief sonication. The mixture was applied to the air−water interface of a 25 μL air bubble by microsyringe. Surface tension was recorded every 10 s for 300 s to establish equilibrium surface tension. The bubble was then pulsated at 10 cycles per minute and minimum surface tension recorded at the fifth pulsation when the bubble was reduced to 80% of its original volume.

Measurement of Tryptophan Fluorescence Emission Spectra. Fluorescence emission spectra were acquired by scanning (300−400 nm) the synthetic SP-B peptides in an SLM-Aminco Bowman Series 2 luminescence spectrometer (Urbana, IL). Excitation wavelength (λ_{EX} = 280 nm) and spectral bandwidths (4 nm) were used for the excitation and emission monochromators. SP-B (2 μM) was added to DPPC/PG liposomes at protein-to-lipid ratios of 1:20 (mol/mol). Liposomes alone had no fluorescence under these conditions. The association of the SP-B synthetic peptides and DPPC/PG liposomes was determined by the shift of the fluorescence emission for tryptophan.

Data Analysis. All data are expressed as mean ± SEM. Differences between the two groups were determined by two-tailed *t*-test analysis.

RESULTS

Fusogenic Activity of SP-B Synthetic Peptides. Synthetic peptides were used to map the fusogenic and lytic activities of SP-B because there is currently no expression system capable of producing recombinant SP-B. Peptides were synthesized to the predicted helices and interhelical loops

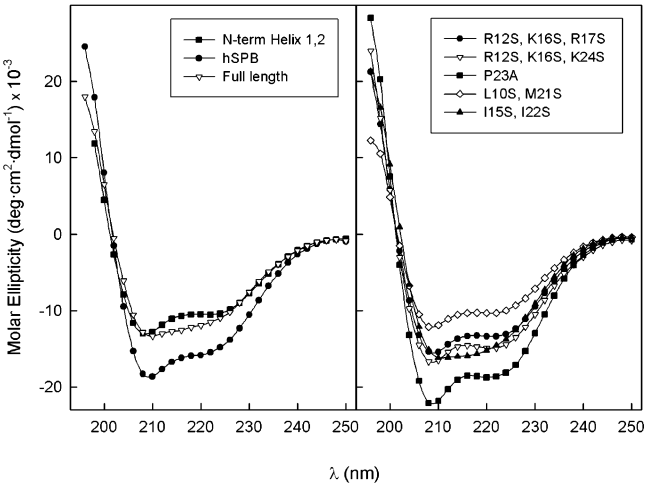

FIGURE 2: Far-UV CD spectra of SP-B synthetic peptides. CD spectra were generated for native human SP-B (hSP-B), the synthetic full-length 79 amino acid protein and the SP-B 1−37 peptide (left panel), and some of the SP-B 1−37 variants (right panel), dissolved in methanol. Spectra for all other peptides listed in Figures 1 and 7 but not shown in the figure fell between the spectrum for native SP-B and full-length synthetic peptide.

of SP-B on the basis of the 3D structure of NK-lysin (*4*, *23*) (Figure 1); it is important to note that the exact boundaries of each domain are not known. CD spectra indicate that each peptide has a relatively high helical content in methanol, as does native SP-B as indicated by molar absorptivity at 222 nm (Figure 2). To assess the fusogenic properties of the synthetic peptides, DPPC/PG (70:30) liposomes with an average diameter of 200 nm were incubated with increasing concentrations of individual SP-B peptides at room temperature. The increase in vesicle size was monitored by an N4+ submicrometer particle size analyzer, which estimates the sizes of uniform vesicles over the range of 3−4000 nm. A peptide encompassing residues 1−37 (N-term helix 1,2) promoted a dose-dependent increase in vesicle size at pH 7.0 (Figure 3) and pH 5.5 (not shown). Helices 3,4,5 also promoted vesicle fusion but to a lesser extent than N-term helix 1,2. Removal of the N-terminal six amino acids from N-term helix 1,2 resulted in decreased fusion to values similar to helix 3,4,5; however, the six amino acid N-term peptide alone was not capable of inducing liposome fusion (not shown). Similarly, helix 2 alone had no fusogenic activity; helix 1 exhibited a small amount of fusion activity at the highest dose of peptide. These results suggest that the entire N-terminal region of SP-B (i.e., residues1−37) is required for liposome fusion.

To compare the fusogenic activity of N-term helix 1,2 to native SP-B, DPPC/PG (70:30) liposomes were incubated with increasing concentrations of native human SP-B (Figure 4). Native SP-B promoted a dose-dependent increase in vesicle size at both pH 7.0 and pH 5.5. Maximum fusion was attained at a concentration of 120 nM native peptide. However, despite very similar α-helical structure the N-term helix 1,2 peptide required a 20-fold higher concentration (2.5 μM) to achieve an increase in vesicle size comparable to native SP-B (Figure 3). The addition of helix 3,4,5 to N-term helix 1,2 did not increase fusogenic activity or decrease the concentration of synthetic peptide needed to achieve maximum fusion (not shown). The fusogenic activity of a full-length, synthetic SP-B peptide (residues 1−79) was significantly less than that of N-term helix 1,2 (Figure 3) despite the similar CD spectra of the peptides (Figure 2). These results suggest that while the N-terminal region of SP-B is important for fusogenic activity, the tertiary and/or quaternary structure of SP-B is important for maximal activity.

