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MINIREVIEW

Surfactant Protein B and C Analogues

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Mammalian lung surfactant is a mixture of phospholipids and four surfactant-associated proteins (SP-A, SP-B, SP-C, and SP-D). Its major function is to reduce surface tension at the air–water interface in the terminal airways by the formation of a surface–active film highly enriched in dipalmitoyl phosphatidylcholine (DPPC), thereby preventing alveolar collapse during expiration. SP-A and SP-D are large hydrophilic proteins, which play an important role in host defense, whereas the small hydrophobic peptides SP-B and SP-C interact with DPPC to generate and maintain a surface-active film. Surfactant replacement therapy with bovine and porcine lung surfactant extracts, which contain only polar lipids and SP-B and SP-C, has revolutionized the clinical management of premature infants with respiratory distress syndrome. Newer surfactant preparations will probably be based on SP-B and SP-C, produced by recombinant technology or peptide synthesis, and reconstituted with selected synthetic lipids. The development of peptide analogues of SP-B and SP-C offers the possibility to study their molecular mechanism of action and will allow the design of surfactant formulations for specific pulmonary diseases and better quality control. This review describes the hydrophobic peptide analogues developed thus far and their potential for use in a new generation of synthetic surfactant preparations.

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The use of lung surfactant dispersions, based on lipid and protein components extracted from animal lung tissue, has significantly reduced mortality and morbidity from respiratory distress syndrome (RDS) in premature infants in the past decade (1,2). However, there is still a need to refine surfactant preparations for therapy. By systematically investigating the components involved in the function of surfactant *in vitro* and *in vivo*, investigators should be able to optimize formulations for treatment of a variety of lung conditions where surfactant replacement therapy would be effective. Peptide analogues of surfactant hydrophobic proteins SP-B and SP-C offer the opportunity to better understand surfactant molecular mechanisms of action, which should allow the design of surfactant formulations for specific applications such as neonatal RDS and adult RDS (ARDS). In addition to the flexibility of the molecular design approach, synthetic peptide–lipid-based formulations permit levels of quality control that cannot be achieved with surfactant components derived from native sources of lung surfactant material. Growing concerns of possible viral and prior contamination from animal sources, as well as overcoming the expense in isolation and formulation of native surfactant components, make the synthetic mimetic approach an attractive alternative to tradi-

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tional lung surfactant replacement therapeutics now available. The objective of this review is to describe many of the hydrophobic peptide analogues that have been used in this approach thus far and, based on the findings of these studies, suggest future areas of investigation.

SURFACTANT PROTEIN B (SP-B) ANALOGUES

The design of most SP-B analogue peptides has been templated on the known SP-B amino acid sequences and/or structural conformations, in attempts to emulate the native proteins' *in vitro* and *in vivo* functional activities. SP-B is a small (amino acid residues 1–79; MW ~8700) lipid-associating protein found in mammalian lung surfactant (3). Recent studies with antibodies raised against human SP-B with the protein from the primitive Australian lungfish *Neoceratodus forsteri* suggest that the protein has had a conserved antigenic structure for ~300 million years (4). SP-B is essential in lung function, as mutations in the SP-B gene cause severe and fatal lung disease (5,6). The use of a knockout-mouse model for SP-B not only shows the absolute requirement for SP-B in the lung, but also suggests that the covalently linked homodimer is essential for optimal function (7). Surfactant preparations containing either synthetic peptides representing N-terminal and C-terminal domains of SP-B or synthetic, full-length SP-B improve oxygenation and lung compliance in surfactant-deficient animal models (8,9). Synthetic and native full-length SP-B analogues also exhibit substantial antimicrobial activity *in vitro* (10,11) and may participate, along with SP-A and SP-D, as a first line of host-defense against pathogenic bacteria and viruses (12). Mature SP-B (Fig. 1) is very hydrophobic and partitions into the surfactant lipid phase when isolated from lung lavage. Plasma desorption mass spectral analysis of native SP-B indicates that, while the protein has no posttranslational modifications, each SP-B monomer contains three intramolecular disulfide bridges, linking cysteine residues (Cys) as follows: Cys-8 to Cys-77, Cys-11 to Cys-71, and Cys-35 to Cys-46 (13). The predominant molecular species of SP-B is a disulfide-linked (through Cys-48) homodimer, although variable amounts of monomer are also been observed in samples isolated from lung surfactant.

