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Antimicrobial Activity of Native and Synthetic Surfactant Protein B Peptides

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Surfactant protein B (SP-B) is secreted into the airspaces with surfactant phospholipids where it reduces surface tension and prevents alveolar collapse at end expiration. SP-B is a member of the saposin-like family of proteins, several of which have antimicrobial properties. SP-B lysed negatively charged liposomes and was previously reported to inhibit the growth of *Escherichia coli* in vitro; however, a separate study indicated that elevated levels of SP-B in the airspaces of transgenic mice did not confer resistance to infection. The goal of this study was to assess the antimicrobial properties of native SP-B and synthetic peptides derived from the native peptide. Native SP-B aggregated and killed clinical isolates of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and group B streptococcus by increasing membrane permeability; however, SP-B also lysed RBC, indicating that the membranolytic activity was not selective for bacteria. Both the antimicrobial and hemolytic activities of native SP-B were inhibited by surfactant phospholipids, suggesting that endogenous SP-B may not play a significant role in alveolar host defense. Synthetic peptides derived from native SP-B were effective at killing both Gram-positive and Gram-negative bacteria at low peptide concentrations (0.15–5.0 μM). The SP-B derivatives selectively lysed bacterial membranes and were more resistant to inhibition by phospholipids; furthermore, helix 1 (residues 7–22) retained significant antimicrobial activity in the presence of native surfactant. These results suggest that the role of endogenous SP-B in host defense may be limited; however, synthetic peptides derived from SP-B may be useful in the treatment of bacterial pneumonias. *The Journal of Immunology*, 2006, 176: 416–425.

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3 Abbreviations used in this paper: DPPC, dipalmitoylphosphatidylcholine; PG, phosphatidylycerol; SP-B, surfactant protein B; SAPLIP, saposin-like family of proteins; h, human; BALF, bronchoalveolar lavage fluid; N-term, N-terminal.
the lytic domain of SP-B to helix 1, an α-helical, amphipathic region containing a net charge of +3 (16). In the present study, native SP-B and lytic peptides derived from the native peptide were tested for antimicrobial activity against both Gram-positive and Gram-negative bacteria.

Materials and Methods

Materials

DPPC and phosphatidylglycerol (PG) were purchased from Avanti Lipids. HEPES buffer was purchased from Cambrex Bioscience, and melittin peptide from bee venom was purchased from Sigma-Aldrich.

Peptide design

Synthetic peptides were designed to the proposed helices and interhelical loops of the mature SP-B peptide (16). Peptides were synthesized by Bio-synthesis Inc. 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 SP-B

Human SP-B was isolated from bronchoalveolar lavage fluid (BALF) of patients with pulmonary alveolar proteinosis, as described by Shen et al. (17). Briefly, surfactant was isolated from BALF 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 a 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 dialyzed (SnakeSkin dialysis tubing; m.w. cutoff, 3500; Pierce Chemical) against chloroform/methanol (2:1 (v/v)) overnight at 4°C to remove HCl and was quantitated by amino acid composition analysis (18).

In vitro bacterial killing assay

Clinical isolates of Klebsiella pneumoniae (KPA1 serotype), Staphylococcus aureus, or Pseudomonas aeruginosa were grown in Luria broth (LB) and group B streptococcus (provided by J. Wright, Duke University Medical Center, Durham, NC) was grown in Todd Hewitt broth at 37°C with continuous shaking to exponential phase. The bacteria were harvested from broth by centrifugation at 500 × g for 10 min, washed, and resuspended in sterile PBS at a concentration of 10^5 CFU/100 μl. The concentration of bacteria was verified by quantitative culture on sheep blood agar plates. One hundred microliters of bacterial suspension was plated in a 96-well microtiter plate and incubated with individual SP-B peptides (2 mg/ml stocks dissolved in methanol) at 2.5, 5.0, and 10.0 μM. Controls for zero and 100% hemolysis consisted of SP-B suspended in PBS and 1% Triton X-100, respectively; additional controls included PBS and PBS containing 0.5 or 1% methanol. The hRBC/peptide suspension was incubated with agitation for 60 min at 37°C. The samples were centrifuged at 800 × g for 10 min, and the release of hemoglobin was measured by measuring the absorbance of the supernatant at 550 nm.

