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Surfactant Protein B Propeptide Contains a Saposin-Like Protein Domain with Antimicrobial Activity at Low pH

Li Yang,* Jan Johansson,† Ross Ridsdale,* Hanna Willander,† Michael Fitzen,† Henry T. Akinbi,* and Timothy E. Weaver*  

Surfactant protein B (SP-B) propeptide contains three saposin-like protein (SAPLIP) domains: a SAPLIP domain corresponding to the mature SP-B peptide is essential for lung function and postnatal survival; the function of SAPLIP domains in the N-terminal (SP-BN) and C-terminal regions of the propeptide is not known. In the current study, SP-BN was detected in the supernatant of mouse bronchoalveolar lavage fluid (BALF) and in nonciliated bronchiolar cells, alveolar type II epithelial cells, and alveolar macrophages. rSP-BN indirectly promoted the uptake of bacteria by macrophage cell lines and directly killed bacteria at acidic pH; the bactericidal activity of BALF at acidic pH was completely blocked by SP-BN Ab. Transgenic mice overexpressing SP-BN and mature SP-B peptide had significantly decreased bacterial burden and increased survival following intranasal inoculation with bacteria. These findings support the hypothesis that SP-BN contributes to innate host defense of the lung by supplementing the nonoxidant antimicrobial defenses of alveolar macrophages.

Materials and Methods

Expression, purification, and refolding of recombinant mouse

SP-BN

SP-BN cDNA (encoding residues 61–146; Fig. 1A) was generated from mouse type II cell RNA by RT-PCR using upstream primer 5′-GGG AAT TCC ATA TGC ATG CAG GAG GTA ATG ACC TG-3′ and downstream primer 5′-CCG CTC GAG CTG CCC ACG TGG GCA CAG GCC-3′; restriction sites for Ndel and XhoI were encoded in the upstream and downstream primers, respectively. The amplified 258-bp fragment was cloned into the Ndel/XhoI sites of PET21a vector (Novagen, Madison, WI). SP-BN was expressed in Escherichia coli BL21 (DE3). Transformed bacteria were grown in Luria-Bertani medium supplemented with 50 µg/ml carbenicillin to an OD600 of 0.6; protein expression was induced by the addition of 0.1 mM isopropyl β-d-thiogalactoside (IPTG) for 3 h at 37°C. Ten to 20% tricine–SDS-PAGE of bacterial lysates expressing SP-BN detected a band, Mw = 9 kDa, following IPTG induction. The broth was centrifuged, and the isolated bacterial pellet was lysed by sonication in 20 mM Tris buffer, pH 7.4, 4°C. Inclusion bodies were recovered by centrifugation, washed in Tris buffer, and solubilized in 20 mM Tris, 6 M urea, 50 mM DTT buffer, pH 7.4. Denatured, solubilized inclusion body protein was diluted (1:10) in 20 mM Tris, 6 M urea, 0.5 M NaCl, 3 mM reduced glutathione, 0.3 mM oxidized glutathione, pH 7.4, and dialyzed three times against 10 volumes of the same buffer in which the urea concentration was
reduced to 2 M followed by dialysis against 10 volumes of 20 mM Tris, 0.5 M NaCl (nicket-nitrilotriacetic acid [Ni-NTA] binding buffer), pH 7.9. After centrifugation, the supernatant was applied to a Ni-NTA agarose column (Novagen). The column was washed and eluted according to the manufacturer’s protocol. Eluted protein was dialyzed against sodium phosphate buffer, pH 7.0, and stored in aliquots at −80°C.

Circular dichroism experiments
Circular dichroism spectra in the far-UV region (250–190 nm) were recorded at 25°C with a J-810-150S spectropolarimeter (Jasco, Tokyo, Japan), using a bandwidth of 1 nm and a response time of 2 s; 10 data points/ nm were collected. Each spectrum shown is the average of three scans. The residual molar ellipticity (θ) is expressed in degree × cm²/mole. Spectra were recorded of 25 μM SP-B*N in PBS, pH 7.4 or in 20 mM NaAc, pH 5.6 with or without 100 μM 1-palmitoyl-sn-glycero-3-phosphocholine (lysoPC, Sigma-Aldrich, St. Louis, MO).

Trypsin digestion and identification of disulfides
Fifty micrograms SP-B*N were treated with 500 ng trypsin (Promega, Madison, WI) in 50 mM Tris buffer, pH 8.0, containing 2 μM at 37°C for 3 h. The reaction was stopped by the addition of trifluoroacetic acid (10% in final concentration). The urea-containing buffer was changed to a 50 mM ammonium bicarbonate buffer, pH 7.4. Digestion SP-B*N was reduced by boiling in 1% β-mercaptoethanol (Sigma-Aldrich) and desalted prior to mass spectrometry (MS) analysis with C2 ZipTips (Millipore, Bedford, MA), according to the manufacturer’s instructions. Peptides were eluted in 75% acetonitrile, 0.1% formic acid directly onto the MALDI target and mixed with α-cyano (Bruker Daltonics, Bremen, Germany) matrix. MALDI-MS spectra were acquired on an Ultraflextreme mass spectrometer (Applied Biosystems, Foster City, CA) operated in reflector mode and analyzed using the Ultraflextreme Data Explorer 3.4 software package.