The polypeptide motif of monomeric SP-B, characterized by six Cys residues paired as disulfides

SP-B (human)

```
FPIPLPYCWL CRALIKRIQA MIPKGALAVA VAQVCRVVPL
1          10          20          30          40

VAGGICQCLA ERYSVILLDT LLGRMLPQLV CRLVLRCSM-COOH
           50          60          70          79
```

$SP\text{-}B_{1\text{-}25}$ N-terminal (human)

```
FPIPLPYCWL CRALIKRIQA MIPKG-COOH
1          10          20     25
```

SP-B disulfide stabilized bend (human)

```
CRVVPLVAGGIC-CONH2
34          46
```

$SP\text{-}B_{49\text{-}66}$

```
LAERYSVILLDTLLGRnLL-CONH2
49                  66
```

KL_4

```
KLLLLKLLLLK LLLLKLLLLK-COOH
1           10          20
```

nL = norleucine (non oxidizable methionine substitute)

FIG. 1. SP-B native protein and synthetic peptide sequences. The amino acids are shown in single-letter code.

and periodic hydrophobic residues, belongs to a class of "saposin-like" proteins that also includes saposins A–D, sulfated glycoprotein-I, acid sphingomyelinase, acyloxyacyl hydrolase, *Entamoeba histolytica* pore-forming peptides (amoebapores), plant aspartic proteases, and NK-lysin (14–17). SP-B, as well as other proteins of the saposin family, uniformly exhibits high levels of α-helix (15,16,18–21), suggesting that this motif plays an important structural role. Based on the crystallographic structure of the related hemerythrin (15,22) and the two-dimensional nuclear magnetic resonance (2D NMR) structure of NK-lysin (23), the saposin fold is apparently characterized by amphipathic α-helices with hydrophilic residues facing solvent, and hydrophobic side chains forming a core stabilized by intramolecular disulfide bonds.

A hypothetical structural model for the native SP-B homodimer has been recently formulated based on the NMR structure for NK-lysin (65). In this model (Fig. 2), the two monomers are covalently linked by a Cys48-Cys48′ disulfide bond and the two subunits are aligned so that complementary ion pairs are made between Glu51 and Arg52′ and Glu51′ and Arg52. This results in a homodimer that