Bacterial aggregation assays

Bacteria were grown until mid-log phase, diluted to an OD_{600} of 0.1, and plated in a 96-well polystyrene plate. Native SP-B or synthetic peptides in methanol were added to bacteria and incubated at 37°C for 3 h. Samples were stained using the fluorescent probe Syto 9 and imaged by fluorescence microscopy to assess bacterial aggregation and changes in propidium iodide (BD Biosciences, Mo- lecular Probes). Bacteria were examined by fluorescence microscopy to assess bacterial aggregation and changes in propidium iodide or Syto 9 staining compared with untreated or methanol-treated controls.

Isolation of pulmonary surfactant

Surfactant was isolated by high-speed centrifugation of cell-free BALF (2 ml in sterile PBS/mouse) obtained from 25-g FVB/NJ mice (6–8 wk old). Phosphorous in total BAL was measured by the Bartlett assay (19).

Data analysis

All data are expressed as mean ± SEM. Differences between groups were determined by ANOVA followed by Student-Newman-Keuls or Dunnett posttests if p < 0.05. Differences between two groups were determined by Student’s t test.

Results

Antimicrobial activity of human SP-B against K. pneumoniae

Sequence alignments revealed that the location of the cysteine residues in SP-B was a common feature among SAPLIP family members, several of which are bacteriolytic (20). To determine whether SP-B was also bacteriolytic, a clinical isolate of K. pneumoniae (10^5 CFU) was incubated with increasing concentrations of purified human SP-B for 6 h at 37°C. Mature SP-B peptide exhibited potent, dose-dependent antimicrobial activity (Fig. 1A), killing >90% of K. pneumoniae at a concentration of 1.0 μM. Incubation of SP-B with bacteria also resulted in dose-dependent detection of the bacterial periplasmic enzyme alkaline phosphatase, consistent with membrane permeabilization (data not shown).

Because SP-B is always associated with membranes, experiments were designed to determine whether SP-B-mediated bacterial killing was altered in the presence of surfactant phospholipids. Surfactant-like liposomes, composed of DPPC/PG (9:1, w/w) or DPPC at a 20:1 lipid:protein ratio, were first mixed with SP-B
followed by incubation with bacteria. Surfactant phospholipids decreased SP-B-mediated killing by ~70% (Fig. 1B); however, removal of PG from the liposomes partially restored activity, resulting in only a 30% decrease in bacterial killing. We previously reported that killing of \textit{P. aeruginosa} and group B streptococcus was not enhanced in transgenic mice in which the concentration of human SP-B were intratracheally instilled with 10^4 CFU of \textit{K. pneumoniae}, and lungs were harvested 24 h postinfection. CFU were counted from plated lung homogenates, and data are expressed as CFU per gram of lung tissue ± SEM; WT vs transgenic SP-B overexpressors, p = 0.7365. n = 8 mice/group.

Effect of SP-B on \textbf{bacterial aggregation}

The hydrophilic surfactant proteins SP-A and SP-D play important roles in lung host defense by inducing bacterial aggregation. To determine whether SP-B could also induce bacterial aggregation, \textit{K. pneumoniae} (OD_{600} 0.1) was incubated with SP-B for 90 min and stained with the vital dyes Syto 9 (green fluorescence indicates live bacteria) and propidium iodide (red fluorescence indicates dead/dying bacteria). Bacteria were examined by fluorescence microscopy to assess bacterial aggregation and to detect changes in propidium iodide or Syto 9 staining compared with untreated or methanol-treated controls. Addition of SP-B (1–3 \mu M) to \textit{K. pneumoniae} induced significant bacterial aggregation compared with controls (Fig. 2). The mean area of bacterial aggregates was 540 m^2, and aggregates as large as 5000 m^2 were detected. Similar results were obtained with other strains of bacteria including \textit{P. aeruginosa}, \textit{S. aureus}, and group B streptococcus (data not shown). Increased propidium iodide staining was detected in SP-B-treated samples but not in untreated or vehicle controls, indicating that the aggregated bacteria were also killed.