For liquid chromatography (LC)-MS, digested SP-B*N was reduced by incubation in 5 mM DTT (Sigma-Aldrich) for 30 min at 37°C, carbamidomethylated by the addition of iodoacetamide (Sigma-Aldrich) to a final concentration of 7 mM, and incubated for 15 min in the dark at 20°C. Electrospinning solution MS and MS/MS data were acquired on a QTOF Premier API instrument (Waters, Manchester, U.K.) equipped with the standard Z-spray source and a nanoAcquity nanoflow LC system. Lockmass reference of (Glu1)phosphopeptide (Sigma-Aldrich) was used for mass scale correction.

SP-B*N Ab
rSP-B*N was injected into guinea pigs to generate polyclonal Abs. SP-B*N Abs were initially characterized by Western blotting of lung homogenates from E18.5 Sftpb–/– mice and wild-type (WT) littersmates. The Ab used in the current study detected a single protein, Mr = 8 kDa, in WT mice and no protein bands in SP-B knockout mice (data not shown and Fig. 5a, right panel, lane 2). All experiments involving the use of animals (guinea pigs, mice, and rats) were approved by the Institutional Animal Care and Use Committee at the Cincinnati Children’s Research Foundation.

Isolation of native rat SP-B*N
Bronchoalveolar lavage fluid (BALF) was collected from rats as previously described (15). BALF was centrifuged at 5000 × g for 30 min, and the supernatant from 10 rats was pooled. The concentrated sample was dialyzed against PBS in the dark.

Analyses of native SP-B*N activity
For phagocytosis assays, RAW264.7 macrophages were maintained as described by Vunta et al. (17). Cells were seeded at 1 × 10⁵/ml (3 ml total volume) on cover slips in 6-well plates and incubated with heat-killed S. aureus. All bacteria were fluorescently labeled with Alexa Fluor 594 (multiplicity of infection = 50), with or without rSP-B*N or heat-denatured SP-B*N (rSP-B*N was heated at 100°C for 1 h in 1% β-mercaptoethanol). Noninternalized particles were removed by extensive washing with PBS; cells were fixed in PBS containing 4% paraformaldehyde at 25°C for 15 min and evaluated by fluorescence microscopy, as described by Shibata et al. (18).

To assess the bacteriostatic property of rSP-B*N, 10⁵ CFU of S. aureus was incubated with rSP-B*N antibiotic-free RPMI 1640/FBS in a volume of 1 ml. After washing with antibiotic-free RPMI 1640, cells were infected with 2 × 10⁵ S. aureus or K. pneumoniae. Infected monolayers were washed with warm PBS twice and incubated with RPMI 1640 containing 100 μg/ml carbenicillin or gentamicin to kill extracellular S. aureus and K. pneumoniae, respectively. Intracellular bacteria were released after 1, 2, 4, 6, and 24 h postinfection by lysing cells in 1% Triton X-100; the number of live bacteria at each time point was assessed by quantitative culture. To assess the bacteriostatic property of rSP-B*N, 10⁵ CFU of K. pneumoniae or S. aureus was resuspended in sterile 100 μl PBS, pH 7.0, or 2 mM sodium acetate buffer, pH 5.6. Serial dilutions of rSP-B*N were added to individual wells in triplicate and incubated for 3 h at 37°C with rocking. Direct bactericidal activity of rSP-B*N was assessed using fluorescent probes (luciferase and propidium iodide [Molecular Probes]) (Concentrations of 6 and 30 μM, respectively). Bacteria were subsequently dispersed, and aliquots were plated on blood agar for quantitative culture. Viable pathogen counts after SP-B*N treatment were determined from the number of colonies obtained on the control plates (0 μM SP-B*N) compared with the number of colonies from SP-B*N-treated samples.

Analyses of native SP-B*N activity
BACTERIA
Heat-killed Staphylococcus aureus fluorescently labeled with Alexa Fluor 594 were purchased from Molecular Probes (Eugene, OR). Biliuminscent Pseudomonas aeruginosa Xen 5 were purchased from Caliper Life Sciences (Hopkinton, MA). K. pneumoniae strain K2 (from Dr. Korfhagen, Cincinnati Children’s Hospital) and a clinical isolate of S. aureus were also used in the current study. To minimize variability in virulence, all bacteria were selected from aliquots of the same passage stored at −70°C in 20% glycerol/PBS. For each experiment, an aliquot of bacteria was thawed and plated on tryptic soy/5% sheep blood agar. A single colony was inoculated into 4 ml Luria-Bertani medium (P. aeruginosa and K. pneumoniae) or brain-heart infusion medium (S. aureus) grown to late log phase. Bacteria were pelleted from the medium by centrifugation at 500 × g for 10 min, washed in sterile PBS, and resuspended in 4 ml sterile PBS. For each experiment, the concentration of bacteria was determined by quantitative culture on sheep blood agar plates.