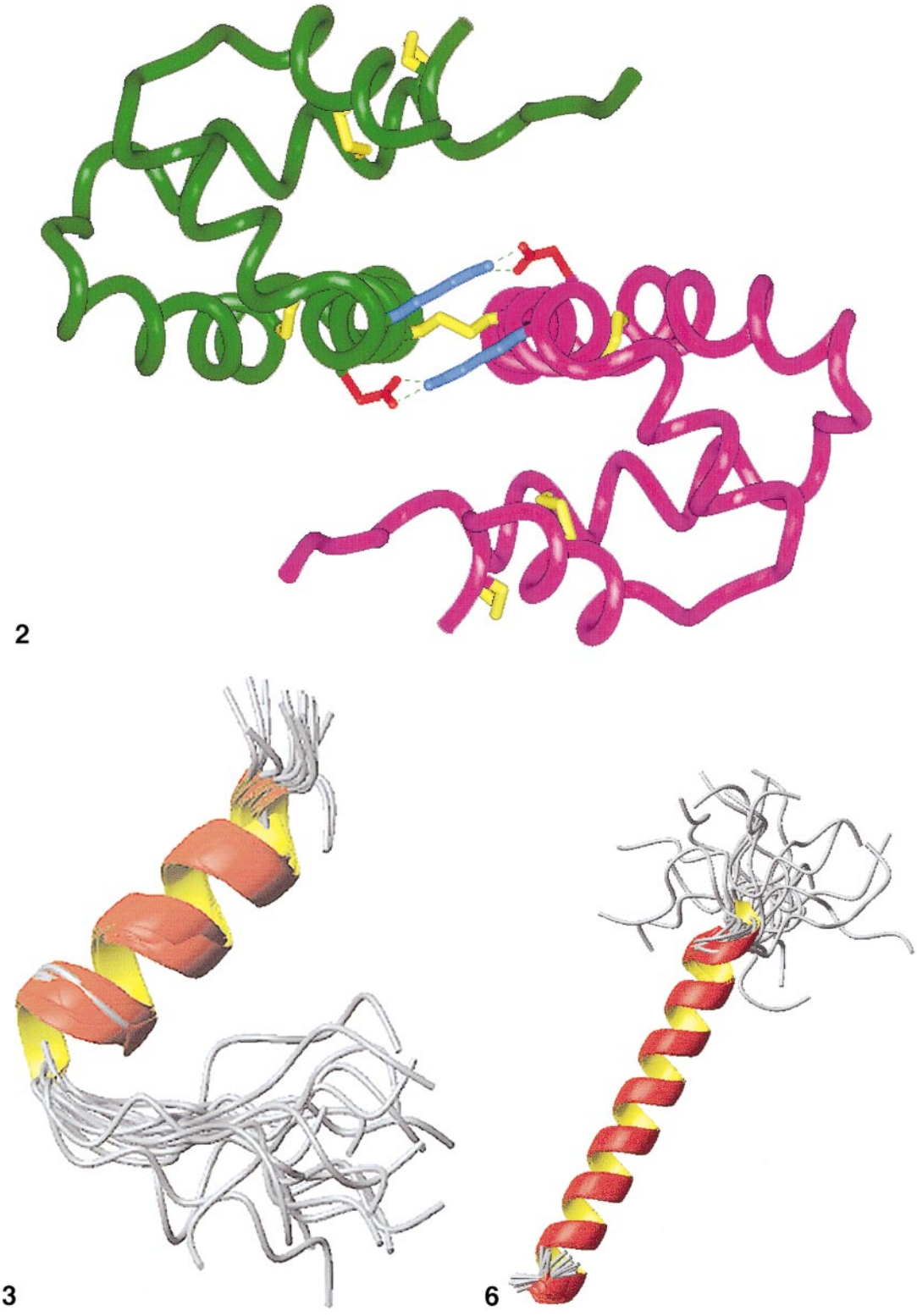

FIG. 2. A hypothetical SP-B native protein structure based on the model of Zaltash *et al.* (65). The two SP-B monomeric backbone conformations are shown in green and magenta. Inter- and intramolecular disulfide connectivities are shown in yellow. The disulfide linkage connecting the SP-B monomers by Cys48, 48′ is in the center of the illustration with the ion pairs Arg52, 52′ in blue and Glu51, 51′ in red. The longer N-termini are in the back and the C-termini are in the foreground.

has the charged residues on one surface of a disk-like structure. It is hypothesized that such molecular topography will facilitate the "cross-talk" of SP-B between monolayers/bilayers that optimizes SP-B function in the lung.

Various model systems have been developed to test the surface activity of SP-B *in vitro* by mimicking the situation within the alveoli using the Wilhelmy balance, the pulsating bubble, or the captive bubble surfactometer. *In vivo* surface activity has been tested in neonatal RDS models such as the ventilated 27-day gestation premature rabbit, and ARDS models such as the ventilated lavaged rat model. When surface films, composed of 1-palmitoyl-2-palmitoyl phosphatidyl choline: 1-palmitoyl-2-oleyl phosphatidyl glycerol (DPPC:PDPC) (8:2, mol:mol) containing SP-B are spread at the interface of an air bubble in the captive bubble surfactometer, SP-B shows a concentration dependence with maximum effects at 0.5 mol% SP-B (24). Neonatal SP-B-deficient mice die because of respiratory failure at birth, and decreased SP-B protein is associated with reduced lung compliance (25). Studies in ventilated premature rabbits have shown that SP-B-containing surfactant improves dynamic and static mechanics of the immature lung (26).

Given the critical *in vivo* role for SP-B in lung function, it is of interest that numerous actions of the full-length protein on surfactant lipids are mimicked by a synthetic peptide representing the N-terminal domain of SP-B (i.e., $SP\text{-}B_{1\text{-}25}$; residues 1–25). Both SP-B and positively charged $SP\text{-}B_{1\text{-}25}$ increase the collapse pressure of surfactant lipid monolayers containing palmitic acid (PA). This suggests that the cationic N-terminus of SP-B interacts with anionic lipids to remove the driving force for lipid squeeze-out from the surface film (27,28). Another indication of specific interactions of proteins with lipids is insertion of the protein into monolayers at varying surface pressures. The critical insertion pressure, an index of the degree of protein association with lipid films and membrane systems, is very high for both $SP\text{-}B_{1\text{-}25}$ and the parent SP-B (19,29). Furthermore, full-length SP-B and $SP\text{-}B_{1\text{-}25}$ each similarly induce a coexistence of buckled and flat monolayers when added to surfactant lipids, which may participate in the reduction of surface tension and respreadability of the surfactant monolayer (30). Last, initial studies indicate that $SP\text{-}B_{1\text{-}25}$ and the disulfide-linked $SP\text{-}B_{1\text{-}25}$ homodimer (see below) mediate lipid–vesicle mixing, as does the native SP-B (14,31–33).