To determine whether surfactant phospholipids altered the ability of SP-B to aggregate bacteria, SP-B was added to DPPC or DPPC/PG liposomes before incubation with bacteria (OD_{600} 0.1) for 90 min. The presence of DPPC liposomes did not affect the ability of SP-B to induce aggregation or alter the number or size of bacterial aggregates (data not shown). SP-B-induced bacterial aggregation also occurred in the presence of DPPC/PG liposomes but to a lesser extent. Propidium iodide staining was reduced in the presence of both DPPC and DPPC/PG lipids consistent with decreased CFU in the bacterial killing assays. Thus, surfactant phospholipids, in particular PG, inhibited both bacterial killing and aggregation.

\textbf{Hemolytic activity of human SP-B}

To determine the specificity of the membranolytic activity of SP-B, native peptide was incubated with hRBC in the presence or absence of DPPC or DPPC/PG liposomes (Fig. 3). SP-B induced a dose-dependent release of hemoglobin from RBC at a concentration of 1.0–7.5 \mu M. Membrane lysis was significantly reduced in the presence of DPPC and was virtually ablated in the presence of DPPC/PG liposomes.

\textbf{Antimicrobial activity of SP-B synthetic peptides against \textit{K. pneumoniae}}

To map the antimicrobial domain(s) of SP-B, synthetic peptides were made to the proposed helices and interhelical loops of human SP-B based on the three-dimensional structure of NK-lysin, as previously described (16) (Fig. 4A). Antimicrobial activity was assessed by incubating individual synthetic peptides with a clinical isolate of \textit{K. pneumoniae} (10^5 CFU) for 6 h at 37°C. Bacteria were subsequently plated on blood agar plates, and the number of colonies was counted after 18 h. SP-B peptides containing helix 1 exhibited potent antimicrobial activity against \textit{K. pneumoniae} (Fig. 4B). A peptide encompassing residues 1–37 (N-terminal (N-term)}
helix 1,2) killed >60% of the bacteria at a concentration of 2.5 μM. Removal of the N-terminal 6 aa from N-term helix 1,2 resulted in significantly higher levels of bacterial killing (>80%). Helix 1 (residues 7–22) killed >80% of the bacteria as did a shorter helix 1 peptide (residues 10–22) (data not shown). In contrast, helix 2 and a peptide encompassing helices 3,4,5 exhibited much lower levels of antimicrobial activity. These results demonstrate that residues 10–22 (helix 1) are sufficient for bacterial killing.

Dose response of SP-B peptides on bacterial killing

To determine the lowest concentration of SP-B peptide required for K. pneumoniae killing, dose-response curves were generated for the most effective synthetic peptides (helix 1 (residues 7–22), N-term helix 1, and helix 1,2). Helix 1,2 was significantly more effective at bacterial killing than helix 1 or N-term helix 1 and exhibited significant antimicrobial activity (30%) at concentrations as low as 0.075 μM (Fig. 5A). Maximal killing was attained at a

% Hemolysis

SP-B concentration

FIGURE 3. Hemolytic activity of native hSP-B. hRBC (4%) were incubated with human SP-B (1–3 μM) or solvent (methanol) controls for 90 min at 37°C. Methanol-treated controls were included in all experiments and had no effect on hemolysis. Results are the mean of four separate experiments ± SEM; *, p < 0.001 vs native SP-B lipid-free; +, p < 0.05 vs SP-B DPPC.
concentration of 2.5 μM for helix 1, 2 and 5.0 μM for helix 1. To further characterize the bacteriolytic activity of the SP-B peptides, increasing concentrations of helix 1 were incubated with *K. pneumoniae* (OD<sub>600</sub> 0.05) for 90 min, and membrane permeability was assessed by alkaline phosphatase detection. Helix 1 (residues 7–22) caused significant membrane permeability in a dose-dependent manner at concentrations as low as 2.5 μM (data not shown).