Identification of endogenous mouse SP-B*N
Mice were lavaged, and the supernatant, cell pellet, and surfactant pellet were analyzed by 10–20% tricine–SDS-PAGE followed by Western blotting. SP-B*N Ab was used at a dilution of 1:5000, and immunoreactive protein was visualized by ECL (Pierce) followed by exposure to Kodak X-Omat AR film.

For immunohistostaining analyses, lungs from 5-wk-old WT mice were inflation-fixed, and immunohistochemistry was performed as previously described (16). Immunostaining for SP-B*N was performed at an Ab dilution of 1:2500. Parallel lung sections were incubated with preimmune guinea pig serum to verify the specificity of immunostaining.

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Heat-killed Staphylococcus aureus fluorescently labeled with Alexa Fluor 594 were purchased from Molecular Probes (Eugene, OR). Biofilms Pseudomonas aeruginosa Xen 5 were purchased from Caliper Life Sciences (Hopkinton, MA). K. pneumoniae strain K2 (from Dr. Korfhagen, Cincinnati Children’s Hospital) and a clinical isolate of S. aureus were also used in the current study. To minimize variability in virulence, all bacteria were selected from aliquots of the same passage stored at −70°C in 20% glycerol/PBS. For each experiment, an aliquot of bacteria was thawed and plated on tryptic soy/5% sheep blood agar. A single colony was inoculated into 4 ml Luria-Bertani medium (P. aeruginosa and K. pneumoniae) or brain-heart infusion medium (S. aureus) grown to late log phase. Bacteria were pelleted from the medium by centrifugation at 500 × g for 10 min, washed in sterile PBS, and resuspended in 4 ml sterile PBS. For each experiment, the concentration of bacteria was determined by quantitative culture on sheep blood agar plates.
bacteria was assessed by measuring luminescence. Results are expressed as the percentage of viable bacteria = 100 × (relative light units from control wells [without treatment] − relative light units from experimental wells)/relative light units from control wells.

We previously described the generation of transgenic mice that express truncated human SP-B (SP-BN) (19). hSP-BΔN is SP-BN (transgenic mice) overexpresses the N-terminal propiece and mature peptide of SP-B (Fig. 5A). Transgenic and WT mice were intranasally inoculated with 3.7 \times 10^7 CFU P. aeruginosa Xen 5 in 50 μL PBS, as previously described (20). In vivo bioluminescent images were acquired under anesthesia with the IVIS system (Caliper Life Sciences) at 1, 8, and 24 h following infection. Images were acquired with a 20-cm field of view and an exposure time of 20 s (data not shown). The experiments were repeated three times, and the data were pooled. Transgenic and WT mice were also challenged with S. aureus and analyzed at 24 or 48 h postinfection. Lungs were weighed and homogenized, and equal amounts of protein were plated for quantitative culture. The numbers of colonies were expressed as CFU/g of lung tissue. Studies were conducted three times (n = 9–10 mice/group), and the results were pooled.

To assess survival, transgenic and WT mice (n = 16 mice/group) were intranasally inoculated with 3.7 \times 10^7 CFU P. aeruginosa Xen 5 or 5 \times 10^6 CFU S. aureus suspended in 50 μL PBS. Water and food were provided ad libitum during the period of observation. The number of surviving mice was documented every 12 h for up to 120 h, at which time surviving mice were sacrificed.

**Statistical analysis**

All data are expressed as mean ± SEM. Difference between groups was analyzed by one-way ANOVA, and post hoc testing for pairwise group differences was conducted using the Student–Newman–Keuls test. Non-parametric survival distribution was estimated to examine the differences in survival among groups and was subsequently analyzed using Kaplan–Meier curve statistics. The differences between the groups of mice were assessed using the log-rank test.

**Results**

**Identification and localization of SP-BN in mouse lung**

Based on alignment of SP-B with other members of the SAPLIP family of proteins (data not shown), the region encoding the saposin-like fold that is stabilized by three disulfide bridges.

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**SP-BN Ab detected a protein,**

90% pure (Supplemental Fig. 1). Circular dichroism analyses indicated that rSP-BN contained mainly an saposin-like fold of SP-BN. rSP-BN was digested with trypsin and identified alanine at position 62 as the N-terminal residue (Fig. 1D, upper lane). The increased electrophoretic mobility of endogenous mouse SP-BN relative to recombinant peptide suggested that the saposin-like domain was smaller than predicted (Fig. 1A). To establish the N terminus of endogenous SP-BN, rat BALF supernatant was concentrated and subjected to preparative iso-electric focusing, followed by analytical size-exclusion HPLC and SDS-PAGE. Edman degradation identified alanine at position 62 as the N-terminal residue (Fig. 1C, first amino acid in the lower line). Trypsin digestion and MS/MS analyses of purified rat SP-BN yielded seven peptides (covering 78% of SP-BN); all mapped within the predicted saposin-like domain (Fig. 1C, lower line). Taking into account the larger size of rSP-BN (which includes an N-terminal histidine and six C-terminal histidines not present in the endogenous peptide), the strict conservation of the six cysteine residues in SAPLIP family members, the identification of Ala62 as the N-terminal residue, and an estimated mass of 8 kDa, it is likely that Cys142 represents the C terminus of endogenous mouse SP-BN (Fig. 1C, upper line).