Using [^{13}C]carbonyl isotope-enhanced Fourier transform infrared (FTIR) spectroscopy, the residue-specific structure of $SP\text{-}B_{1\text{-}25}$ has been solved in POPG liposomes (34). The peptide has a short, distinctive extended β-sheet residues (residues 1–6), α-helical residues (residues 8–22), and random residues (residues 23–25) (Fig. 3). These conformations are close to those predicted for the full-length protein (35), and is analogous to the structural motifs observed in the corresponding homologous N-terminal regions of the saposin family of proteins. Since β-sheet segments require hydrogen bonding to adjacent strands, it is likely that the peptide self-associates in surfactant lipid films via the N-terminal 6 residues, as hypothesized by Perez-Gil and Keough (36). Further support for this type of hydrogen-bonded "biochemical Velcro" hypothesis is implied by the extensive peptide network formed in surfactant lipid films as a function of surface pressure (28).

The $SP\text{-}B_{1\text{-}25}$ peptide has been additionally engineered to more closely resemble the "saposin-fold" structure found in full-length SP-B. By substituting an Ala for Cys-11 in $SP\text{-}B_{1\text{-}25}$, a unique disulfide-linked homodimer analogue of $SP\text{-}B_{1\text{-}25}$ (i.e., dimeric $SP\text{-}B_{1\text{-}25}$) was prepared (Fig. 4). Captive bubble surfactometry of monomeric and dimeric $SP\text{-}B_{1\text{-}25}$ indicates that the dimeric $SP\text{-}B_{1\text{-}25}$ has elevated dynamic respreading properties that accurately mimic those observed for full-length human SP-B. Consequently, the covalent-linking of $SP\text{-}B_{1\text{-}25}$ monomers in the dimeric peptide may act to enhance the monolayer buckling structures and membrane crosslinking im-

FIG. 3. Conformational dynamic model of 10 solution structures calculated for $SP\text{-}B_{1\text{-}25}$ based on FTIR measurements of Gordon *et al.* (34). The disordered molecular C-terminal residues 23–25 are pictured at the top of the illustration in gray tube format, the α-helical residues 8–22 are in red-orange ribbon representation, and the N-terminal β-sheet (residues 1–6) are in gray shown at the bottom of the model. The Protein Data Bank Accession No. for the atomic coordinate set for $SP\text{-}B_{1\text{-}25}$ is 1DFW.

FIG. 6. SP-C backbone representation of porcine SP-C in chloroform based on NMR studies of Johansson *et al.* (51). The N-terminal residues 1 to 8 are in gray tube format at the top of the model, the hydrophobic α-helix spanning residues 9 to 33 is shown in orange-yellow ribbon, and the remaining disordered C-terminal segment is in gray tubes. The Protein Data Bank Accession No. for the coordinates calculated for SP-C conformers is 1SPF.

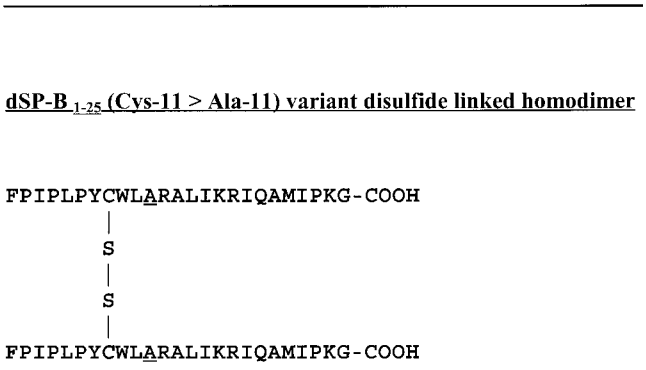

FIG. 4. SP-$_{B1-25}$ dimer sequence with amino acids in single letter code. The disulfide linkage is shown with –S–S– at Cys8 (33).

