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The role of membrane remodeling in surfactant protein B (SP-B) function

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ABSTRACT

Surfactant protein B (SP-B) is a hydrophobic, 79 amino acid peptide that regulates the structure and function of surfactant phospholipid membranes in the airspaces of the lung. Addition of SP-B to liposomes composed of DPPC/PG (7:3) leads to membrane binding, destabilization and fusion ultimately resulting in rearrangement of membrane structure. The hypothesis underlying this work is that the fusogenic and/or lytic properties of the 79 residue mature SP-B peptide are critical for (1) SP-C maturation, (2) organization of surfactant phospholipids in the distal secretory pathway, (3) formation and maintenance of a surface film and (4) maintenance of a sterile gas exchange surface. As a first step in determining the functional importance of SP-B mediated fusion and lysis, the fusogenic and lytic domains of SP-B were mapped. Synthetic peptides were generated to the predicted helices of SP-B and tested for liposome fusion and lysis. The molecular basis of these properties was determined by systematically introducing amino acid substitutions into the SP-B sequence to identify specific residues important for membrane fusion and lysis. Finally, the importance of these properties for SP-B function was assessed by analyzing the affect of altered fusion and lysis on the surface tension reducing properties of surfactant (Chapter II) and determining the importance of the lytic property of SP-B in its ability to kill airway pathogens (Chapter III).
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>SECTION</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td></td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td></td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td></td>
</tr>
<tr>
<td>CHAPTER I</td>
<td></td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>Figure 1.1 Cross-section of an individual alveolus</td>
<td></td>
</tr>
<tr>
<td>Figure 1.2 SP-B structure and surfactant homeostasis</td>
<td></td>
</tr>
<tr>
<td>Figure 1.3 The Saposin-like family of proteins</td>
<td></td>
</tr>
<tr>
<td>Figure 1.4 Model of SP-C processing</td>
<td></td>
</tr>
<tr>
<td>Figure 1.5 Ultrastructure analysis of alveolar type II cells from wildtype and SP-B(^{-}) mice</td>
<td></td>
</tr>
<tr>
<td>CHAPTER II</td>
<td></td>
</tr>
<tr>
<td>Mapping and Analysis of the Lytic and Fusogenic Domains of Surfactant Protein B (SP-B)</td>
<td></td>
</tr>
<tr>
<td>Figure 2.1 Amino acid sequences of SP-B synthetic peptides</td>
<td></td>
</tr>
<tr>
<td>Figure 2.2 Far U.V. CD Spectra of SP-B synthetic peptides</td>
<td></td>
</tr>
<tr>
<td>Figure 2.3 Effect of SP-B synthetic peptides on liposome size</td>
<td></td>
</tr>
<tr>
<td>Figure 2.4 Effect of native SP-B on liposome size</td>
<td></td>
</tr>
<tr>
<td>Figure 2.5 Leakage of DPPC/PG liposome contents in the presence of native SP-B and synthetic peptides</td>
<td></td>
</tr>
<tr>
<td>Figure 2.6 Surface activity of synthetic SP-B peptides</td>
<td></td>
</tr>
<tr>
<td>Figure 2.7 Helical wheel prediction of SP-B (amino acids 1-25) showing the relative location of each residue with respect to the phospholipid bilayer</td>
<td></td>
</tr>
<tr>
<td>Figure 2.8 Fusogenic activity of the SP-B synthetic peptides (amino acids 1-37) containing polar and non-polar substitutions in Helix 1 and 2</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.9  Leakage of DPPC/PG liposome contents in the presence of SP-B synthetic peptides containing polar substitutions in helix 1 .................................................................48
Figure 2.10  Surface activity of synthetic SP-B peptides containing polar and non-polar substitutions..................................................................................................................49
Figure 2.11  Model of interactions of SP-B helix 1 and 2 with phospholipid Membranes..............57