Lytic Activity of SP-B Synthetic Peptides. The lytic activity of SP-B was mapped to individual helices by measuring leakage of liposome contents in the presence of synthetic peptides. Disruption of liposome membranes was monitored by recording the release of the fluorescent probe ANTS and its quencher DPX from encapsulated DPPC/PG (70:30) liposomes at baseline and after the addition of synthetic peptide (Figure 5). Native SP-B was very effective in disrupting liposome membranes and caused a rapid increase in fluorescence (comparable to the Triton-X 100 control) at a concentration of 60 nM peptide (Figure 5A). The smallest synthetic peptide capable of inducing liposome lysis was helix 1 (residues 7−22 of SP-B); maximal lytic activity was achieved at a concentration of 0.5 μM peptide (Figure 5B,D). Full-length synthetic SP-B peptide also exhibited maximal lytic activity at a concentration of 0.5 μM peptide (Figure 5B). Helix 2 alone had no lytic activity whereas helices 3,4,5 exhibited reduced lytic activity compared to helix 1 (Figure 5C). Collectively, these results indicate that native SP-B is

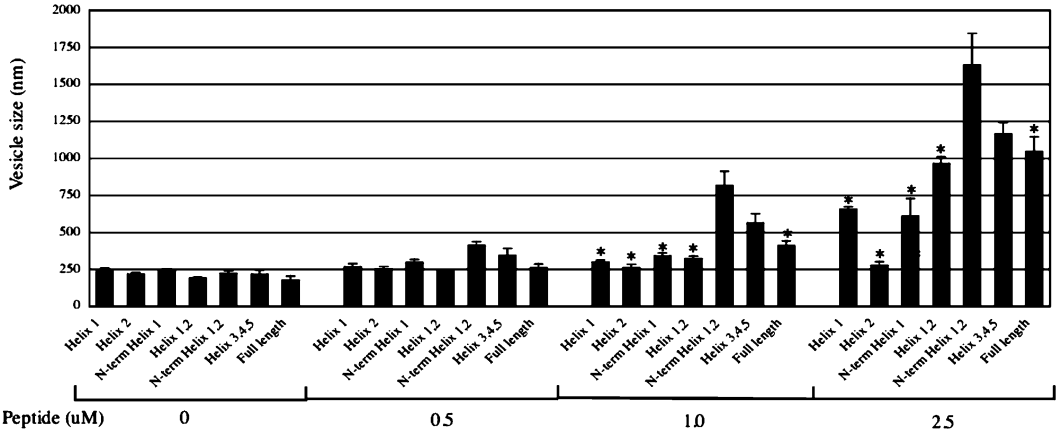

FIGURE 3: Effect of SP-B synthetic peptides on liposome size. Liposomes (20 μg/mL) composed of DPPC/PG (7:3 by weight) with an average diameter of 200 nm were suspended in 50 mM MOPSO/140 mM NaCl/0.1 mM EDTA buffer, pH 7.0, and incubated with increasing concentrations of individual SP-B synthetic peptides. SP-B-mediated increase in vesicle size was determined using an N4 particle size analyzer at room temperature. Results ($n = 3$) are expressed as mean ± SEM; * = $p < 0.05$ for synthetic peptides versus N-term helix 1,2.

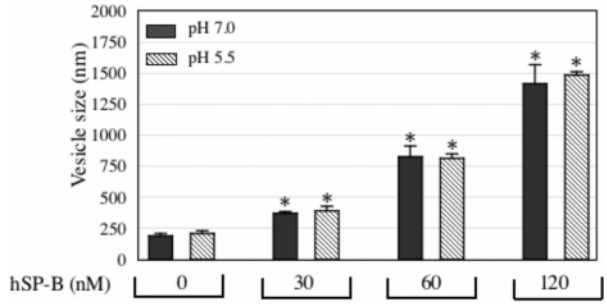

FIGURE 4: Effect of native SP-B on liposome size. Liposomes (20 μg/mL) composed of DPPC/PG (7:3 w/w) with an average diameter of 200 nm were suspended in 50 mM MOPSO/140 mM NaCl/0.1 mM EDTA buffer, pH 5.5 or 7.0, and incubated with increasing concentrations of native human SP-B. The increase in vesicle size was determined using an N4 particle size analyzer at room temperature. Results ($n = 3$) are expressed as mean ± SEM. * = $p < 0.05$ for liposomes with SP-B versus liposomes alone.

significantly more lytic and fusogenic than any of the synthetic peptides.

Surface Activity of SP-B Synthetic Peptides. To assess the contribution of individual SP-B helices to surface tension reduction, the surface properties of synthetic peptides were assayed by captive bubble analysis (Figure 6). N-term helix 1,2 (i.e., residues 1−37 of SP-B) and full-length SP-B (residues 1−79) were the only synthetic peptides that reduced minimum surface tension to values <5 mN/m. Helix 1,2 exhibited poor surface tension reducing properties (>15 mN/m), indicating that the nonhelical, N-terminal six amino acids were essential for surface tension reduction. All other peptides, including the highly lytic helix 1, generated elevated minimum surface tensions (≥15 mN/m), indicating that the lytic activity of SP-B was not sufficient to reduce surface tension. Generation of low minimum surface tension (<5 mN/m) required at least 1.5% dimeric, native SP-B peptide or 4% monomeric, N-term helix 1,2 synthetic peptide; the minimum surface tension attained with 3% N-term helix 1,2 peptide was >10 mN/m (not shown).

Identification of Amino Acids Critical for Fusogenic Activity. The domain mapping experiments indicated that helix 1 was absolutely required for the fusion, lytic, and surface activities of SP-B. To further examine the molecular basis for these properties, amino acid substitutions were introduced into the N-term helix 1,2 synthetic peptide (i.e., residues 1−37 of SP-B) (Figure 7). Substitution of individual, positively charged amino acids in helix 1 (R12A or K16A) or the N-terminus of helix 2 (K24) did not affect the fusion activity of N-term helix 1,2 (not shown). Likewise, combinatorial substitution of a positively charged residue in helix 2 (K24A) and helix 1 (R12A) did not alter fusogenic activity. However, substitution of two positively charged residues in helix 1 (R12S,K16S or K16S,R17S) partially inhibited liposome fusion (Figure 8A). Fusogenic activity was further inhibited by combinatorial substitution of R12S and K16S in helix 1 and K24S in helix 2. The largest effect occurred when all three positively charged amino acids in helix 1 (R12S,K16S,R17S) were substituted. Paired substitution of serines for hydrophobic residues in the nonpolar face of helix 1 (L10S,M21S, I14S,L18S, or I15S,I22S) also inhibited liposome fusion (Figure 8B). None of the peptide variants of helix 1 showed significantly different secondary structure compared with the native sequence, as assessed by CD (Figure 2). Thus, the changes in the polarity of either subdomain of helix 1 likely account for the altered fusogenic activity of SP-B peptides.

