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Hydrophobic Surfactant Proteins and Their Analogues

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Pulmonary surfactant · Synthetic surfactant · Surfactant protein B · Surfactant protein C

Abstract
Lung surfactant is a complex mixture of phospholipids and four surfactant-associated proteins (SP-A, SP-B, SP-C and SP-D). Its major function in the lung alveolus is to reduce surface tension at the air-water interface in the terminal airways by the formation of a surface-active film enriched in surfactant lipids, hence preventing cellular collapse during respiration. Surfactant therapy using bovine or porcine lung surfactant extracts, which contain only polar lipids and native SP-B and SP-C, has dramatically improved the therapeutic outcomes of preterm infants with respiratory distress syndrome (RDS). One important goal of surfactant researchers is to replace animal-derived therapies with fully synthetic preparations based on SP-B and SP-C, produced by recombinant technology or peptide synthesis, and reconstituted with selected synthetic lipids. Here, we review recent research developments with peptide analogues of SP-B and SP-C, designed using either the known primary sequence and three-dimensional (3D) structure of the native proteins or, alternatively, the known 3D structures of closely homologous proteins. Such SP-B and SP-C mimics offer the possibility of studying the mechanisms of action of the respective native proteins, and may allow the design of optimized surfactant formulations for specific pulmonary diseases (e.g., acute lung injury (ALI) or acute respiratory distress syndrome (ARDS)). These synthetic surfactant preparations may also be a cost-saving therapeutic approach, with better quality control than may be obtained with animal-based treatments.

Introduction

Lung surfactant is a mixture of lipids, mostly phospholipids, and proteins that is essential for normal breathing due to its ability to reduce alveolar surface tension to extremely low values, thus preventing alveolar collapse during expiration and reducing the work of breathing. Surfactant is synthesized and secreted into the alveolar fluid by alveolar type II cells and is composed of approximately 80% phospholipids, 10% neutral lipids, and 10% proteins. Although dipalmitoyl phosphatidylcholine and phosphatidylglycerol constitute the main phospholipid components in surfactant, its biophysical activity in the lung depends to a large extent on the presence of the hydrophobic surfactant protein B (SP-B) and to a lesser ex-

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tent on the extremely hydrophobic surfactant protein C (SP-C). The water-soluble surfactant proteins SP-A and SP-D are important for host defense [1, 2], but the presence of SP-B is critical for reducing surface tension within the alveolus and maintaining lung volume at end expiration. Hereditary SP-B deficiency is lethal in humans [3], whereas mutations in the gene encoding SP-C may cause interstitial lung disease and increase susceptibility to infection [4].

Neonatal surfactant therapy has dramatically reduced mortality and morbidity from respiratory distress syndrome (RDS) among preterm infants [5], but it has also been shown to be effective in full-term infants with meconium aspiration syndrome [6] and group B streptococcal pneumonia/sepsis [7]. Meconium aspiration and group B streptococcal infection both can lead to severe respiratory failure (acute respiratory distress syndrome, ARDS) through surfactant inactivation by proteins and phospholipases. Treatment of ARDS with commercial surfactant preparations, consisting of (phospho)lipids with SP-B and SP-C harvested from animal lung lavages or lung extracts, has shown encouraging results in animal studies, but not always in clinical trials. Curstedt and Johansson [8] have succinctly reviewed earlier work on the development of synthetic surfactant preparations for biomedical applications. More recently, substantial progress has also been made in the testing of novel synthetic lipids for use in surfactant preparations [9–11]. Here, we discuss our latest work on the design and testing of various peptides based on the structures of SP-B and SP-C, with the aim of developing fully synthetic mixtures of SP-B and SP-C analogues and lipids for use in both neonatal RDS and patients with ARDS. We have designed and tested various SP-B and SP-C peptides with the aim of developing synthetic surfactant mixtures with SP-B and SP-C peptides and lipids that are able to withstand inactivation by proteins and phospholipases in patients with ARDS.