Table 2.1  Emission spectra of SP-B synthetic peptides in the absence or presence of DPPC/PG liposomes..................................................................................................................50
Table 2.2  Summary of the fusogenic, lytic and surface properties of the SP-B synthetic peptides ...............................................................................................................................58

CHAPTER III
Antimicrobial Activity of Native and Synthetic Surfactant Protein B (SP-B) Peptides...............60
Figure 3.1  Effect of SP-B on K. pneumoniae viability..................................................................69
Figure 3.2  SP-B mediated bacterial aggregation and membrane permeabilization ......................71
Figure 3.3  Hemolytic activity of native hSP-B..............................................................................73
Figure 3.4  SP-B synthetic peptides containing helix 1 kill Klebsiella pneumoniae .......................74
Figure 3.5  Dose-dependent and surfactant inhibition of Klebsiella pneumoniae killing by SP-B...............................................................................................................................76
Figure 3.6  Dose-dependent and surfactant inhibition of Staphylococcus aureus killing by SP-B ...............................................................................................................................78
Figure 3.7  Effect of cationic amino acid substitutions on SP-B peptide-mediated killing of Klebsiella pneumoniae.................................................................................................80
Figure 3.8  Effect of synthetic SP-B peptides on bacterial aggregation and membrane permeabilization .................................................................................................................................81
Figure 3.9  Hemolytic activity of SP-B peptides .............................................................................83
Figure 3.10 SP-B helix 1 kills bacteria in the presence of native surfactant.................................84

CHAPTER IV
Discussion/Future Directions ........................................................................................................91
Figure 4.1  Interfacial adsorption of SP-B peptides ................................................................. 94
Figure 4.2  Insertion of SP-B peptides into phospholipid monolayers ................................. 95
Figure 4.3  Effect of SP-B peptides on the compression dynamics of a surface film .......... 95
Figure 4.4  Hydropathy-at-interface profile using the SP-B peptides (residues 1-37) .............. 97
Figure 4.5  Model of two SP-B subunits ................................................................................ 98
Figure 4.6  Western blot analysis of fetal lung homogenates from wildtype and transgenic
positive pups ...................................................................................................................... 100
Figure 4.7  Ultrastructural analysis of alveolar type II cells from wildtype and transgenic
positive pups ...................................................................................................................... 102
Figure 4.8  Airway ultrastructure in wildtype and transgenic positive fetal mice .................. 103
Figure 4.9  Expression of hSP-B^{C248S/R252A} mice in the presence of doxycycline .......... 106
Figure 4.10 SP-B mediated _Klebsiella pneumoniae_ killing ................................................. 110

REFERENCES .................................................................................................................... 115
ABBREVIATIONS

DPPC, dipalmitoylphosphatidylcholine
PG, phosphatidylglycerol
SP-, surfactant protein
MVB, multivesicular body
LB, lamellar body
SAPLIP, saposin-like family of proteins
RDS, respiratory distress syndrome
AMP, antimicrobial peptide
ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid
CD, circular dichroism
DPX, \( N,N'-(p\text{-phenylenedimethylene})\text{bis(pyridinium bromide)} \)
MOPSO, 3-(\( N\)-morpholino)-2-hydroxypropanesulfonic acid
NBD-PE, \( N-(7\text{-nitro-2,1,3-benzoxadiazol-4-yl})\text{phosphatidylethanolamine} \)
POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol
RH-PE, \( N\)-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine
CFU, colony forming unit
hRBC, human red blood cells
BALF, bronchoalveolar lavage fluid
CHAPTER I