The N-terminal hydrophobic sequence (residues 1−6) was previously reported to be important for anchoring SP-B to phospholipid bilayers (*28*). In particular, tryptophan residues are known to play an important role in the interaction of

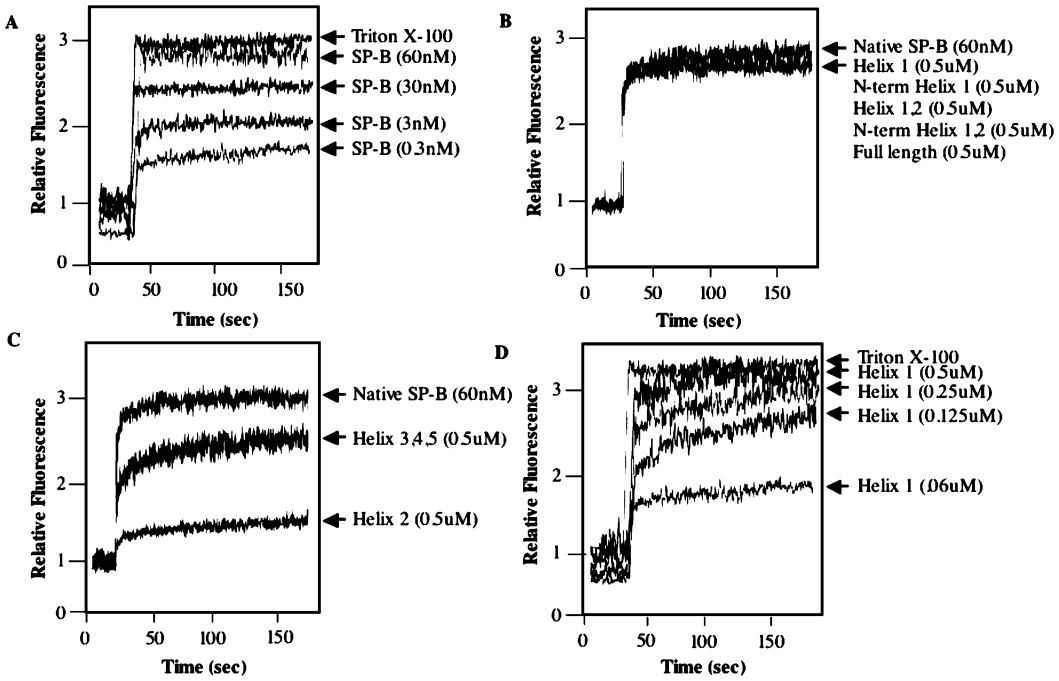

FIGURE 5: Leakage of DPPC/PG liposome contents in the presence of native SP-B and synthetic peptides. Liposomes (20 μg/mL) containing encapsulated fluorescent probes were added to a 2.0 mL cuvette containing MOPSO buffer, pH 7.0, and stirred continuously. Fluorescence was recorded at baseline and after the addition of increasing concentrations of native SP-B, synthetic peptide (1 mg/mL stock solution in methanol), or vehicle control for 180 s. Maximum fluorescence was detected after the addition of Triton X-100. Each curve is representative of three experiments.

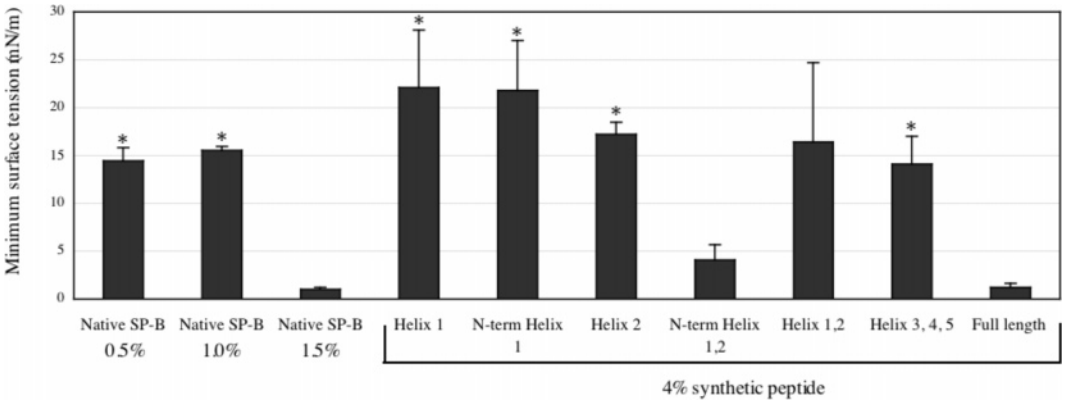

FIGURE 6: Surface activity of synthetic SP-B peptides. Surface properties of mixtures of synthetic peptide (4%) and DPPC/POPG (7:3 by weight) were assessed by captive bubble analysis. The bubble was pulsated at 10 cycles per minute, and the minimum surface tension was recorded at the fifth pulsation, when the bubble was reduced to 80% of its original volume. The values shown are the average of three separate experiments ± SEM; * = $p < 0.05$ for synthetic peptides versus 1.5% native SP-B.