**Immunohistochemical analyses of lung sections with SP-BN Ab detected staining in nonciliated bronchiolar epithelial cells (Clara cells) and alveolar type II epithelial cells, which could represent proSP-B and/or SP-BN (Fig. 1D, upper panels). Strong immunoreactivity was detected in macrophages in uninfected lungs and 24 h following challenge with S. aureus (Fig. 1D, right panels). SP-BN, M_r = 8 kDa, was the only form of SP-B detected in cells and BALF supernatant isolated from the airspaces of uninfected and infected mice (Fig. 1E).**

**rSP-BN enhances uptake and killing of bacteria in vitro**

Localization of endogenous SP-BN to alveolar macrophages (Fig. 1D) suggested that the peptide might facilitate uptake and/or intracellular killing of bacteria by alveolar macrophages. This hypothesis was initially tested in vitro by incubating heat-killed fluorescent-labeled S. aureus with rSP-BN prior to incubation with primary mouse lung macrophages or the mouse macrophage cell line RAW 264.7. Phagocytosis after 1 h was not increased compared with untreated bacteria, suggesting that SP-BN did not directly promote the uptake of bacteria (data not shown). In contrast, preincubation of macrophages (data not shown) or RAW264.7 cells (Fig. 2A) with rSP-BN for 1 h prior to the addition of bacteria resulted in a dramatic increase in intracellular S. aureus; similar results were observed in MHS cells, a mouse alveolar macrophage cell line (data not shown). Heat-inactivation of rSP-BN blocked the increase in uptake of S. aureus (data not shown). FITC-labeled rSP-BN colocalized with internalized, heat-killed S. aureus (Fig. 2B) and with the lysosomal marker Lamp-1 (data not shown). Colocalization of SP-BN with internalized bacteria suggested that SP-BN might play a role in the intracellular killing of pathogens. To test this hypothesis, RAW264.7 cells were incubated with rSP-BN for 1 h prior to incubation with S. aureus or K. pneumoniae for 1–24 h. Quantitative culture of cell lysates 24 h later indicated that bacterial killing was significantly increased (Fig. 2C). Taken together, these data suggest that SP-BN indirectly promotes the uptake of bacteria and facilitates intracellular killing.

**rSP-BN directly kills bacteria at low pH**

To determine whether SP-BN directly killed bacteria, S. aureus was incubated with rSP-BN for 90 min and stained with Syto9 (stains living bacteria green) and propidium iodide (stains dead/dying
bacteria red). Fluorescence microscopy detected a dose-dependent increase in propidium iodide staining in SP-BN–treated samples at pH 5.6 but not at pH 7.4 (Fig. 3A), consistent with killing in a lysosomal compartment. Subsequently, clinical isolates of *K. pneumoniae* or *S. aureus* (10^3 CFU) were incubated with rSP-BN for 3 h at pH 5.6 (Fig. 3B) or pH 7.4 (data not shown). rSP-BN exhibited antimicrobial activity against Gram-positive and -negative organisms at pH 5.6 but not at pH 7.4. At 0.5 μM SP-BN, growth inhibition of *K. pneumoniae* and *S. aureus* was >50%. *K. pneumoniae* or *S. aureus* (10^3 CFU) was also incubated with purified native rat SP-BN (native refers to endogenous SP-BN isolated from rat BALF) for 3 h at pH 5.6 (Fig. 3C) or pH 7.4 (data not shown). Native SP-BN inhibited the growth of both bacteria, but it was more effective against *K. pneumoniae* (>50% inhibition at 0.1 μM native SP-BN).
FIGURE 2. SP-B\textsuperscript{N} is internalized by RAW264.7 cells, resulting in increased uptake of bacteria and killing activity. A, RAW264.7 cells were preincubated with (treated; upper panel) or without (untreated; lower panel) 18 \mu g/ml of rSP-B\textsuperscript{N} for 1 h. Cells were washed twice with warm PBS buffer to remove SP-B\textsuperscript{N}, and heat-killed, Alexa fluorescent-labeled S. aureus were added to the media for 1 h. DAPI (nuclear stain) was added to the mounting reagent. Bacterial uptake was assessed by fluorescence microscopy (original magnification \times 63). B, RAW264.7 cells were preincubated for 1 h with 36 \mu g/ml of FITC-labeled SP-B\textsuperscript{N} followed by incubation with heat-killed S. aureus (Alexa Fluor 594) for 1 h, as described in A (original magnification \times 63). Subcellular localization of SP-B\textsuperscript{N} and bacteria was assessed by fluorescence microscopy. C, RAW264.7 cells were preincubated with rSP-B\textsuperscript{N} for 1 h. Cells were washed twice with PBS, and S. aureus (1.5 \times 10^6 CFU) or K. pneumoniae (1 \times 10^6 CFU) was added to the media. After an additional hour, the media was replaced, and 100 \mu g/ml carbenicillin or gentamicin was added to kill extracellular S. aureus or K. pneumoniae. Cells were lysed with 1% Triton X-100 at the indicated time points postinfection, and the number of viable bacteria was assessed by quantitative culture of cell lysates. Data represent mean \pm SEM of three independent experiments. *p = 0.007 versus untreated RAW264.7 cells at 24 h postinfection.
FIGURE 3. SP-B-N directly kills *S. aureus* or *K. pneumoniae* at acidic pH. A, *S. aureus* (OD₆₀₀ = 0.1) was incubated without (a) or with 5 μg (c) or 10 μg (b and d) rSP-B-N for 90 min at 37°C. Bacteria were suspended in buffer, pH 7.4 (b) or pH 5.6 (a, c, and d) and stained with the vital dye Syto9 (stains living bacteria green) and propidium iodide (stains dead/dying bacteria red), followed by fluorescence microscopy to assess viability (original magnification ×40). B, Increasing amounts of rSP-B-N were added to 10⁷ CFU of *S. aureus* or *K. pneumoniae* suspended in 100 μl of 2 mM sodium acetate buffer, pH 5.6, and incubated for 3 h at 37°C. The number of viable bacteria was assessed by quantitative culture and results expressed as the mean ± SEM of three independent experiments; *p* < 0.05 versus untreated (0 μM) group. C, Purified native rat SP-B-N was added to 10⁷ CFU of *S. aureus* or *K. pneumoniae* suspended in 100 μl of 2 mM sodium acetate buffer, pH 5.6, and incubated for 3 h at 37°C. The number of viable bacteria was assessed by quantitative culture and results expressed as the mean ± SEM of three independent experiments. *p* < 0.05 versus untreated (0 μM) group. D, 10 μg rSP-B-N was added to mouse BALF at pH 7.4 (upper panel) or pH 5.6 (lower panel), incubated at RT for 2 h, and then centrifuged to separate surfactant lipids and supernatant. Supernatants and lipid pellets were analyzed by SDS-PAGE/Western blotting with SP-B-N Ab. E, 10⁷ CFU of *S. aureus* was suspended in buffer, pH 7.4 or 5.6, incubated with 10 μg rSP-B-N for 2 h at RT, centrifuged to separate the bacterial pellet and supernatant, and analyzed as in D. One-tenth microgram rSP-B-N was loaded as a positive control (C).
was significantly decreased in transgenic mice at 8 and 24 h postinfection (Fig. 5B), consistent with a protective effect of SP-BN. Survival studies indicated that all WT mice died by 48 h postinfection, whereas only 20% of transgenic mice died (Fig. 5C). Fifty percent of transgenic mice survived at 120 h, the longest time point analyzed. Collectively, these data support the hypothesis that SP-BN confers resistance to infection by *P. aeruginosa*.