Surfactant Protein B (SP-B)

SP-B is a small (79 amino acids; monomer MW of 8.7 kDa) protein that is found in the mammalian lung as a covalently linked homodimer (through a disulfide bridge between cysteine residues at position Cys-51, Cys-51′). SP-B is also a lipid-associating protein that belongs to the saposin protein superfamily. Previous x-ray crystallographic or two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopic analyses on saposins such as NK-lysin [12], saposin B [13], saposin C [14, 15], granulysin [16], and amoebapore [17] indicate that the characteristic 'saposin' fold consists of 4–5 α-helical domains (residues 10–20, 25–38, 41–52, 59–63, and 68–75) joined together by 2–3 intramolecular disulfide bridges. The helical bundle for saposins is folded into two leaves, with one leaf having α-helices 1 and 5 and the second leaf composed of α-helices 2 and 3, with flexible hinges between helices 1 and 2 and also between helices 3 and 5. Despite the helical elements being highly conserved, three-dimensional (3D)-structural determinations indicate that saposins exhibit either closed or open tertiary conformations. For the saposins NK-lysin, granulysin or saposin C (fig. 1a) in aqueous environments, the peptide backbone folds in a closed conformation [18], with the two leaves in close juxtaposition such that the amphipathic α-helices with hydrophilic (charged, neutral) residues face the solvent, while the hydrophobic side chains form a core stabilized by intramolecular disulfide bonds. Contrarily, aqueous dimeric saposin B or saposin C bound to detergent micelles (fig. 1b) each exhibit an open conformation, in which the leaves of the V are now far apart having expanded at the flexible joints. The respective open conformations permit saposin-B protein to form non-covalent dimers interacting through their exposed hydrophobic cores [13], while saposin C unmasks its hydrophobic core to bind fatty acyl chains in detergent micelles [15]. These saposin findings support a proposal [19], based on their early modeling studies, that increases in the hydrophobicity of the protein's environment (e.g., association with membranes or lipids) may generally produce a greater splay between the saposin leaves, thereby exposing more hydrophobic residues.

As has been noted in several recent reviews [20, 21], neither x-ray crystallographic nor 2D-NMR spectroscopic analyses have experimentally determined the 3D structure of SP-B. Nevertheless, several lines of evidence suggest that SP-B will share the 'saposin fold' described above. First, the primary sequence of SP-B is highly homologous with those of other known saposins [18, 19, 22]. Second, the characteristic intrachain disulfide-linkage pattern observed with SP-B [23] has been conserved for an estimated 300 million years [24], and is also exhibited by other saposins [18]. Third, circular dichroism and Fourier transform infrared (FTIR) spectroscopy of native SP-B in membrane mimics indicated high levels of α-helix [25–27], consistent with SP-B assuming the classic saposin fold. Fourth, past molecular modeling of homodimeric SP-B, based on templating the primary sequence of SP-B onto the known 3D structure of NK-lysin, indicated

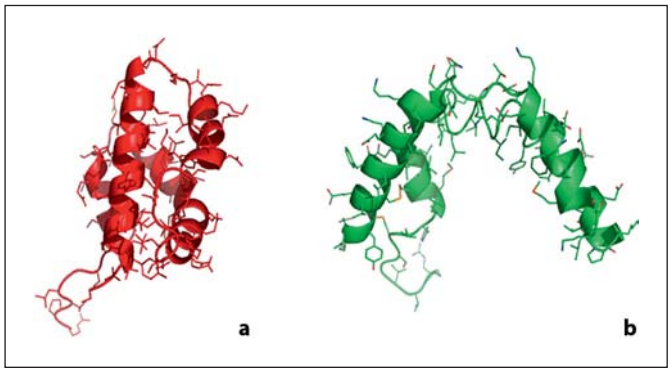

Fig. 1. Molecular graphics illustration of the closed structure of saposin C in solution (**a**) ([11]; PDB: 1M12; in red) compared with saposin C bound to an SDS micelle (**b**) ([12]; PDB: 1SN6; in green). Helical elements are shown as ribbons.

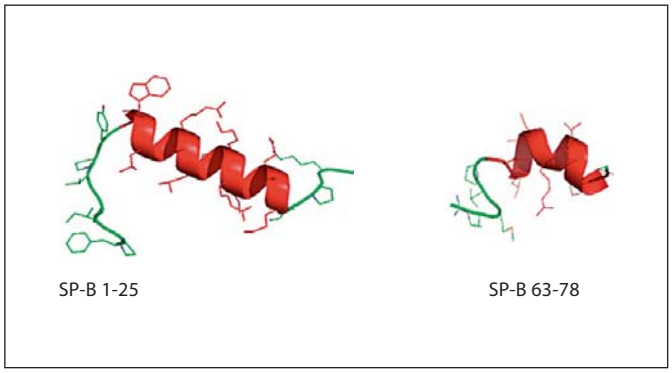

Fig. 2. Molecular graphics of the N-terminal SP-B_{1-25} (PDB: 1DFW) and C-terminal SP-B_{63-78} (PDB: 1RG4) peptides.