INTRODUCTION
Background

The lung is composed of numerous branching tubes that terminate distally in approximately 300 million alveoli providing an extensive gas exchange surface. The extremely thin alveoli-capillary barrier composed of alveolar epithelial cells and red blood cells facilitates rapid exchange of oxygen and carbon dioxide. The two cell types that form the epithelium of the alveolus are called type I cells and type II cells. The type I cell covers 90% of the cell surface and is the cell type across which gas exchange occurs. Type I cells are characterized by their extremely flat shape which minimizes the distance across which gasses diffuse and increases the surface area of the gas exchange surface. Due to high surface tensions generated by a thin liquid lining layer on the surface of the alveolar epithelium, there is a natural tendency for the alveolus to collapse at end expiration. Alveolar structure is preserved by formation of a surface film, commonly referred to as pulmonary surfactant, at the air-liquid interface. The type II cell, which covers the remaining 10% of the surface of the alveolus, synthesizes, stores and secretes pulmonary surfactant into the airspaces (Figure 1.1).

Pulmonary surfactant is composed of 90% phospholipids and 10% protein. The primary lipid component is dipalmitoylphosphatidylcholine (DPPC), a disaturated, zwitterionic phospholipid able to reduce surface tension at an air-liquid interface to near zero. DPPC packs tightly in a monolayer but respreads poorly during the expansion phase and requires an unsaturated charged lipid such as phosphatidylglycerol (PG) to provide the necessary fluidity during inhalation/exhalation cycling. The protein component of surfactant consists of the surfactant proteins (SP-) A, B, C and D. SP-A and SP-D are hydrophilic proteins that have structural homology to members of the calcium dependent carbohydrate binding collectin family. These proteins function in the lung as host defense molecules that opsonize, aggregate and enhance clearance of microorganisms (Mcneely et al., 1993; Kabha et al., 1997; Ofek et al., 2001). SP-B and SP-C are small hydrophobic proteins that facilitate the formation and maintenance of a
surface active film in the airspaces. SP-B deficiency results in lethal respiratory distress indicating that this peptide plays a critical role in surface film dynamics (Clark et al., 1995; Melton et al., 2003).

Figure 1.1. Cross-section of an individual alveolus. Picture represents a type I cell (1) that is aligned closely with the endothelium of the underlying capillary network (2) to allow for gas exchange to occur and a type II cell (3) which synthesizes, stores and secretes pulmonary surfactant (4) into the lining of the airspaces.
Surfactant Homeostasis

Human SP-B is synthesized as a 381 amino acid preproprotein in alveolar type II cells. The mature, 79 residue peptide is flanked by an N-terminal propeptide (200 amino acids) and a C-terminal peptide (102 amino acids). Cleavage of the N- and C-terminal peptides of SP-B occurs in the late endosome/multivesicular body (MVB) by processing enzymes located in the lumen of the MVB (Voorhout et al., 1992; Brasch et al., 2003). Removal of the propeptides most likely involves multiple enzymes and cleavage sites; both napsin and cathepsin H have been implicated in pro-SP-B processing (Ueno et al., 2004). Mature SP-B is stored with surfactant phospholipids as concentric membrane bilayers in specialized secretory granules called lamellar bodies. SP-B is transferred to the lamellar body when a late endosome/MVB fuses with a lamellar body (Stahlman et al., 2000). The phospholipid vesicles within the MVB are incorporated into the concentric membrane sheets of the lamellar body. The contents of the lamellar body are secreted into the airspaces where the membranes unravel to form a square lattice network called tubular myelin (Weibel et al., 1968). This form of surfactant may serve as a reservoir of surfactant phospholipids and proteins for the surface film (Perez-Gil et al., 1998). Surfactant phospholipids are removed from the surface film and are recycled back to the type II cell via the small aggregate form or degraded by the alveolar macrophage (Figure 1.2).