Increased expression of SP-BN in transgenic mice enhances bacterial clearance and survival following infection with *S. aureus*

To determine whether SP-BN also protected against a Gram-positive bacterium, WT and transgenic mice were intranasally inoculated with *S. aureus*, and the bacterial burden was assessed after 8, 24, and 48 h (Fig. 6A). Overexpression of SP-BN modestly, but significantly, enhanced bacterial killing at 8 and 24 h postinfection, consistent with an early protective effect of SP-BN. Intranasal inoculation with a larger dose of *S. aureus* (5 × 10⁸ CFU) was also protective, consistent with other reports (28, 29).

**Figure 4.** Native SP-BN in BALF inhibits growth of *P. aeruginosa*. A, BALF supernatant (containing 8–32 μg protein) was added to 10⁷ CFU of bioluminescent *P. aeruginosa* Xen 5 suspended in 100 μl of 2 mM sodium acetate buffer, pH 5.6, and incubated overnight at 37°C. The number of viable bacteria was assessed by measuring luminescence. Results are expressed as mean ± SEM of three independent experiments. *p < 0.05 versus buffer (0 μg BALF); †p < 0.004 versus 32 μg BALF; ‡p < 0.0001 versus 32 μg BALF. C, One × 10⁷ CFU of bioluminescent *P. aeruginosa* Xen 5 in 100 μl of 2 mM sodium acetate buffer, pH 5.6, was incubated with IgG (0–20 μg) directed against SP-BN and/or 32 μg of BALF supernatant. The number of viable bacteria was assessed by measuring luminescence. Results are expressed as mean ± SEM of three independent experiments. *p < 0.05 versus buffer (0 μg BALF); †p < 0.004 versus 32 μg BALF; ‡p < 0.0001 versus 32 μg BALF; ‡p < 0.02 versus 32 μg BALF + 20 μg IgG.