SP-B may fold as a closed saposin [28, 29]. Lastly, synthetic peptides based on the N- and C-terminal regions have had their residue-specific structures determined with either ^{13}C-FTIR or 2D-NMR spectroscopy, and indicate α-helical domains in regions earlier predicted on sequence homology with other saposins [12, 18, 22]. For example, 2D-NMR spectroscopy of the C-terminal SP-B_{63-78} peptide in the membrane mimic hexafluoroisopropanol (HFIP) showed α-helix for residues 68–77 (fig. 2; Protein Data Bank [PDB] Accession code: 1RG4; www.rcsb.org), while ^{13}C-FTIR of the N-terminal SP-B_{1-25} in HFIP indicated α-helix for residues 9–21 (fig. 2; PDB: 1DFW) [31].

Furthermore, ^{13}C-FTIR of a disulfide-linked construct (i.e., 'Mini-B') in HFIP, based on the N- and C-terminal domains, also showed α-helices for residues 9–21 and residues 68–74 (fig. 3; PDB: 1SSZ) [32]. Given the above structural homologies between SP-B and other saposins, it is tempting to speculate that SP-B will adopt an open saposin conformation (e.g., fig. 1b) when it associates with surfactant lipids. Here, the exposed amphipathic helices of SP-B would bind to lipid monolayers or bilayers by inserting their hydrophobic residues to interact with fatty acyl chains, while polar and neutral residues would associate with the more polar lipid headgroup region [33].

Although SP-B typically is observed as the disulfide-linked homodimer in mammalian lung, questions have arisen concerning the respective in vivo activities of the monomeric versus dimeric protein [34–36]. Transgenic mice expressing only monomeric SP-B have normal longevity, but a significantly decreased lung hysteresis, and surfactant from these mice is less able to reduce surface tension on a Wilhelmy balance [34]. Also, the membrane activity of monomeric SP-B increased on dimerization [36].

Fig. 3. Molecular graphics of Mini-B (PDB: 1SSZ) structure in structure promoting solvent hexafluoroisopropanol [32]. Helical elements in figures 2 and 3 are shown in red while bend and disordered segments are in green. The Mini-B disulfide linkages between the N-terminal helix in the foreground and C-terminal helix in the background are highlighted in yellow.

SP-B Analogues

One of the most interesting features of the SP-B structure is the localization of cationic residues in the amphipathic helices of the N- and C-terminal regions. These positively-charged segments likely provide important protein surfaces for interactions with lung surfactant lipids (e.g., phospholipids with anionic head groups [37]), especially in light of the above model suggesting that SP-B interacts with lipids in an open saposin conformation.

Table 1. Family of SP-B peptides designed and synthesized for structural and functional studies

SP-B79	FPIPLPYCWLCRALIKRIQAMIPKGALAVAVAQVCRVVPLVAGGICQCLAERYSVILLDTLLGRMLPQLVCRLVLRCSM
B1-25	FPIPLPYCWLCRALIKRIQAMIPKG
B11-25	CRALIKRIQAMIPKG
B8-25	CWL**A**RALIKRIQAMIPKG
B63-78	GRMLPQLVCRLVLRCS
Mini-B	CWLCRALIKRIQAMIPKG----------GRMLPQLVCRLVLRCS
dB1-25	FPIPLPYCWL**A**RALIKRIQAMIPKG
	FPIPLPYCWL**A**RALIKRIQAMIPKG
dB63-78	GRMLPQLV**A**RLVLRCS
	GRMLPQLV**A**RLVLRCS
B35-46	CRVVPLVAGGIC

Since isolated peptides based on the N- and C-terminal domains are highly α-helical in membrane mimics (see above), we have designed and synthesized a family of SP-B peptides for further structural and functional studies (table 1).