SP-B Structure

Mature SP-B is a cationic peptide that consists of 4-5 amphipathic alpha helices. The peptide is extremely hydrophobic and is always associated with lipids. The presence of 6 conserved cysteine residues places SP-B in the saposin-like family of proteins (SAPLIP) (Patthy, 1991) (Figure 1.3).
Figure 1.2. **SP-B structure and surfactant homeostasis.** SP-B is synthesized as a proprotein consisting of an N-terminal and a C-terminal domain which is proteolytically cleaved in the multivesicular body (MVB). Mature SP-B consists of 4-5 amphipathic alpha helices and forms homodimers through helix 3 (dotted line). The MVB fuses with the LB and the surfactant lipids with associated proteins are stored in the lamellar body (LB) as membrane sheets or concentric lamellae. The LB releases its contents into the airspaces where the lamellae unravel to form tubular myelin. This form of surfactant serves as the extracellular reservoir of surfactant lipids and proteins that will be incorporated into the active surface film. Surfactant phospholipids are recycled within the type II cell or degraded via the alveolar macrophage.
Due to the constraints imposed by the disulfide bridges, these proteins likely have a similar 3D structure or ‘saposin fold’ (Andersson et al., 1995). SP-B contains a seventh cysteine residue at position 48 that is involved in dimerization of two SP-B subunits. The role of SP-B homodimerization in lung function is not clear. Transgenic mice expressing a monomeric form of SP-B (substitution of cysteine 48 for serine) were indistinguishable from wildtype animals indicating that the transgene had no significant effect on lung function (Beck et al., 2000). However, additional studies demonstrated that SP-B isolated from these mice still dimerized through an ionic interaction between glutamic acid 51 of one SP-B subunit and arginine 52 of another subunit (Zaltash et al., 2000). The salt bridge formed by this interaction likely contributed to dimer stability in the transgenic monomer mice.

**Figure 1.3. The Saposin-like family of proteins.** Sequence alignments reveal the presence of 6 conserved cysteine residues in SP-B which places this peptide in the saposin-like family of proteins. Members include saposins A-D, Nk-lysin, granulysin, amoebapore and acid sphingomyelinase.

**SP-B Function**

The importance of SP-B in the airway is underscored by the fact that deficiency of SP-B in both mice and humans results in lethal neonatal respiratory distress syndrome (Clark et al., 1995; Melton et al., 2003). The principle cause of RDS in infants is surfactant insufficiency due to inadequate lung development at birth. Little surfactant is secreted leading to increased surface tension in the airspaces,
alveolar collapse, and ultimately compromised gas exchange. Surfactant replacement therapy has improved the survival of premature infants with RDS by maintaining alveolar structure during lung maturation (Halliday et al., 1984). SP-B deficient infants with mutations in the SP-B gene that result in the absence of the mature peptide in the airway (Nogee et al., 1994; Ballard et al., 1995; Williams et al., 1999; Somaschini et al., 2000) suffer from lethal RDS despite surfactant replacement and respiratory support (Nogee et al., 1993). Further evidence of the importance of SP-B within the type II cell and in the airway was evident in the SP-B−/− mouse model (Clark et al., 1995). Characteristics of SP-B deficiency include (1) lethal respiratory distress syndrome due to alveolar collapse and inability to inflate the lungs, (2) misprocessing of SP-C proprotein resulting in decreased mature SP-C peptide, (3) disorganized lamellar bodies containing vesicles and electron dense inclusions and (4) absence of tubular myelin. Further, acute deficiency of SP-B in the adult mouse lung resulted in lethal respiratory distress syndrome similar to the neonatal SP-B deficiency model (Melton et al., 2003; Nesslein et al., 2005) demonstrating an important role for SP-B in both neonatal and adult lung function.

**SP-B-mediated membrane fusion and lysis**

SP-B has been shown to have a dramatic effect on membrane structure. Addition of SP-B to lipid vesicles composed of naturally occurring phospholipids found in surfactant (DPPC/PG) results in lipid fusion and significant leakage of liposome contents (Oosterlaken-Dijksterhuis et al., 1992; Chang et al., 1998). Electron microscopy of SP-B containing liposomes revealed the presence of large stacked membrane sheets further demonstrating the effect of SP-B on membrane organization (Suzuki et al., 1989; Poulain et al., 1992). A peptide encompassing residues 1-25 of SP-B promoted lipid mixing (fusion) and lysis indicating that these properties may be contained within the N-terminal domain of SP-B (Veldhuizen et al., 2000). The fusogenic and lytic properties of SP-B are likely important for (1) SP-C processing, (2)
organization of phospholipids in lamellar bodies, (3) formation of tubular myelin, (4) formation and/or maintenance of a surface film and (5) alveolar sterility.