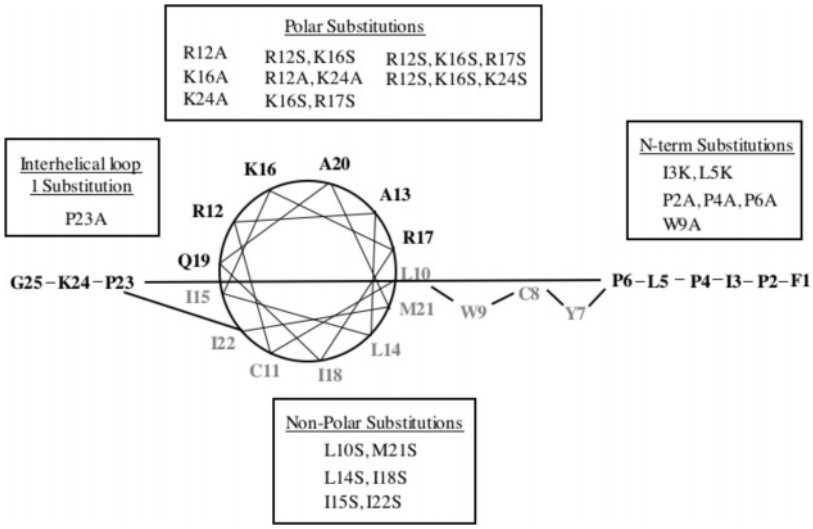

FIGURE 7: Helical wheel prediction of SP-B (amino acids 1−25) showing the relative location of each residue with respect to the phospholipid bilayer [adapted from Wang, Rao, and Demchuk (*30*)]. The diagram shows the amphipathic nature of helix 1 with positively charged residues R12, K16, R17, and K24 at the surface of the membrane and hydrophobic residues Y7, C8, W9, M21, L14, I18, C11, I22, and I15 embedded in the lipid bilayer (gray). The horizontal line represents the surface of the phospholipid bilayer. Alanine, serine, or lysine substitutions were introduced at the indicated positions (boxes).

peptides with lipid membranes (*29*). SP-B has one tryptophan residue at position 9 that has previously been reported to be embedded in the phospholipid bilayer (*30*, *31*). Substitution of tryptophan 9 to alanine did not affect the fusogenic activity of SP-B (not shown). The helix-breaking amino acid proline has also been shown to be important for the activity of some fusogenic peptides (*32*); however, substitution of P2,4,6 to alanine did not affect fusogenic activity; likewise, increasing the polarity of the N-terminal domain, by substituting lysine for I3 and L5, did not alter liposome fusion (not shown). Therefore, although deletion of the N-terminal domain (residues 1−6) significantly decreased fusogenic activity (Figure 3), the molecular basis for this effect is not clear.

In addition to the N-terminal domain and helix 1, the proline residue between helix 1 and 2 was shown to be important for fusogenic activity (Figure 8B). Substitution of P23 to alanine dramatically inhibited liposome fusion, suggesting that a bend between helix 1 and 2 may be required for this activity. Interestingly, the peptide with the substitution P23A had a CD spectrum that was consistent with a significant increase in the proportion of an α-helical conformation.

Identification of Amino Acids Critical for Lytic Activity. Positively charged amino acids have been shown to be critical for liposome lysis and disruption of bacterial membranes (*33*). Substitution of individual, positively charged amino acids in helix 1 (R12A or K16A) or helix 2 (K24A) did not affect liposome lysis (not shown). In addition, substitution of two positively charged amino acids in helix 1 (R12S,-K16S or K16S,R17S) or helix 1 and helix 2 (R12A,K24A) did not alter the lytic activity of SP-B (not shown). However, substitution of three positively charged amino acids (R12S,-K16S in helix 1 and K24S in helix 2) partially reduced liposome lysis (Figure 9). Lytic activity was inhibited to an even greater extent by substitution of all three positively charged residues in helix 1 (R12S,K16S,R17S). Compared to N-term helix 1,2, liposome leakage induced by R12S,-K16S,K24S or R12S,K16S,R17S peptides was very slow and required higher concentrations of peptide. None of the other substituted SP-B peptides had any effect on membrane lysis (not shown), indicating that positively charged residues were the major determinant of this property.

Identification of Amino Acids Critical for Surface Activity. To determine if amino acids critical for fusion and lysis were

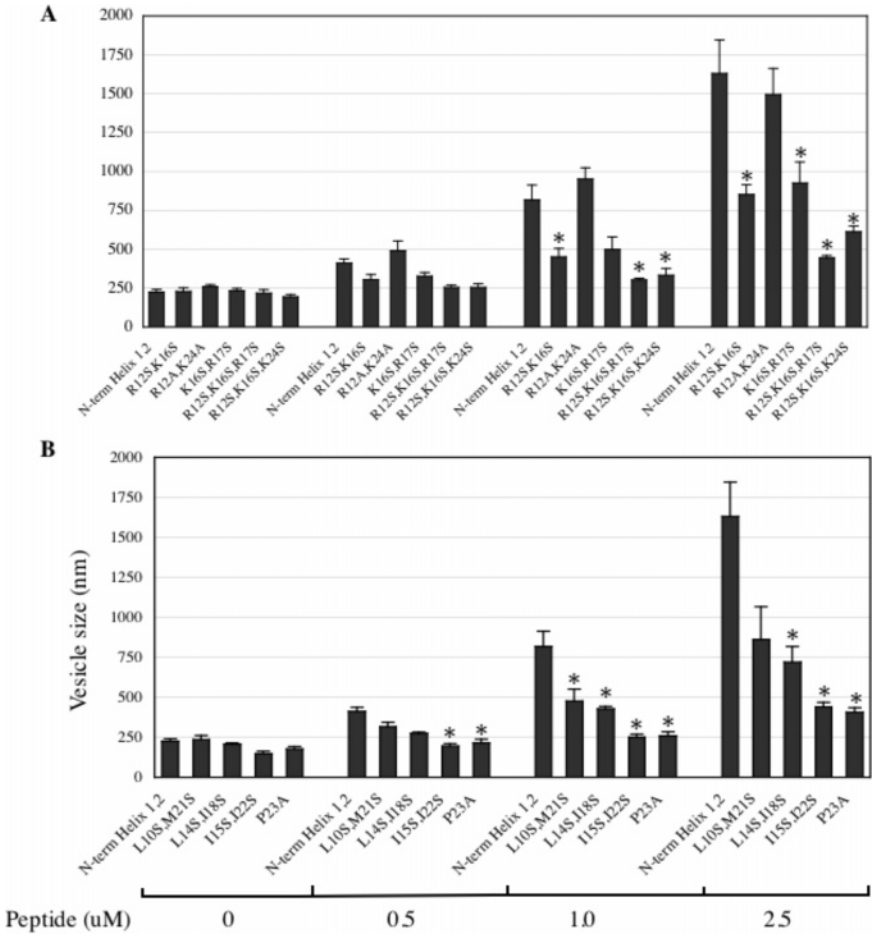

FIGURE 8: Fusogenic activity of the SP-B synthetic peptides (amino acids 1−37) containing polar and nonpolar substitutions in helix 1 and 2. DPPC/PG (70:30) liposomes (200 nM) were incubated with increasing concentrations of 37 amino acid synthetic peptides containing substitutions at positively charged residues (A) or hydrophobic residues (B). Fusion was determined by the increase in vesicle size using an N4 particle size analyzer. Results ($n = 3$) are expressed as means ± SEM; * = $p < 0.05$ for synthetic peptides versus N-term helix 1,2.