Surfactant preparations containing synthetic peptides representing either N- or C-terminal segments of SP-B show surface activity in vitro and improve oxygenation and lung compliance in ventilated premature rabbits and lavaged, surfactant-deficient rats. The conformation of monomeric SP-B_{1-25}, the N-terminal domain of SP-B, in phospholipid mixtures containing 1–2% peptide is predominantly α-helical. The N-terminal disulfide-linked homodimer (dimeric SP-B_{1-25}), designed to probe the mechanism of action of dimeric constructs that resemble the full-length native dimeric SP-B, retains helical content similar to that of monomeric SP-B_{1-25} in surfactant lipids [38]. With in vitro testing, dimeric SP-B_{1-25} shows enhanced lipid-vesicle mixing activity and surface absorption compared with monomeric SP-B_{1-25} [39], and matches the surface activity of native SP-B in the captive bubble surfactometer [39]. Studies of monolayer films using atomic force microscopy indicated that dimeric SP-B_{1-25} is able to effectively create a lipid reservoir attached to the monolayer upon compression [40]. These attached multilayer structures, termed 'nanosilos', are formed from the more fluid phase and have heights and sizes similar to those observed for surfactant dispersions that contain native SP-B protein [41], further suggesting that the dimeric peptide efficiently emulates much of the mechanistic character of the full-length protein. In both the ventilated preterm rabbit and the lavaged rat model, oxygenation and lung volumes were consistently higher after tracheal instillation of dimeric SP-B_{1-25} than after monomeric SP-B_{1-25} surfactant [38]. Comparing 1–2% dimeric SP-B_{1-25} in a standard phospholipid mixture in lavaged rats showed that improvements in oxygenation and lung volume are concentration-dependent [42].

Since the N- and C-terminal domains of SP-B are in large part the active sites of interaction of the protein with surfactant lipids, we designed, chemically synthesized and folded, a peptide construct having this motif. This 34-residue peptide (fig. 3, 4), which we call 'Mini-B', incorporates residues 8–25 and 63–78 of native human SP-B as a single linear peptide. The peptide is designed to join the critical N- and C-terminal amphipathic helixes using a β-sheet-loop domain to promote folding into a helix hairpin structure. The construct is folded in organic solvent buffer (trifluoroethanol) to give a secondary structure that is stabilized by oxidation of the cysteine residues to form disulfide connectivities between Cys-8 and Cys-78 and Cys-11 and Cys-71 (residue numbers refer to the full protein sequence), similar to that observed in the native protein. The disulfide connectivity of the Mini-B construct has been confirmed by mass spectral analysis of the trypsin-cleaved fragments of the oxidized peptide.

Oxidized Mini-B has high α-helical levels in membrane mimics, containing approximately 53.6% α-helix, 8.1% β-sheet, 15.7% turn, and 23.5% random structures. Since physical biochemical studies indicated high α-helical content for peptide fragments based on the N- and C-terminal domains of SP-B (see above), it is likely that these regions will also be highly helical in the Mini-B construct. Indeed, residue-specific analysis using isotope-enhanced FTIR spectroscopy confirmed that Mini-B has the same three-dimensional saposin fold as the predicted full-length SP-B protein in the N- and C-terminal

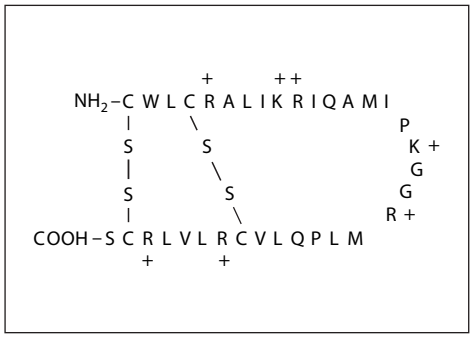

Fig. 4. Sequence, disulfide connectivities and charge distribution of Mini-B.

regions [32, 43]. Molecular modeling indicated the following preliminary conformation map for the Mini-B structure in a hydrophobic environment: residues 1–15, α-helix; residues 18–22, β-sheet; residues 24–34, α-helix. These findings suggest that the synthetic Mini-B construct faithfully reproduces the topographical organization of the charged, α-helical N- and C-terminal domains in the full-length SP-B [32].

Surface activity measurements of Mini-B in a lipid mixture demonstrated that Mini-B reduced the surface tension to values near zero and re-spread efficiently even after collapse. Comparison with reduced Mini-B (same primary sequence but without the disulfide linkages), SP-$B_{8–25}$ + SP-$B_{63–78}$ (disconnected Mini-B peptide components), and the mutant Mini-B(thr) (with polar, uncharged threonines replacing positively-charged arginine and lysines) verified that the good in vitro performance was due to the unique structure of Mini-B [32]. Mini-B was compared to porcine SP-B, reduced Mini-B, SP-$B_{8–25}$ + SP-$B_{63–78}$ (the Mini-B peptide components), threonine mutant Mini-B, and synthetic lavage lipids alone in surfactant-deficient, ventilated rats [32]. Rats demonstrated a rapid recovery of oxygenation (PaO_2) and dynamic compliance after rescue surfactant treatment with Mini-B, porcine-B, reduced Mini-B, and SP-$B_{8–25}$ + SP-$B_{63–78}$, but not with mutant Mini-B or lipids alone. PaO_2 values for Mini-B and porcine-B surfactant exceeded those of the other surfactant preparations or lipids alone ($p < 0.001$ and $p < 0.05$, respectively). Dynamic compliance values for Mini-B and porcine-B surfactant exceeded those of the other surfactant preparations and lipids alone, but the differences between Mini-B and porcine-B surfactant were not significant.