portant for optimal lipid–protein surface activity. (33). Monomeric SP-B_{1-25}, formulated in DPPC: POPG:PA (69:22:9 wt/wt/wt), quickly improves lung function in either premature rabbits or lavaged, surfactant-deficient rats, and is relatively insensitive to plasma and fibrinogen inhibition (37). When monomeric and dimeric SP-B_{1-25} were compared in the premature rabbit and lavaged rat models, dimeric SP-B_{1-25} was more efficient in improving lung function than monomeric SP-B_{1-25} (38,39).

Other SP-B domains that have been modeled with synthetic peptides include the disulfide-stabilized bend region (SP-B_{34-46}; SP-B residues 34 to 46) and the C-terminal helical segments. SP-B_{34-46} shows a turn-fold propensity that is solvent dependent (40). In trifluoroethanol (TFE):phosphate buffer, SP-B_{34-46} exhibits a turn-like structure, as determined with circular dichroism (CD) and FTIR spectroscopy (40). This turn propensity is confirmed by the formation of a disulfide-linkage between Cys-34 and Cys-46 for SP-B_{34-46} suspended in the TFE solvent system. On the other hand, SP-B_{34-46} is only partially soluble in the Tanaka lipid formulation (41), and shows poor surface activity. These findings suggest that the bend region (residues 34–46) in full-length SP-B functions as a hinge region between the N-terminal and C-terminal domains, and does not directly perturb surfactant lipid structure, as do other segments of the SP-B protein.

Peptides based on the C-terminal sequence of SP-B have also been studied extensively. Almost any amphipathic peptide will exhibit some surface activity in surfactant lipids. However, optimal *in vitro* and *in vivo* emulation of the full-length SP-B protein in surfactant lipids may be viewed as the best "gold standard" for comparing the effectiveness of any mimic peptides. With this in mind, investigators have synthesized segments of increasing length in order to establish a minimally functional SP-B fragment. The surface activity for a family of peptides beginning at the C-terminus has been tested *in vitro* and *in vivo* with an isolated lung system (42). Results from these studies indicate that lengthening the sequence toward the N-terminus of SP-B will increase the surfactant activity of the peptide fragment (42). Mixtures of peptides representing the amphipathic helical regions of both the N-terminus (i.e., SP-B_{1-25}; residues 1–25) and the C-terminus (i.e., SP-B_{48-66}; residues 48–66) also show reasonable surface activity *in vitro* and *in vivo* (8), although they are not as effective as full-length SP-B (42). CD measurements indicate that these N- and C-terminal SP-B segments retain their amphipathic, helical structures, and that antibodies raised against these peptides cross-react with the native SP-B protein (44).

Another synthetic surfactant design strategy that uses the C-terminal region of SP-B as a molecular template is the KL and RL series of peptides. The KL and RL peptides are based on the Leu-Leu repeat sequence found in the C-terminal helical region of the SP-B protein (residues 57 to 63) (45,46). Although these C-terminal peptides definitely exhibit *in vitro* and *in vivo* surface activities, their surface properties do not accurately replicate those observed for native and synthetic SP-B sequences. Specifically, the KL and RL peptides do not produce the low surface tensions and dynamic respreading properties typical of SP-B peptides in *in vitro* studies; these peptides also do not show the same *in vivo* efficacy when compared with native SP-B sequences in animal model systems (9) using the Tanaka lipid formulation (41). Significant discrepancies are also observed in the respective molecular topographies for the KL/RL peptides and native SP-B peptides when bound to lipids. For example, the helical axes for the KL/RL peptides in surfactant lipid films are perpendicular to the lipid monolayer and bilayer surfaces. This result is exactly opposite (47) to that seen for the corresponding helix axes of both the native SP-B protein (18) and a synthetic peptide having the native sequence (48). Molecular redesign of the KL sequence to adjust its orientation in the lipid film, so that it would lie closer to the native parallel orientation, did not enhance the peptide's surface activity