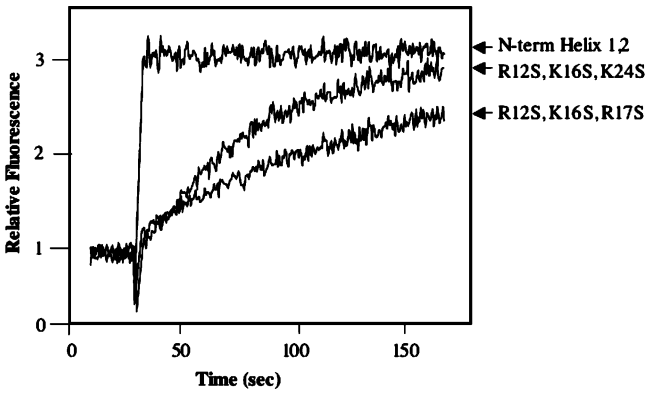

FIGURE 9: Leakage of DPPC/PG liposome contents in the presence of SP-B synthetic peptides containing polar substitutions in helix 1. Liposome leakage was measured as described in Materials and Methods. Liposomes (20 μg/mL) containing encapsulated fluorescent probes were added to a 1.0 mL cuvette containing MOPSO buffer, pH 7.0, and stirred continuously. Fluorescence was recorded at baseline and after the addition of substituted synthetic peptides (1.0 μM) or N-term helix 1,2 (0.5 μM) for 180 s. Maximum fluorescence was detected after the addition of Triton X-100. The data shown are representative of three separate experiments.

also required for surface tension reducing activity, the surface properties of synthetic SP-B peptides were assayed by captive bubble analysis. Substitution of individual, positively charged amino acids in helix 1 (R12A or K16A) or helix 2 (K24A) did not alter the surface properties of SP-B (not shown). Similarly, substitution of two positively charged amino acids in helix 1 (R12S,K16S and K16S,R17S) or in helix 1 and helix 2 (R12A,K24A) resulted in peptides that retained excellent surface activity (Figure 10A). However, substitution of three positively charged amino acids in helix 1 (R12S, K16S,R17S) or in helix 1 and 2 (R12S,K16S,K24S) significantly increased minimum surface tension (≥20 mN/m) (Figure 10A). These results suggest that the positively charged residues in helix 1 and 2 are important for fusion, lysis, and surface activity.

The importance of proline residues (located in N-term and interhelical loop 1) for surface tension reducing activity was also assessed. Substitution of P2,4,6 or P23 to alanine resulted in significantly increased minimum surface tension (≥10 mN/m) (Figure 10B); likewise, substitution of W9, located within the lipid bilayer, resulted in elevated minimum surface tension (≥15 mN/m) (Figure 10B). These analyses revealed substitutions (tryptophan 9 and prolines 2, 4, and 6) that selectively inhibited the surface activity without affecting liposome fusion or lysis.

Paired substitution of hydrophobic residues (I15S,I22S or L10S,M21S) located in the nonpolar face of helix 1 resulted in elevated minimum surface tension (>10 mN/m) (Figure 10B). Interestingly, low minimum surface tension was attained when L14,I18 was substituted to serine, indicating that a decrease in the hydrophobicity of the nonpolar face was tolerated.