**Figure 5.** Increased expression of SP-BN in transgenic mice enhances bacterial clearance and survival following infection with *P. aeruginosa*. A, The concentration of SP-BN in the airspaces was assessed in three groups of mice: WT mice (*lane 1*), transgenic mice expressing truncated human SP-B proprotein (SP-BΔC) in the mouse SP-B+/− background (hSP-BΔC/mSP-B+/−, *lane 2*), and transgenic mice expressing SP-BΔC in the WT background (hSP-BΔC/mSP-B+/+, *lane 3*). Twenty micrograms of BALF supernatant was fractionated by SDS-PAGE under nonreducing (*left panel*) or reducing conditions (*right panel*), blotted onto nitrocellulose, and incubated with Ab that detects human and mouse SP-B (23) (*left panel*) or mouse-specific SP-BN Ab (*right panel*). B, Transgenic (hSP-BΔC/mSP-B+/+) and WT mice were intranasally inoculated with 3.7 × 10⁷ CFU of bioluminescent *P. aeruginosa* Xen 5. In vivo bioluminescence was assessed under anesthesia at 1, 8, and 24 h postinfection and quantitative analysis of bioluminescence of mice. Values are means ± SEM. The experiments were repeated three times, and data were pooled (*n* = 12–15 mice/group per time point). *p < 0.01, transgenic versus WT mice. C, Survival after intranasal instillation of *P. aeruginosa* Xen 5. Transgenic or WT mice (*n* = 16 for each genotype) were infected by intranasal instillation of 3.7 × 10⁷ CFU of *P. aeruginosa* Xen 5. The number of surviving mice was documented every 12 h until 120 h postinfection.

**Figure 6.** Increased expression of SP-BN in transgenic mice enhances bacterial clearance and survival following infection with *S. aureus*. A, The concentration of SP-BN in the airspaces was assessed in three groups of mice: WT mice (*lane 1*), transgenic mice expressing truncated human SP-B proprotein (SP-BΔC) in the mouse SP-B+/− background (hSP-BΔC/mSP-B+/−, *lane 2*), and transgenic mice expressing SP-BΔC in the WT background (hSP-BΔC/mSP-B+/+, *lane 3*). Twenty micrograms of BALF supernatant was fractionated by SDS-PAGE under nonreducing (*left panel*) or reducing conditions (*right panel*), blotted onto nitrocellulose, and incubated with Ab that detects human and mouse SP-B (23) (*left panel*) or mouse-specific SP-BN Ab (*right panel*). B, Transgenic (hSP-BΔC/mSP-B+/+) and WT mice were intranasally inoculated with 3.7 × 10⁷ CFU of bioluminescent *P. aeruginosa* Xen 5. In vivo bioluminescence was assessed under anesthesia at 1, 8, and 24 h postinfection and quantitative analysis of bioluminescence of mice. Values are means ± SEM. The experiments were repeated three times, and data were pooled (*n* = 12–15 mice/group per time point). *p < 0.01, transgenic versus WT mice. C, Survival after intranasal instillation of *P. aeruginosa* Xen 5. Transgenic or WT mice (*n* = 16 for each genotype) were infected by intranasal instillation of 3.7 × 10⁷ CFU of *P. aeruginosa* Xen 5. The number of surviving mice was documented every 12 h until 120 h postinfection.
 leads to an ~3-fold increase in SP-B\textsuperscript{N} and SP-B mature peptide in the airspaces. Although it is possible that the mature peptide contributes to the antimicrobial effect of SP-B in transgenic mice, we believe that this is unlikely. Purified, nonlipid-associated mature SP-B is a potent membrane-lytic (5–7) and antimicrobial (25, 26) peptide; however, mature SP-B nonselectively disrupted prokaryotic and eukaryotic membranes and, importantly, this activity and its antimicrobial activity was completely blocked in the presence of surfactant lipids (26). Mature SP-B is always lipid associated, whereas SP-B\textsuperscript{N} does not associate with surfactant lipids in the airspaces; further, SP-B\textsuperscript{N} localizes to lysosomes in phagocytic cells and directly kills bacteria at acidic pH. Taken together, these data suggest that SP-B\textsuperscript{N} promotes macrophage-mediated killing of bacteria in the alveolar airspaces.

We previously reported that mice expressing the SP-B\textsuperscript{NC} transgene were not protected following infection with a lower dose (1 × 10\textsuperscript{3}) of P. aeruginosa (20). In the current study, a significant protective effect and survival benefit were associated with expression of the transgene following infection with a higher dose (3.7 × 10\textsuperscript{3}) of P. aeruginosa. Bacterial clearance and survival of transgenic mice were also modestly, but significantly, increased following infection with S. aureus. These results are similar to a recent study in which constitutive overexpression of cathelicidin in transgenic mice conferred protection against bacterial skin infection (27). Taken together, these findings suggest that exogenous administration of SP-B\textsuperscript{N} may be of therapeutic benefit.

Unlike the vast majority of antimicrobial peptides that are cationic (28), SP-B\textsuperscript{N} has a net negative charge: endogenous mouse SP-B\textsuperscript{N} has a net negative charge of ~2, and human SP-B\textsuperscript{N} has a negative charge of ~6 at neutral pH. The antimicrobial database (29) lists 1034 antibacterial peptides, of which only 68 are anionic. The anionic character of SP-B\textsuperscript{N} may confer a significant advantage over cationic antimicrobial peptides in the alveolar environment. Pulmonary surfactant is rich in anionic phospholipids, principally phosphatidylglycerol, which can serve as a sink for cationic peptides; for example, the bactericidal activity of two cathelicidins was inhibited by surfactant phospholipids (30). In contrast, SP-B\textsuperscript{N} does not bind surfactant lipids at neutral pH and, thus, escapes sequestration in the airspaces. The association of SP-B\textsuperscript{N} with bacterial membranes and its bactericidal activity is dramatically increased only in an acidic environment.