Surfactant Protein C (SP-C)

SP-C is a short (35 amino acids; MW of 4.2 kDa in humans) protein in lungs that is highly enriched in valine, leucine and isoleucine residues, making it much smaller and more hydrophobic than SP-B. For SP-C suspended in a membrane mimic, earlier 2D-NMR analysis indicated that the middle and C-terminal regions (i.e., residues 9–34) are α-helical (PDB: 1SPF) [44]. When incorporated into lipids, FTIR spectroscopy also showed that SP-C is principally α-helical, with its long molecular helix axis parallel to the phospholipid acyl chains [25]. The N-terminal region of human SP-C contains two cysteine-linked palmitoyl groups at positions 5 and 6, which may either extend into the bilayer or interact with adjacent bilayers [45]. Although SP-C is not effective when used to treat ARDS in single protein-based formulations [46], SP-C may nonetheless play key secondary roles in surfactant function. An ancillary participation of SP-C in lung activities is emphasized by the observation that animals with mutated SP-C survive, in sharp contrast to those with the lethal SP-B knockout [47], but still have disease that disrupts lung development [48].

In vitro studies also suggest that SP-C stabilizes phospholipid bilayer ensembles that remain attached to the monolayer at the air-water interface [49, 50]. Such ensembles may constitute a surfactant lipid 'reservoir' that is important in maintaining the lipid monolayer throughout the respiration cycle. The presence of SP-C in lung surfactant also enhances the resistance to surfactant flow by increasing surface viscosity [51], unlike SP-B which has little effect. Furthermore, the shelf-life of surfactant dispersions may be partially due to the self-aggregation state of SP-C proteins. Earlier studies indicate that SP-C in lipid environments may convert from predominately α-helix into insoluble and inactive 'amyloid-like' fibrils consisting of β-sheets [45, 52, 53], and similar such conversions may be associated with stored commercial preparations losing surfactant activity. Finally, joint use of SP-C with SP-B in surfactant formulations may produce synergistic effects in terms of surfactant activities, as well as immune functions and longer-term lung development.

SP-C Analogues

In recent work, we have synthesized, purified and tested SP-Cff, an SP-C construct in which phenylalanines are substituted for palmitoylated cysteine residues in the N-

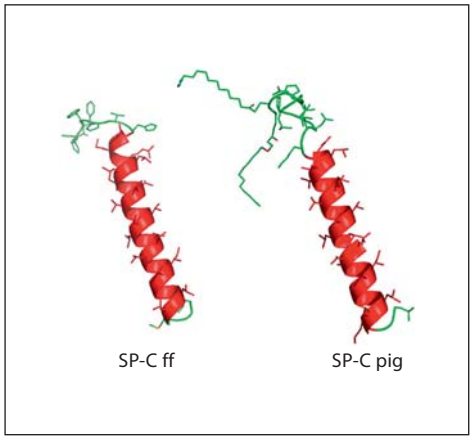

Fig. 5. Computer graphics models of SP-Cff and SP-C proteins. The SP-Cff structure is based on the molecular coordinates of the solution NMR structure of recombinant SP-C [54]. The structure of pig SP-C was derived by linking palmitic acid to the Cys residues of the coordinates from the solution NMR structure of pig SP-C in organic solvent ([44]; PDB: 1SPF) by thioester bonds. Both structures were then placed in a simulated methanol solvent box and minimized by steepest decent followed by 1 ns equilibrium molecular dynamics at 300 K using the GROMACS version 3.3 force-field to simulate a final approximate molecular conformation for each of the proteins. The molecular graphics illustrations show the structural similarities of the two surfactant molecules. The hydrophobic (polyvaline) helical sequences are shown as a red ribbon, while the more flexible N-terminal segment that includes either the vicinal phe in the case of SP-Cff or the vicinal Cys-Cys with thioester-linked palmitate for SP-C pig is colored in green.