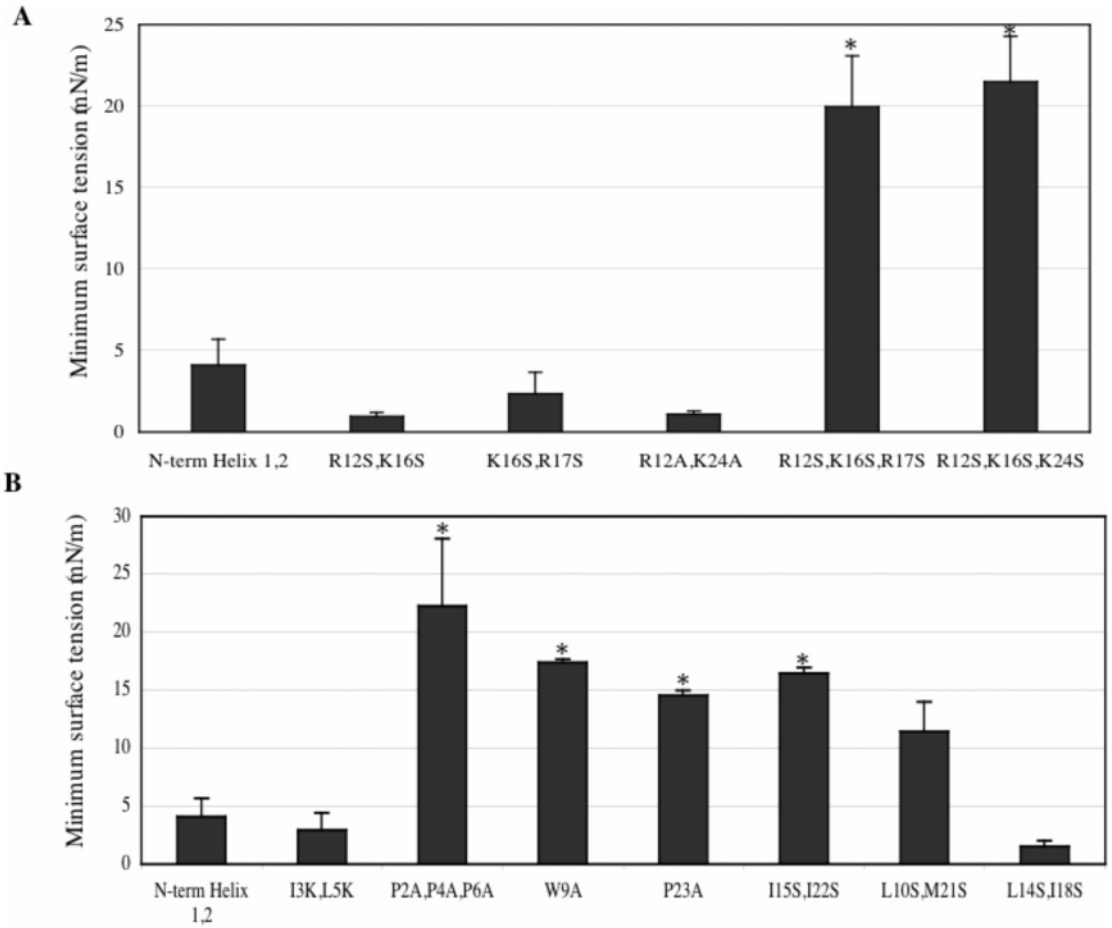

FIGURE 10: Surface activity of synthetic SP-B peptides containing polar and nonpolar substitutions. Surface properties of mixtures of synthetic peptide (4%) and DPPC/POPG (7:3 by weight) were assessed by captive bubble analysis. The bubble was pulsated at 10 cycles per minute, and the minimum surface tension was recorded at the fifth pulsation, when the bubble was reduced to 80% of its original volume. Substituted SP-B peptides were compared to N-term helix 1,2. The values shown are the average of three separate experiments ± SEM; * = $p < 0.05$ for synthetic peptides versus N-term helix 1,2.

Membrane Interactions of SP-B Synthetic Peptides. To determine if amino acid substitutions altered the interaction of SP-B with membranes, tryptophan fluorescence emission spectra were monitored in the presence or absence of DPPC/PG liposomes. Changes in the environment of the tryptophan residue (hydrophilic to hydrophobic) were detected by monitoring the maximum emission wavelength and intensity (*34*). The fluorescence emission spectra for N-term helix 1,2, P23A, R12S,K16S,R17S, and R12S,K16S,K24S were obtained and compared to measurements in the presence of lipids. Maximal emission wavelength shifts to the blue direction were recorded for all four peptides, suggesting that amino acid substitutions in these peptides did not alter peptide/membrane interaction (Table 1).

Table 1: Emission Spectra of SP-B Synthetic Peptides in the Absence or Presence of DPPC/PG Liposomes[a]

SP-B peptides (1−37)	emission maxima (nm) −DPPC/PG	emission maxima (nm) +DPPC/PG	emission shift
N-term helix 1,2	347	335	blue
R12S,K16S,R17S	340	335	blue
R12S,K16S,K24S	340	335	blue
P23A	347	335	blue

[a] The spectra were acquired at λ_{EX} = 280 nm in 1 mM Hepes and 3 mM NaCl, pH 7.0 at room temperature. The protein:lipid ratio was 1:20 with 2.0 μM synthetic peptide and 40 μM lipids.

DISCUSSION

Addition of SP-B to liposomes composed of DPPC/PG (7:3) leads to membrane binding, destabilization, and fusion, ultimately resulting in rearrangement of membrane structure. The goal of this study was to map the fusogenic and lytic domains of SP-B and assess the effects of altered fusion and lysis on surface activity. Synthetic peptides were generated to predicted helices and/or interhelical loops of SP-B and tested for fusion, lytic, and surface activities. The N-terminal half of SP-B (residues 1−37), which includes the nonhelical N-terminal amino acids in addition to helices 1 and 2, promoted rapid liposome fusion whereas shorter peptides were significantly less effective. The requirements for optimal surface tension reduction were similar to those for fusion; in contrast, helix 1 (residues 7−22) alone was sufficient for liposome lysis. The C-terminal half of SP-B (residues 43−79), which includes helices 3, 4, and 5, exhibited significantly lower levels of fusogenic, lytic, and surface tension reducing activities compared to the N-terminal region. These results indicate that SP-B fusion, lytic, and surface activities map predominantly to the N-terminal half of SP-B.

Native SP-B promoted liposome fusion and lysis at significantly lower concentrations than the most effective synthetic peptide. Maximal liposome fusion and lysis re-

quired 20-fold and 8-fold more synthetic peptide than native peptide. The results of a previous study indicated that a 25 residue SP-B peptide (SP-B 1−25) promoted lipid mixing nearly as well as native SP-B (*35*). In the current study, N-term helix 1 (residues 1−22) promoted lipid mixing, as assessed by FRET (not shown), similar to results reported by Veldhuizen et al. (*35*); however, only peptides containing helix 2 produced an increase in vesicle size consistent with liposome fusion. The fusogenic activity of the N-terminal half of SP-B was not enhanced in the presence of the C-terminal peptide; further, the fusogenic activity of full-length synthetic SP-B peptide was less than that of the N-terminal domain of SP-B. Synthetic full-length SP-B had similar secondary structure to the native protein, as assessed by circular dichroism. The present results suggest therefore that the tertiary and/or quaternary structure of native SP-B significantly influenced the fusogenic property of the peptide. Native SP-B is a homodimer that is stabilized by an intersubunit disulfide bridge between cysteines at position 48; a salt bridge between glutamic acid 51 of one subunit and arginine 52 of the other subunit may also contribute to dimer stability (*36*). Since SP-B interacts with the membrane surface, it is possible that each subunit of the homodimer initially resides on separate membranes. Membrane cross-linking by SP-B could promote fusion by bringing opposing membranes into close proximity (cross-linking) prior to lipid mixing as described for the influenza hemagluttinin fusion protein (*32*). If this is the case, 37 amino acid N-terminal synthetic peptides would be less effective in promoting fusion because they lack residues involved in subunit association/stabilization. Consistent with this hypothesis, a dimeric form of SP-B 1−25 was shown to be more fusogenic than the monomeric peptide (*35*).