**Effect of surfactant phospholipids on SP-B-mediated bacterial killing**

Experiments were designed to determine whether SP-B-mediated bacterial killing was altered in the presence of surfactant phospholipids. Liposomes, composed of DPPC/PG (9:1, w/w) at lipid:peptide ratios of 20:1 or 10:1, were first mixed with synthetic peptide followed by incubation with bacteria. Preincubation of liposomes...
To determine whether the SP-B peptides could also kill Gram-positive bacteria, dose-response curves were generated by incubating helix 1 (residues 7–22), N-term helix 1, or helix 1,2 peptide with S. aureus (Fig. 6A). Helix 1,2 killed bacteria at a concentration as low as 0.15 μM (55% killing) with maximal bacterial killing at a concentration of 0.6 μM, indicating that this peptide was significantly more effective than helix 1 (residues 7–22) or N-term helix 1. Helix 2 alone was much less effective at killing bacteria (<10% at 2.5 μM, data not shown) providing further evidence that helix 1 was required for bacterial killing. Domain-mapping experiments demonstrated that the shorter helix 1 peptide (residues 10–22) was much less effective at killing S. aureus than the longer peptide (residues 7–22) (<45% killing at 2.5 μM for residues 10–22 compared with >95% killing for residues 7–22) (Fig. 6B). These results suggest that the hydrophobic residues tyrosine 7, cysteine 8, and tryptophan 9 may be important for disrupting membranes of Gram-positive bacteria. SP-B-mediated killing of S. aureus was also inhibited by PG-containing liposomes at a 20:1 lipid:peptide ratio (Fig. 6C); however, decreasing the lipid:peptide ratio to 10:1 dramatically improved bacterial killing (>90%) with helix 1 (residues 7–22). Both helix 1,2 and helix 1 (residues 7–22) were effective at killing S. aureus (>95%) in the presence of DPPC vesicles at both the 20:1 and 10:1 lipid:peptide ratios.

Identification of amino acids important for bacterial killing

The domain-mapping experiments demonstrated that the SP-B peptides containing helix 1 were antimicrobial. To further examine the structural basis for this property and to identify specific residues involved in bacterial killing, amino acid substitutions were introduced into helix 1 in the context of N-term helix 1,2 (i.e., residues 1–37) (Fig. 7). N-term helix 1,2 was previously shown to be the smallest SP-B peptide that promoted surface tension reduction (16). Positively charged amino acids have been shown to be important for the bactericidal activity of several antimicrobial peptides (21). To determine whether these residues were also important for the antimicrobial activity of SP-B, positively charged residues located in helix 1 and 2 were systematically substituted with uncharged amino acids. We have previously shown that single alanine or multiple serine substitutions did not alter the secondary structure of the peptides (16). Substitution of a single positively charged amino acid (R12, K16, or K24) with alanine had no effect on the antimicrobial activity of SP-B; however, substitution of two or three positively charged residues significantly inhibited killing of K. pneumoniae (<25%) (Fig. 7) and S. aureus (data not shown). In particular, substitution of serine for R12 and K16 in helix 1 virtually ablated bacterial killing. These results indicate that at least two positively charged residues in helix 1 are required for the antimicrobial activity of SP-B peptides.

Effect of SP-B synthetic peptides on bacterial aggregation

To assess the ability of synthetic peptides to aggregate bacteria, helix 1 (residues 7–22) or helix 1,2 were added to K. pneumoniae (OD₆₀₀ 0.1) and incubated for 90 min. Bacteria were stained with the vital dyes Syto 9 and propidium iodide and analyzed by fluorescence microscopy (Fig. 8). Helix 1 (residues 7–22) did not induce bacterial aggregation but caused a significant increase in propidium iodide staining (Fig. 8, D–F) compared with control (A–C). Helix 1,2 (residues 7–37) induced bacterial aggregation, but the
majority of aggregates were significantly smaller than those induced by native SP-B (mean aggregate area, 50 ± 10 μm²; p < 0.001) (Fig. 8G-I). In a few fields, larger bacterial aggregates were observed with sizes similar to those induced by native SP-B (Fig. 8, J–L). Virtually all of the bacteria within the aggregates were positive for propidium iodide staining consistent with dead/dying bacteria (Fig. 8K). Helix 1,2 exhibited similar activity toward other strains of bacteria including P. aeruginosa and group B streptococcus (data not shown).