SP-C(human)

```
FGIPCCPVHL KRLLIVVVVV VLIVVVIVGA LLMGL-COOH
1         10          20          30
```

SP-C(Pig)

```
LRIPCCPVNL KRLLVVVVVV VLVVVVIVGA LLMGL-COOH
1         10          20          30
```

SP-C(dog)

```
GIPCFPSSLK RLLIIVVVIV LVVVVIVGAL LMGL-COOH
1         10          20          30
```

SP-C*ff*

```
FGIPFFPVHL KRLLIVVVVV VLIVVVIVGA LLMGL-COOH
1         10          20          30
```

SP-C(Ala>Val)

```
FGIPCCPVHLK RLLAVAVAVA LAVAVAVGAL LMGL-COOH
1          10          20          30
```

SP-C(Leu>Val)

```
FGIPSSPVHL KRLLILLLL LLILLLILGA LLMGL-COOH
1         10          20          30
```

SP-C(LKS)

```
FGIPSSPVHL KRLKILLLL KLILLLKLGA LLMGL-COOH
1         10          20          30
```

FIG. 5. SP-C native protein and synthetic peptide sequences. The amino acids are in single-letter code.

in surfactant lipids (49). Nevertheless, KL_4 has shown some promise as an airway lavage for improving lung function in meconium aspiration syndrome (50).

SURFACTANT PROTEIN C ANALOGUES

Most SP-C analogues have been designed using as a template the known hydrophobic protein sequence and conformation. Native lung surfactant protein C (i.e., SP-C) is one of the most hydrophobic proteins characterized thus far. The mature protein has an N-terminus with a pair of vicinal Cys residues that are covalently linked to palmitoyl moieties via thioester bonds (Fig. 5), making this protein a true "proteolipid" (66). The only exception to this vicinal Cys pairing is that found in dog SP-C, which has a phenylalanine substituted as a surrogate for one of the palmitates. The palmitoylated groups in SP-C are adjacent to a short polar segment, characterized either by cationic residues such as lysine and arginine in the human or by polar nonionic residues such as serine in the dog (Fig. 5). SP-C isolated from native sources is polymorphic, and often has truncations in the N-terminal residues varying from one to three residues. The polar headgroup, N-terminal region of SP-C is followed by an extremely hydrophobic polyvaline sequence that spans for 25 residues (residues 9–33) to near the C-terminus.

The secondary structure for SP-C is conformationally heterogeneous, and is sensitive to the acylation state of the protein. The solution structure for palmitoylated, porcine SP-C has been solved with 2D NMR spectroscopic studies of peptide in chloroform (51) (Fig. 6). In this solvent, porcine SP-C has a relatively unstructured N-terminus (residues 1 to 8), with residues 9 to 33 forming a stable α-helix. Deacylated versions of porcine SP-C may also fold as antiparallel β-sheets; these structures constitute ~10% of native surfactant (52), and appear to form hexameric ensembles in surfactant lipids. Delipidated SP-C from a patient with pulmonary alveolar proteinosis has been shown to form amyloid-like fibrils (53), and has attenuated surface activity (54).

Molecular topographic studies of bovine SP-C in surfactant lipid films (55) indicate that the helix is roughly parallel to the fatty acyl chains, with the N-terminus near the lipid polar headgroups. It is not clear where the palmitoyl fatty acyl chains of SP-C are located in this ensemble. However, the most likely arrangement for the palmitate residues may be an orientation that allows them to cross-talk to the adjacent bilayer. Recent atomic force microscopy (AFM) and low-angle X-ray diffraction results indicate that SP-C forms multilayer stacks of surfactant lipid that are attached to the monolayer, providing evidence that the palmitate may couple to bilayer lipid ensembles adjacent to the monolayer (56). For DPPC monolayers containing SP-C, imaging studies using scanning force microscopy (57) indicate that SP-C promotes the formation of layered stacks of lipid–protein, suggesting that the protein connects a lipid reservoir to the monolayer that can be reincorporated as a function of surface pressure. However, if the lipid component contains PA and an unsaturated lipid (POPG), the SP-C protein promotes the formation of fluid-phase domains that are not squeezed out but remain at the monolayer interface. These observations suggest that SP-C acts to eliminate squeeze-out of the unsaturated lipid necessary for good absorption and respreading, while SP-B induces a reversible folding transition at monolayer

collapse that allows all components of surfactant to remain at the interface during respreading (57).