Both native SP-B and a synthetic peptide encompassing the N-terminal half of SP-B exhibited maximal fusion and lytic activity at concentrations below those required to achieve low surface tension in vitro. Amino acid substitutions that significantly impaired the fusogenic or lytic activities of SP-B also inhibited the surface tension reducing property of the peptide; further, SP-B-mediated liposome fusion and lysis proceeded rapidly at pH 7.0. These findings support the hypothesis that SP-B-mediated fusion and lysis play an important role in the formation and maintenance of the alveolar surface film. SP-B-mediated fusion and lysis was also active at pH 5.5. Both the late endosome/MVB and lamellar body have an acidic interior (approximately pH 5.5) (*37*), and the estimated concentration of SP-B in the lamellar body approximates that required for liposome fusion and lysis in vitro (*38*). These observations are consistent with the hypothesis that fusion and/or lysis play(s) a role in the incorporation of internal vesicles of the MVB into the internal membranes of the lamellar body.

Amino acid substitutions were introduced into a synthetic peptide encompassing the N-terminus, helix 1, interhelical loop 1, and helix 2 (i.e., residues 1−37) in order to assess the contribution of individual structural domains to the fusogenic, lytic, and surface properties of SP-B (summarized in Table 2). On the basis of the NMR structure of NK-lysin, the N-terminal domain was predicted to consist of six amino acids (*23*); however, the results of a recent study by Wang et al. (*30*) suggested that this domain may extend to tryptophan at position 9. The six to nine amino acid

Table 2: Summary of the Fusogenic, Lytic, and Surface Properties of the SP-B Synthetic Peptides[a]

Domain	Peptide	Fusion	Lysis	Surface activity
N-term	I3L5K	NC	NC	NC
	P2,4,6A	NC	NC	↓↓
	W9A	NC	NC	↓↓
Helix 1 Non-polar face	L10M21S	↓	NC	↓
	L14I18S	↓	NC	NC
	I15I22S	↓↓	NC	↓↓
Helix 1 Polar face	R12K16S	↓	NC	NC
	K16R17S	↓	NC	NC
	R12K16R17S	↓↓	↓↓	↓↓
	R12K16K24S	↓↓	↓	↓↓
Interhelical loop 1	P23A	↓↓	NC	↓

[a] NC indicates no change in activity compared to N-term helix 1,2. ↓ indicates partial activity; ↓↓ indicates minimal activity compared to N-term helix 1,2.

N-terminal domain likely forms a highly kinked motif, as it contains three regularly spaced proline residues. Substitution of alanines for prolines completely ablated the surface tension reducing property of SP-B; likewise, substitution of alanine for tryptophan (W9A) resulted in elevated surface tension in vitro. The tryptophan residue is embedded in the phospholipid bilayer (*30*, *31*) and may serve to anchor SP-B to the membrane, as proposed for other lytic peptides (*39*, *40*). However, the W9A substitution did not alter the ability of SP-B to lyse or fuse liposomes. Similarly, the proline residues in the N-terminal domain were not required for SP-B-mediated fusion or lysis. Together, these findings indicate that the N-terminal domain is absolutely required for the surface tension reducing property of SP-B but is completely dispensable for lytic activity. Removal of the N-terminal domain moderately impaired fusogenic activity, but the molecular basis for this effect remains unclear. Finally, these results clearly indicate that fusion and lysis are necessary but not sufficient to impart surface tension reducing ability.

Helix 1, extending from residue 7−10 through residue 22, is absolutely required for fusion, lysis, and surface activity. Helical wheel projections clearly illustrate the amphipathic nature of this domain (*4*). The polar face contains three positively charged amino acids at positions 12, 16, and 17 that interact with the anionic headgroups at the surface of the membrane. Numerous studies have shown that positively charged residues are important for the lytic properties of antimicrobial peptides (*33*), and both charge and hydrophobicity were reported to affect the helical content and lipid association of SP-B 1−25 (*41*). In the current study, substitution of serine for a single positively charged amino acid in helix 1 had no effect on the fusogenic, lytic, or surface properties of SP-B. Substitution of two positively charged residues altered fusogenic activity without any effect on lytic or surface activities. In the absence of any positively charged amino acids in helix 1, the peptide bound to liposomes (Table 1) but failed to fuse or lyse membranes; further, the surface tension reducing ability of the peptide was abrogated. These