SP-B\textsuperscript{N} shares some features with dermacidin, an anionic, antimicrobial peptide secreted into sweat (31). In addition to a net negative charge (~1 for dermacidin), both peptides are constitutively expressed. However, with respect to structure and function, SP-B\textsuperscript{N} is most similar to the cytolytic peptides of Entamoeba histolytica (14). Amoebas ingest bacteria and kill them in acidic phagolysosomes via the action of the cytolytic peptides, amoebapore A, B, and C. Amoebapore B and C exhibit optimal antibacterial activity in the acidic range and very little activity above pH 6.0 (32, 33), unlike dermacidin, which is active over a broad pH range (31). Mouse SP-B\textsuperscript{N} is 24% identical to amoebapore B and 21% identical to amoebapore C; amoebapore B and C share 35% identity. Importantly, SP-B\textsuperscript{N} and the amoebapore peptides belong to the SAPLIP family of proteins and are predicted to have very similar secondary structures. The three-dimensional structure of amoebapore A was solved by nuclear magnetic resonance, which showed that it contains five helices connected by three disulfide bonds (34). Mouse SP-B\textsuperscript{N} is predicted to contain four amphipathic helices (residues L6-K22, A25-I40, V47-S67, and P70-V77) and three disulfide bridges arising from six invariant cysteine residues. This so-called “saposin-fold” confers resistance to denaturation and proteolysis, likely promoting protein stability/antimicrobial activity in an acidic environment (11, 14, 35).
Although the findings of this study support the hypothesis that SP-BN plays an important role in innate defense of the airspaces, a number of questions remain unanswered. First, the mechanism by which SP-BN kills bacteria is not known. Amoebopores form a stable transmembrane pore that disrupts the cytoplasmic membrane, ultimately leading to cell death (32). Given the striking similarities between SP-BN and amoebopores, it is possible that SP-BN also depolarizes membranes via formation of pores. Second, the range of SP-BN cytolytic activity is unclear. Mature SP-B peptide (SP-B; Fig. 1A) displays potent cytolytic activity toward eukaryotic cells that is completely inhibited in the presence of surfactant phospholipids (26). It is possible that SP-BN may also be cytolytic for eukaryotic cells but that this activity is only unmasked in an acidic environment, thus protecting alveolar cells. Third, the spectrum of SP-BN microbial activity remains to be defined: only representative Gram-positive and -negative organisms were tested in this study. Whether the protective effect of SP-BN extends broadly to other bacteria remains to be determined. Fourth, the mechanism by which SP-BN promotes phagocytosis of bacteria by macrophages in culture and the relevance of this activity to clearance of pathogens from the airspaces are still unclear. Finally, the SP-B proprotein contains another saposin-like domain (SP-BC, Fig. 1A) that has yet to be assessed for potential antimicrobial properties. Overall, based on the results of in vitro and in vivo studies, we conclude that SP-BN contributes to innate defense of the lung by supplementing the nonoxidant antimicrobial defenses of alveolar macrophages.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Data

Figure S1. Recombinant SP-B$^N$ on 10-20% Tricine-SDS PAGE gel. 5 μg of purified recombinant SP-B$^N$ were analyzed by Tricine-SDS PAGE on a 10-20% gradient gel under nonreducing condition and stained with Imperial blue. Lane 1, SeeBlue Plus 2 (Invitrogen) pre-stained standard; Lane 2, unreduced SP-B$^N$
Table 1. The α-helical content in SP-B\textsuperscript{N} at pH 7.4 or 5.6 with or without lysoPC at θ\textsubscript{222}. The α-helical content was calculated with the following equation: (θ\textsubscript{222}-3000/-39000) x 100 (Barrow CJ, Yasuda A, Kenny PT, Zagorski MG (1992) J Mol Biol. 225:1075-1093. Solution conformations and aggregational properties of synthetic amyloid beta-peptides of Alzheimer's disease. Analysis of circular dichroism spectra). The results agree with a predicted saposin-like fold of SP-B\textsuperscript{N}, considering that the recombinant protein used in this analysis contained a His-tag.

<table>
<thead>
<tr>
<th>Condition</th>
<th>θ\textsubscript{222}</th>
</tr>
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<tbody>
<tr>
<td>SP-B\textsuperscript{N}, pH 7.4</td>
<td>63 %</td>
</tr>
<tr>
<td>SP-B\textsuperscript{N}, pH 5.6</td>
<td>58 %</td>
</tr>
<tr>
<td>SP-B\textsuperscript{N} + lysoPC, pH 7.4</td>
<td>55 %</td>
</tr>
<tr>
<td>SP-B\textsuperscript{N} + lysoPC, pH 5.6</td>
<td>53 %</td>
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</table>
Table 2. Peptides verified by ESI-MS/MS from tryptic digest of SP-B\textsuperscript{N}. Recombinant mouse SP-B\textsuperscript{N} was digested with trypsin and reduced and non-reduced peptides analyzed by MALDI-MS.