Developers of SP-C analogues have focused on both the N-terminal segment of the protein containing the lipid–protein covalent linkage and the hydrophobic C-terminal helical segments. One of the most challenging areas of synthesis has been attachment of palmitoyl groups to the vicinal Cys residues. The acylation of the vicinal Cys residues (or, alternatively, surrogates substituting for Cys) is critical for optimizing the structure and function of SP-C, since deacylated species form irreversible β-sheet aggregates that have little or no activity in surfactant lipids (53,54). Succinylamidyl palmitate derivatives were the first to be used on synthetic SP-C sequences (58). More recently, synthesis protocols for thioester linkages that closely mimic the native SP-C-lipid covalent bonds have been developed, permitting more meaningful structural and functional comparisons between native SP-C and SP-C analogues (59). We have synthesized a dipalmitoylated SP-$C_{1\text{-}35}$ peptide, and found it to confer surface activity to Tanaka lipids (DPPC:POPG:PA) and Survanta on the Wilhelmy balance (9,60). Formulated with synthetic lipids, the dipalmitoylated SP-$C_{1\text{-}35}$ peptide improved lung function in ventilated, lavaged rats (9) and premature rabbits (61). Another approach to the synthesis of the proteolipid SP-C mimics has been to substitute Phe residues as replacements for the palmitoyl groups (i.e., SP-C*ff*), prepared using either chemical methods or recombinant technology (62). The rationale behind this strategy is that dog SP-C has a Phe replacing one of the palmitates (64). SP-C*ff* reportedly has *in vitro* structures and *in vivo* surface activities that closely resemble those of the native SP-C (63). However, in our hands the Phe > Cys-Pal (Fig. 5, SP-C*ff*) substituted SP-C analogue has low solubility in chloroform–methanol, and is difficult to formulate with Tanaka lipids in an active α-helical conformation. Yet another strategy for reengineering the Cys–palmitate moieties has been the substitution of a vicinal Ser sequence for the native amino acid–lipid derivative (Fig. 5, SP-C[Leu > Val]) (43). This substitution, along with changes in the hydrophobic sequence in the C-terminal regions (see below), produced SP-C analogues with helical conformation and surfactant activity that resemble those of native SP-C.

The hydrophobic polyvaline sequence of SP-C has also been manipulated using molecular design strategies. The overall rationale used here for synthesizing functional SP-C analogues is to maximize helical conformations, while minimizing β-sheet structures, which leads to irreversible peptide aggregation. One approach has been to periodically substitute Ala for Val in the hydrophobic region in order to optimize α-helical content (60) (Fig. 5). This Ala-Val-Ala-Val template was based on the protein signal sequence for LamB that inserts into membrane lipids as an α-helix. The succinamidyl-palmitate derivative of this SP-C mimic appears to share many of the *in vitro* and *in vivo* activities of native SP-C in surfactant lipids (58,64). Nevertheless, surface film measurements suggest that this SP-C analogue may not integrate well into DPPC, compared with native and SP-C*ff* peptides. Another SP-C mimic, SP-C(LKS), limits peptide aggregation by replacing Val residues with Leu, and also adding Lys every fifth residue (43). Alone, or in combination with native SP-B, SP-C(LKS) has a secondary structure and biophysical activity similar to that of native SP-C (43). Although all of the SP-C analogues appear to mimic activities of native SP-C, more detailed AFM and ^{13}C-FTIR analysis will be required before we can determine if these SP-C surrogates fully emulate the structure and function of the native SP-C protein. The performance of SP-C analogues during *in vivo* testing for resistance or reversal of various surfactant inhibition states will also be of interest.

CONCLUSIONS

Although much progress has been made in the development of synthetic surfactant peptides, further optimization of these potential therapies will require knowledge of the residue-specific conformations for native SP-B and SP-B mimic peptides in surfactant lipids. Relatively little information is currently known about the detailed tertiary structure of SP-B proteins and peptides in clinically relevant preparations. In particular, future studies are required to more fully define the saposin-folding pattern that has been attributed to SP-B proteins and mimic peptides. Analogous experiments are also needed to elucidate the structures of dimeric surfactant proteins (i.e., dimer SP-B or dimer SP-C), which reportedly have enhanced surfactant activities and may prove useful in synthetic surfactant therapies for RDS and ARDS. Further work is also needed to develop formulations of surfactant-mimic peptides with specific lipid mixtures for particular biomedical applications.

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