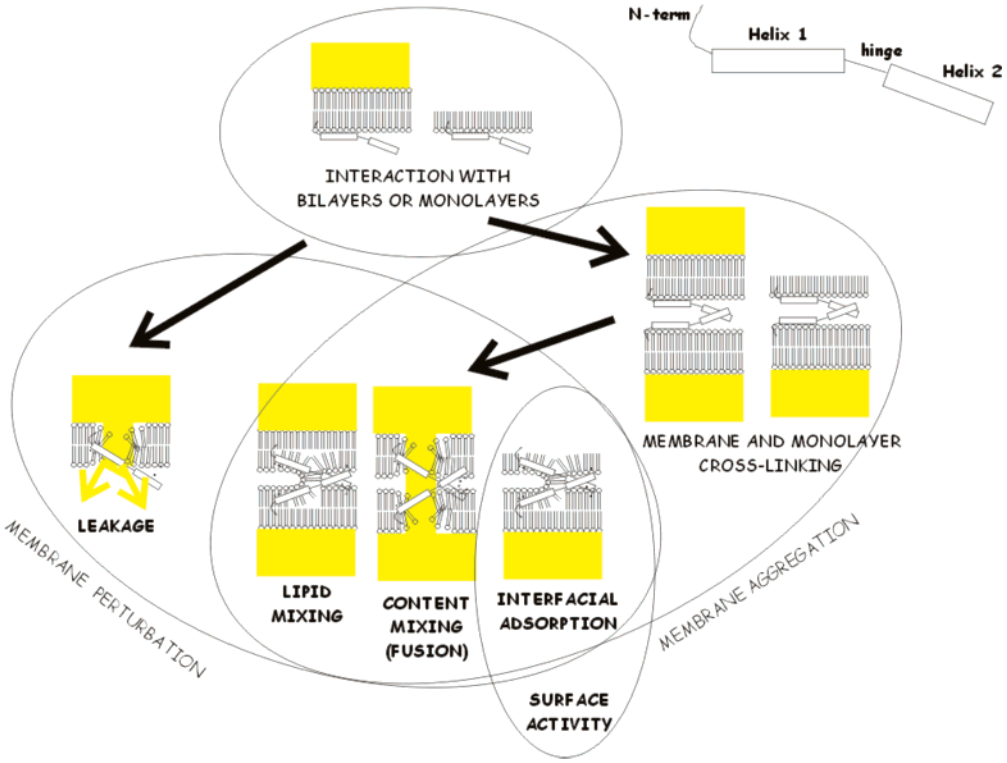

FIGURE 11: Model of interactions of SP-B helix 1 and 2 with phospholipid membranes. Association of helix 1 with the membrane surface induces deep perturbation in the phospholipid bilayer and monolayer. Helix 2 promotes membrane fusion or surface activity but not liposome lysis, suggesting that this domain may be a membrane aggregation motif. In the absence of membrane cross-linking, perturbations induced by helix 1 at the lipid surface lead to content leakage. In the context of aggregated liposomes, membrane perturbation leads to lipid mixing and eventual fusion. Membrane-to-monolayer association followed by lipid perturbation would be the basis for surface activity, which seems to require additional specific motifs at the N-terminal end of SP-B.

results underscore the importance of the positively charged amino acids in helix 1 for SP-B function.

Serine substitutions were introduced into the nonpolar face of helix 1 in order to assess the importance of this hydrophobic subdomain for SP-B function. Each pair of serine substitutions altered the fusogenic activity of the peptide, indicating that both the charge and hydrophobicity of helix 1 were important for membrane fusion; in contrast, lytic activity was not affected, supporting the hypothesis that positively charged residues are more important for this property. Surface activity was maintained when serine was substituted for L14 and I18, indicating that some alteration in hydrophobicity was tolerated. Overall, helix 1 substitutions that significantly inhibited membrane fusion (e.g., I15S, I22S) or lysis (e.g., R12S, K16S, R17S) were invariably associated with elevated minimum surface tension; however, moderate impairment of fusion alone did not affect the surface tension reducing property of SP-B.

The loop between helix 1 and helix 2 contains a proline residue which may serve as a molecular hinge. The helix−hinge−helix motif was reported to be important for the lytic activity of several antimicrobial peptides including melittin, cecropin, and brevinins (*42*−*46*). A recent study of the topographical organization of the N-terminal segment of SP-B (residues 1−25) in phospholipid bilayers indicated that the formation of a sulfhydryl-dependent dimer (via C8) would require rotation of helix 1, consistent with a flexible hinge between helix 1 and helix 2 (*30*). In the current study, substitution of alanine for proline (P23) impaired both the fusogenic and surface tension lowering properties of SP-B, implying that a flexible hinge may also be important for these properties. The ellipticity of the P23A peptide was actually greater than that of the original fully synthetic sequence or of native SP-B (Figure 2). Estimations of the contribution of α-helical conformation from the CD spectra are consistent with the substitution P23A producing an extension of helix 1 that continues through helix 2. Surprisingly, the lytic activity of SP-B was not affected by the P23A substitution.

Helix 2 is predicted to extend from residue 26 through residue 37 and is more hydrophobic than helix 1. The importance of helix 2 for SP-B function was evident from the results of domain mapping experiments, which indicated that a 22 amino acid synthetic peptide lacking this domain (i.e., N-term helix 1) was deficient in membrane fusion and surface tension reducing activity. This result was unexpected because a peptide consisting of the first 25 amino acids of SP-B (SP-B 1−25) was reported to promote surface tension reduction in vitro and improve lung compliance in two animal models of surfactant deficiency (*41*, *47*). This discrepancy may be due in part to the fact that SP-B 1−25 includes the putative molecular hinge (P23) and the first two residues of helix 2 (K24 and G25). However, the SP-B 1−25 peptide also required the presence of palmitic acid in the lipid mixture to achieve low γ_{min} values (*35*). In the current study, 37 amino acid peptides that included helix 2 were able to achieve γ_{min} values of less than 5 mN/m (Figure 10) in the absence of palmitic acid, consistent with an important role for this domain in surface tension reduction. Collectively, these results support the hypothesis that helix 2 is required for membrane fusion and surface tension reduction but do not indicate if part or all of the helix is required for SP-B function.

In summary, this study demonstrates that a synthetic peptide corresponding to the N-terminal 37 amino acids of human SP-B promotes liposome lysis and fusion as well as rapid surface tension reduction in vitro. Association of helix 1 alone (residues 7−22) with the membrane surface through ionic and hydrophobic interactions results in membrane permeabilization but not aggregation (Figure 11). We propose that helix 2 (residues 24−37) mediates membrane cross-linking (aggregation) which, in turn, facilitates lipid mixing, membrane fusion, and interfacial adsorption/surface tension reduction. Helix 2 may promote membrane cross-linking via peptide−peptide interaction (i.e., peptides anchored to separate membranes associate via helix 2; see Figure 11) or via peptide−lipid interactions (i.e., helix 1 interacts with one membrane and helix 2 interacts with a separate membrane, not shown). In the presence of helix 2, the membrane perturbing properties of helix 1 might promote lipid mixing between bilayers or between bilayers and a monolayer, preceding either fusion or interfacial adsorption, respectively. In the absence of the membrane cross-linking activity of helix 2, perturbation of membranes by helix 1 produces lysis but not fusion or surface activity. This model is consistent with the finding that a flexible hinge between helix 1 and 2 is important for lipid mixing/fusion and surface activities but not membrane lysis. The N-terminal domain (residues 1−9) is critical for the surface tension reducing property of SP-B but plays little or no role in membrane fusion or lysis. We speculate that the N-terminal structural motif, including prolines 2, 4, and 6 and tryptophan 9, might be required to sustain deep association of SP-B peptides with surface films supporting higher lateral pressures than those existing in membrane bilayers. This would explain why membrane fusion and lysis alone are not sufficient to impart surface tension reducing activity. Overall, decreased lytic or fusogenic activities of SP-B were associated with altered surface properties of the peptide.

ACKNOWLEDGMENT

The authors thank Gary Ross and Ward Rice for helpful suggestions.

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BI0485575