The role of SP-B mediated fusion and lysis in SP-C processing

The lytic activity of SP-B may be required for intracellular maturation of SP-C proprotein. Like SP-B, SP-C is synthesized as a proprotein containing N-terminal and C-terminal peptide domains that flank the mature peptide. Generation of the mature form of SP-C is dependent upon the expression of SP-B. Within type II cells, SP-B and SP-C proproteins are processed to their mature forms by proteolytic enzymes located in the late endosome/MVB (Voorhout et al., 1992; Vorbroker et al., 1995b). SP-B resides in the lumen of the MVB whereas the SP-C proprotein is a transmembrane protein that initially resides on the limiting membrane of the MVB (Conkright et al., 2001) (figure 1.4). Inward vesiculation of the limiting membrane results in relocation of SP-C on internal vesicles, with the N-terminal propeptide in the lumen of an internal vesicle and the C terminal peptide in the lumen of the MVB (Figure 1.4). Processing enzymes located in the lumen of the MVB cleave the N- and C-terminal peptides of SP-B (Brasch et al., 2003; Ueno et al., 2004) and the C-terminal peptide of SP-C but cannot access the N-terminus of SP-C. It has previously been proposed that the newly processed hydrophobic mature SP-B peptide associates with internal vesicles and this association leads to membrane lysis ultimately resulting in entry of processing enzymes and completion of SP-C processing (Weaver et al., 2001). In the absence of SP-B, the internal vesicles of the MVB are not lysed and the proteolytic enzymes do not have access to the N-terminal propeptide of SP-C. The misprocessed form of SP-C (N-term SP-C) accumulates in both SP-B deficient mice and humans (Nogee et al., 1994; Clark et al., 1995; Vorbroker et al., 1995a) and has been shown to lack surface properties (Li et al., 2004). This model of SP-C processing suggests that the lytic properties and possibly the fusogenic properties of SP-B are required for maturation of SP-C.
Figure 1.4. Model of SP-C processing. Schematic represents a MVB with the SP-C propeptide (green/blue) residing on the limiting membrane and the SP-B propeptide within the lumen of the MVB (black/red) (A). Inward vesiculation of the membrane carries SP-C into the MVB resulting in SP-C on the membrane of an internal vesicle (B). The N-terminal propeptide of SP-C is located in the lumen of an internal vesicle while the C-terminal peptide is in the lumen of the MVB. Proteolytic enzymes located in the lumen of the MVB cleave both propeptides of SP-B and the C-terminal peptide of SP-C but do not have access to the N-terminal propeptide (C). Mature SP-B dimerizes and associates with the internal vesicles of the MVB leading to membrane fusion (D) and lysis (E) allowing proteolytic enzymes to cleave the N-terminal propeptide of SP-C.

The role of SP-B mediated fusion and lysis in lamellar body structure

SP-B mediated membrane fusion may be important for organization of phospholipids in the distal secretory pathway. Mature SP-B and SP-C peptides are stored with surfactant phospholipids in specialized secretory granules called lamellar bodies. SP-B is transferred to the lamellar body when a late endosome/multivesicular body fuses with a lamellar body (Stahlman et al., 2000) (Figure 1.5). Fusion of these two organelles also results in the transfer of the SP-C containing internal vesicles of the MVB to the lumen of the lamellar body where they are incorporated into the surfactant membranes by a process that
likely involves membrane fusion and perhaps lysis. Loss of SP-B in type II cells results in the appearance of highly disorganized lamellar bodies containing numerous MVB-derived vesicles that have not been incorporated into the surfactant membranes indicating that vesicle fusion within the lamellar body is dependent upon SP-B (Clark et al., 1995; Stahlman et al., 2000) (Figure 1.5).