**Hemolytic activity of the SP-B synthetic peptides**

To determine the specificity of SP-B peptides for prokaryotic cell membranes, N-term helix 1,2, helix 1 (residues 7–22), helix 1 (residues 10–22; data not shown), helix 1,2, N-term helix 1, and melittin were incubated with hRBC for 1 h. All of the SP-B peptides tested exhibited very low levels of hemolytic activity compared with melittin (<15% hemolysis at the highest concentration) (Fig. 9). Incubation of A549 cells with 5 μM helix 1 (residues 7–22) for...
1 h resulted in death of 33.3 ± 6.2% of cells; incubation of cells with the solvent (methanol) control resulted in death of 28 ± 5.8% of cells (p = 0.3977).

Effect of surfactant phospholipids on SP-B peptide-mediated bacterial killing

Helix 1 (residues 7–22) was more effective at killing S. aureus than K. pneumoniae in the presence of lipids compared with helix 1,2 or N-term helix 1 (Figs. 5B and 6C); in particular, helix 1 killed S. aureus much more effectively than helix 1,2 in the presence of DPPC/PG (>90% killing for helix 1 at the 10:1 ratio compared with <5% killing for helix 1,2) (Fig. 6C). We next determined whether helix 1 could augment the ability of native surfactant to kill bacteria. Bronchoalveolar lavage was performed on wild-type FVB/N mice, cells were removed using low-speed centrifugation, and surfactant phospholipids and associated proteins were pelleted at 18,000 × g for 15 min. Increasing amounts of helix 1 peptide were added to 0.75 μg of total surfactant lipid followed by incubation with K. pneumoniae or S. aureus (10^3 CFU) for 6 h at 37°C. Bacteria were plated on blood agar plates, and colonies were counted after 18 h. In the presence of native surfactant, SP-B helix 1 killed both K. pneumoniae and S. aureus at concentrations of 5–10 μM (Fig. 10).

Discussion

SP-B is a member of the SAPLIPS, which include the potent antimicrobial peptides NK-lysin, granulysin, and amoebapore. SAPLIPS are characterized by a conserved disulfide bond pattern and likely share a similar tertiary structure. All SAPLIPS interact with lipids and several, including the antimicrobial peptides, have membrane-lytic activity. The interaction of peptides with membranes is mediated in part by cationic residues usually located in the polar face of an amphipathic helix. SP-B interacts with the surface of the lipid bilayer via four or five amphipathic α helices (20). Positively charged amino acids, located predominantly in the N-terminal domain of SP-B, facilitate interaction of the mature peptide with the negatively charged head groups of PG (22–24). Domain-mapping experiments demonstrated that a cationic peptide corresponding to helix 1 (residues 7–22) was sufficient to lyse negatively charged vesicles (16). The results of the current study indicate that native SP-B and synthetic peptide derivatives containing helix 1 killed Gram-positive and Gram-negative bacteria in vitro.

In the present study, native SP-B, isolated from human BALF, killed clinical isolates of Gram-positive and Gram-negative bacteria in a dose-dependent manner. Antimicrobial activity was detected at concentrations between 0.06 and 1.0 μM, comparable to other potent antimicrobial SAPLIP peptides and other well-characterized α-helical, cationic peptides (12, 25–27). Native SP-B killed bacteria by permeabilizing the bacterial cell membrane, as indicated by detection of alkaline phosphatase activity and increased propidium iodide staining. Peptide-mapping experiments demonstrated that the antimicrobial activity of SP-B mapped with the lytic activity to helix 1 (residues 7–22). This finding supports the observation of Kaser and Skouteris (9), who noted that residues 12–34 of SP-B are 68% homologous to residues 48–72 of the frog peptide antibiotic dermaseptin bI.