<table>
<thead>
<tr>
<th>Peptide Nr.</th>
<th>MS/MS Sequencing Result</th>
<th>Monoisotopic Mass</th>
<th>Observed Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unreduced</td>
<td>Reduced+CAM</td>
</tr>
<tr>
<td>1</td>
<td>MHAGANDLCQECEDIVHLLTK</td>
<td>2340.669</td>
<td>2451.669</td>
</tr>
<tr>
<td>2</td>
<td>MTKEDAFQEAIR</td>
<td>1437.6923</td>
<td>1438.6761*</td>
</tr>
<tr>
<td>3</td>
<td>EDAFQEAIR</td>
<td>1077.493</td>
<td>1077.493</td>
</tr>
<tr>
<td>4</td>
<td>EDAFQEAIRK</td>
<td>1205.604</td>
<td>1205.604</td>
</tr>
<tr>
<td>5</td>
<td>KFLEQECIDLPLK</td>
<td>1574.86</td>
<td>1631.895</td>
</tr>
<tr>
<td>6</td>
<td>FLEQECDILPLK</td>
<td>1446.7429</td>
<td>1503.7644</td>
</tr>
<tr>
<td>7</td>
<td>FLEQECDILPLKLLVPR</td>
<td>2025.155</td>
<td>2082.1548</td>
</tr>
<tr>
<td>8</td>
<td>CRQVLDVYLPLVIDYFQSQINPK</td>
<td>2750.468</td>
<td>2807.468</td>
</tr>
<tr>
<td>9</td>
<td>CR **</td>
<td>277.3413</td>
<td>334.3413</td>
</tr>
<tr>
<td>10</td>
<td>QVLDVYLPLVIDYFQSQINPK</td>
<td>2491.3362</td>
<td>2491.3362</td>
</tr>
<tr>
<td>11</td>
<td>AICNHVGLCP</td>
<td>1182.472</td>
<td>1296.4721</td>
</tr>
</tbody>
</table>

Disulfide-linked peptides

<table>
<thead>
<tr>
<th>Monoisotopic Mass</th>
<th>Observed Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHAGANDLCQECEDI/VHLLTK - AICNHVGLCP</td>
<td>3519.141</td>
</tr>
<tr>
<td>FLEQECDILPLK - CR</td>
<td>1722.0842</td>
</tr>
</tbody>
</table>

* Deamination of Q
** Sequence could not be verified by MS/MS
Figure S2. CD spectra of SP-B<sup>N</sup>. A. The structure is mostly α-helical and similar at pH 7.4 and 5.6. B. CD spectra of SP-B<sup>N</sup> in lysoPC at pH 7.4 or 5.6 was mostly α-helical and essentially unaffected by change in pH. The residual molar ellipticity (θ) is expressed in kdeg x cm<sup>2</sup> x dmol<sup>-1</sup>.
Figure S3A. MALDI-MS spectrum of reduced SP-B tryptic digest. To identify potential disulfide-links between tryptic fragments of SP-B\textsuperscript{N}, digested SP-B\textsuperscript{N} was analyzed by MALDI-MS under reducing. It was found that reduction leads to detection of tryptic fragments with not more than one missed cleavage that covers the entire SP-B\textsuperscript{N} sequence (Table 2).
Figure S3B. MALDI-MS spectrum of unreduced SP-B tryptic digest. Under non-reducing conditions, peaks corresponding to the combined masses of peptides 1 and 11, and of the peptides 6 and 9 were observed. With the exception of AICNHVGLCPR (peptide 11), none of these peptides are detectable as free peptides under non-reducing conditions while all can be detected after reduction (Table 2). This suggests the existence of disulfide bonds between peptides 1 and 11, and peptides 6 and 9.

The observed mass for peptide 11 in MALDI spectra of unreduced SP-B digest is 2 Da lower than the expected theoretical mass. A possible explanation is the formation of an intramolecular disulfide bridge between the two Cys. Comparison of the relative abundances of the peptide ions in Figure S3A and S3B reveals that only a minor amount of peptide 11 is affected. This is further supported by the observation that no free peptide 11 could be detected during ESI-MS analysis of the unreduced SP-B digest.
Figure S4. Verification of disulfide-linked peptides by LC-MS/MS. A. Digested peptides were separated by reverse-phase chromatography and subjected to MS
sequencing. In the unreduced sample, a peak was observed corresponding to the 3519.1 Da ion with 5 charges ($m/z$ 704.8). MS/MS analysis confirmed the presence of a series of y-ions with the sequence PR, corresponding to the last 2 amino acids of peptide 1. The MS/MS data of the same peak were also found to contain a series of y-ions with the sequence DIVHLLTK (upper panel) and PR (lower panel), revealing the presence of peptides 1 and 11. The indicated molecular mass of 3519.1 Da for the monoisotopic peak suggests the existence of two disulfide bridges between peptides 11 (mass: 1182.47) and peptide 1 (mass: 2340.66), corresponding to the loss of 4 hydrogen atoms. B. Similarly, a peak was observed corresponding to the 1722.1 Da ion with 2 charges ($m/z$ 862.1). MS/MS analysis confirmed the presence of the y-ion R and the y-ion sequence DILPLK, corresponding to the last amino acid of peptide 9 and to the last 6 amino acids of peptide 6. It was concluded that the 1722.1 Da ion represents the disulfide-linked peptides 6 and 9.