Table 2. Amino acid sequence of porcine SP-C and synthetic SP-Cff

Native porcine SP-C LRIPCCPVNLKRLLVVVVVVVLVVVVIVGALLMGL	35
SP-Cff synthetic peptide GIPFFPVHLKRLLIVVVVVVLIVVVIVGALLMGL	34

terminal region (i.e., Phe-4 > Cys-4, Phe-5 variant) (table 2, fig. 5). Therefore, the SP-Cff construct is similar to the native SP-C found in dog [55], and also to the recombinant SP-C [i.e., rSP-C(FFI)] [56] used in Venticute® surfactant. In our laboratory, FTIR measurements of SP-Cff indicated that the peptide has dominant helical properties in surfactant lipids including DPPC and POPG, similar to that reported for the peptide rSP-C(FFI) studied by solution NMR spectroscopy in chloroform-methanol [57]. The SP-Cff peptide emulates many of the in vitro and in vivo surface activities of native SP-C. Our preliminary studies with FTIR spectroscopy indicate that, upon storage in organic solvent or in surfactant lipids for 4–6 weeks, the peptide self-associates to form β-sheet aggregations that eventually result in inactive 'amyloid-like' fibrils. However, SP-Cff is an extremely good experimental mimic for SP-C despite its limited shelf-life, if used immediately after formulation.

Surface activity and atomic force microscopy images of SP-Cff in surfactant lipid-peptide mixtures spread at the air-water interface indicate that there is a thickening of the fluid phase [50, 58], similar to that observed by Grunder et al. [59] for native SP-C. This thickening is proportional to the concentration of SP-Cff, and has multilayer morphology characteristic of native SP-C containing surfactant mixtures. More recently, we have measured the viscosity of model surfactant lipid monolayers with varying concentrations of SP-Cff [60]. SP-Cff enhances the resistance to surfactant flow by altering the ratio of solid to fluid phase in the monolayer, leading to a jamming transition. The accompanying 3 orders of magnitude increase in surface viscosity induced by SP-Cff, which is not observed for SP-B protein or peptides, suggests that SP-C (and SP-C mimics such as SP-Cff) may be regulating lung surfactant surface viscosity [60]. This SP-C induced modulation of surfactant surface viscosity may be an important element in surfactant dispersion efficacy, particularly in non-cholesterol containing formulations such as Curosurf®, a lipid extract from whole minced porcine lung tissue.

We examined whether the addition of 1% SP-Cff improves surface activity of 2 mol% Mini-B formulated in synthetic lung lavage lipids. For comparison purposes, we used porcine SP-C, Infasurf® as positive control and lipids alone as negative control. In vitro surface activity measured on a captive bubble surfactometer showed excellent surface activity for Infasurf and Mini-B + SP-C or SP-Cff, but not for SP-C or SP-Cff alone. In vivo surface activity of SP-Cff was tested in ventilated, lavaged, surfactant-deficient rats. Oxygenation and lung compliance improved quickly after instillation of Infasurf, Mini-B + SP-C or SP-Cff, Mini-B, and SP-C surfactant, but not with SP-Cff surfactant or lipids alone. Throughout the experiments, oxygenation and lung compliance in the Infasurf and Mini-B + SP-C or SP-Cff surfactants was marginally better than Mini-B or SP-C surfactant.

Conclusions

Results of recent studies using SP-B and SP-C peptide families indicate that optimal lung surfactant preparations for biomedical applications require that the peptide analogues retain the lipid-interactive domains of the respective native proteins. Specifically, the surfactant activity of peptides based on SP-B depends on positively-charged (cationic), amphipathic α-helices that bind to the water-surfactant lipid interface, while the corresponding activity of SP-C peptides requires that the mimics assume an α-helix conformation nearly parallel to the long-molecular axis of surfactant lipids. In the SP-C family, it is clear that although the α-helical SP-Cff analogue (i.e., phenylalanines replacing the two palmitoyl groups) has reasonable functional activity in in vitro and in vivo testing, its propensity to form inactive β-sheet aggregates may limit the utility of SP-Cff to specific applications. In the SP-B family, synthetic adducts with disulfide-linked α-helical peptides based on the N- and/or C-terminal SP-B domains (i.e., dimeric SP-$B_{1–25}$ or Mini-B) show dramatically increased surfactant activity over that observed with simple single-helix peptides. The enhanced surfactant activities of the dSP-$B_{1–25}$ and Mini-B adducts may be due to these constructs more closely emulating the protein networking observed when native SP-B interacts with surfactant lipids. The ultimate goal of these studies is to create effective second-generation surfactants consisting of synthetic lipids and SP-B and SP-C analogues.

Acknowledgments

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