In addition to direct bacterial killing, SP-B also induced significant aggregation of Gram-positive and Gram-negative bacteria. Bacterial aggregation facilitated by the collectins SP-A and SP-D likely plays a role in enhancement of phagocytosis, complement activation, and/or inhibition of microbial colonization and invasion (3). SP-A and SP-D bind polysaccharides located on the surface of bacteria through their C-terminal carbohydrate recognition domains. Domain-mapping experiments of SP-B indicated that, although helix 1 was sufficient for bacterial killing, aggregation required both helix 1 and helix 2. This finding agrees well with a previous study (16) that implicated helix 2 in membrane cross-linking (aggregation) and fusion (promoting lipid transfer between lipid bilayers and the surface active monolayer): SP-B peptides anchored to separate membranes by helix 1 may cross-link membranes by interacting through helix 2 (peptide-peptide interaction).
alternatively, the SP-B peptide may form a “bridge” in which helix 1 interacts with one membrane and helix 2 interacts with a separate membrane (peptide-lipid interaction). It is interesting to note that only a fraction of the bacteria aggregated by native SP-B were killed (Fig. 2), whereas virtually all of the bacteria aggregated by peptide helix 1,2 were stained by propidium iodide (Fig. 8). This suggests that the synthetic peptide aggregates bacteria through “lethal” domains, presumably helix 1, whereas native SP-B may induce bacterial aggregation via multiple motifs, some of which lack killing activity. However, although SP-B clearly promoted bacterial aggregation in vitro, the importance of this property for bacterial killing and/or clearance in vivo is less certain.

SP-B is very hydrophobic and is likely always associated with surfactant phospholipids in vivo. Although lipid-free SP-B was bactericidal in vitro, this activity was dramatically inhibited in the presence of surfactant phospholipids, particularly PG. We previously reported (28) that the content of SP-B in the alveolar spaces was 5–6 μg, and the total surfactant phospholipid content was 275 μg (estimated for a 25-g mouse at 6–8 wk of age). Thus the lipid:protein ratios used in the current study (20:1) were much lower than the estimated ratio in vivo (50:1). These data strongly suggest that PG will inhibit the antimicrobial activity of SP-B in vivo. Furthermore, lipid-free SP-B exhibited hemolytic activity comparable to melittin, and this activity was completely inhibited in the presence of surfactant phospholipids, indicating the importance of maintaining native SP-B in a lipid-associated form. We cannot exclude the possibility that, in vivo, some SP-B may exist in microdomains that are enriched in DPPC and are relatively poor in PG content. Such a microenvironment would minimize the hemolytic activity of SP-B while preserving at least some of its antibacterial properties. We also cannot exclude the possibility that native SP-B may be proteolytically cleaved into smaller peptide fragments, similar to the synthetic peptide derivatives described in the current study, i.e., peptides that retain antimicrobial activity in the presence of surfactant and have little or no hemolytic activity. The generation of antimicrobial peptides from precursor proteins has been reported previously. For example, buforin I, a peptide that is important for innate host defense of the intestinal epithelium, is generated by enzymatic cleavage of the non-antimicrobial precursor histone H2A (29). SP-B peptide fragments could be generated by a similar process; alternatively, SP-B peptide fragments could be generated within alveolar macrophages following uptake from the airspace. However, the results of studies in transgenic mice are not consistent with the generation of bactericidal peptides or specialized lipid microdomains. Increased expression of SP-B in transgenic mice did not enhance bacterial killing of P. aeruginosa, group B streptococcus (10), or K. pneumoniae (current study), and, importantly, susceptibility to bacterial infection was not increased in mice in which the concentrations of SP-B in the airspaces was decreased by 50% (10). Thus, although a role for native SP-B in host defense remains a formal possibility, the experimental evidence to support this hypothesis is currently lacking.

Synthetic peptide derivatives of SP-B exhibited little to no hemolytic activity and selectively lysed bacterial membranes. The difference in membrane selectivity between native SP-B and the peptide derivatives could be due to the mechanism of action that has been proposed for a large number of cationic antibiotic peptides. According to the carpet model proposed by Shai (30), cationic, amphipathic, α-helical peptides act on bacterial membranes in four main steps including 1) interfacial partitioning with accumulation of monomeric peptides on the target membrane (limiting step); 2) peptide rearrangement, usually via oligomerization; 3) membrane permeabilization/denpolarization associated with adoption of a transient transmembrane orientation of peptide oligomers; and 4) spontaneous deinsertion of peptide with redistribution on both sides of the membrane, permitting access of peptides to intracellular targets. Accumulation of cationic SP-B peptides at levels sufficient to initiate translocation and membrane permeabilization would be critically dependent on electrostatic interactions and interfacial hydrophobicity. Only negatively charged membranes would attract enough of the smaller, cationic, synthetic peptides to form permeabilizing oligomers; in contrast, native SP-B, which is intrinsically oligomerized, may be competent to permeabilize both anionic and zwitterionic membranes, even at low protein densities. The shortest membranolytic SP-B peptide, helix 1, was more resistant to inhibition by phospholipids than the native peptide and retained significant antimicrobial activity in the presence of native surfactant. A synthetic peptide containing helix 2 (helix 1,2) killed bacteria at lower concentrations than helix 1 alone and aggregated bacteria similarly to the native peptide; however, this peptide was more sensitive to lipid inhibition. The lipid vesicle aggregates induced by native SP-B or helix 1,2 may hide a significant fraction of the peptide, thereby decreasing transfer to the bacterial membrane. Because helix 1 does not aggregate membranes, it may be fully exposed on the surface of the vesicles where it can be readily transferred to bacterial membranes.

Residues 7–9 of SP-B were required for efficient killing of Gram-positive bacteria but not Gram-negative bacteria. This difference may be related to the intrinsically different structure of the target membranes of these microorganisms. Permeabilization of Gram-negative bacteria would require translocation through the external LPS containing envelope and the periplasmic space before reaching the target plasma membrane. Both the external envelope and the plasma membrane have anionic surfaces and could accumulate peptide through electrostatic affinity. The presence of competing anionic membranes (DPPC/PG vesicles) would inhibit partitioning of peptides into both layers. Gram-positive bacteria such as S. aureus have a single membrane with a thick external, negatively charged wall containing peptidoglycan and teichoic acid. Electrostatic interactions would facilitate peptide accumulation at the membrane surface, but penetration of the phospholipid bilayer would be dependent on interfacial hydrophobicity, conferred predominantly by the aromatic side chains of Tyr7 and Trp9. This model would explain why 1) lipid vesicles are less able to inhibit the antibiotic activities of SP-B peptides toward S. aureus than K. pneumoniae, and 2) removal of aromatic residues Tyr7 and Trp9 produced a substantial decrease in the anti-staphylococcal properties of helix 1. Consistent with this model, Serrano et al. (31) recently demonstrated that residues 7–9 exhibited the highest affinity for phospholipid interfaces of any motif in SP-B.

In summary, although a significant role for endogenous SP-B in innate host defense of the lung may be limited, synthetic peptides derived from native SP-B may be very useful as antimicrobial agents. SP-B peptides encompassing helix 1 (helix 1, N-term helix 1, and helix 1,2) exhibited potent antimicrobial activity against clinical isolates of K. pneumoniae, S. aureus, group B streptococcus, and P. aeruginosa at low peptide concentrations in vitro. The properties of bacterial killing in the presence of surfactant phospholipids and selectivity for bacterial membranes suggest that helix 1 (residues 7–22) may be useful as an adjunct for treatment of bacterial pneumonias.

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Disclosures
The authors have no financial conflict of interest.
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