The role of SP-B mediated fusion and lysis in tubular myelin formation

The fusogenic and lytic properties of SP-B may also be important in restructuring surfactant membranes following secretion. The contents of the lamellar body are secreted into the airspaces where the surfactant membranes unravel and rearrange into a square tubular lattice structure called tubular myelin (Weibel et al., 1968). This form of surfactant may serve as an extracellular surfactant reservoir or intermediate in the transition to the surface film at the air/liquid interface (Perez-Gil et al., 1998). Formation of tubular myelin is both an SP-B and SP-A dependent process. Addition of these peptides to liposomes composed of DPPC/PG (7:3) in vitro resulted in the formation of tubular myelin-like structures (Suzuki et al., 1989; Poulain et al., 1992). SP-B incubated with liposomes in the absence of SP-A resulted in the formation of numerous tightly packed parallel membranes indicating that the appearance of the square lattice network of tubular myelin required SP-A (Suzuki et al., 1989). Further, SP-A gene targeted animals had little tubular myelin in the airspaces (Korfhagen et al., 1998). Tubular myelin was also not detected in human infants with hereditary SP-B deficiency or in SP-B<sup>-/-</sup> mice (deMello et al., 1994; Clark et al., 1995) indicating that the formation of tubular myelin is in part an SP-B dependent process that likely involves lipid membrane fusion and/or lysis.
Figure 1.5. **Ultrastructure analysis of alveolar type II cells from wildtype and SP-B \(-/-\) mice.** Electron micrograph from wildtype type II cells depicts a MVB (circled in red) and a MVB fusing with a lamellar body (circled in blue). Lamellar bodies from wildtype mice are characterized by tightly packed, well organized concentric membrane sheets. The SP-B \(-/-\) type II cell is characterized by highly disorganized LBs that contain vesicles from the MVB that have failed to fuse and reorganize into concentric lamellae.
The role of SP-B mediated fusion and lysis in surface film formation

Both membrane fusion and lysis are likely important in the formation and/or maintenance of a surface active film. Surface film formation involves adsorption of newly secreted phospholipids from an extracellular surfactant pool (consisting of tubular myelin and less organized forms of surfactant) to the air-liquid interface of the alveolus (Figure 1.2). Maintenance of a stable surface film is essential for respiration. Insertion of phospholipids into the expanding film is required during inhalation followed by phospholipid packing and exclusion from the contracting surface film during exhalation. Both SP-B and SP-C facilitate the transfer of phospholipids between the large aggregate surfactant pool and the surface film; however, only SP-B deficiency results in lethal respiratory distress syndrome indicating that this peptide plays a critical but poorly understood role in surface film dynamics (Clark et al., 1995). Recent studies in infants, children, and adults suffering from acute lung injury have shown that instillation of a natural surfactant containing high levels of SP-B (calfactant) was associated with decreased mortality (Willson et al., 2005). Furthermore, a synthetic peptide corresponding to residues 1-25 of SP-B improved lung function in 2 mouse models of surfactant insufficiency (Bruni et al., 1991; Bruni et al., 1998) indicating the domain responsible for surface activity of SP-B may map to the N-terminal half of the peptide.

The role of SP-B mediated lysis in alveolar sterility

The lytic activity of SP-B may also be important in maintaining the sterility of the gas exchange surface. Pulmonary surfactant serves as a physical barrier to inhaled pathogens and may help prevent pathogen colonization. The hydrophilic surfactant proteins, SP-A and SP-D, belong to the family of collectins and are secreted into the airspaces where they serve as the first line of defense against invading microorganisms. Both SP-A and SP-D interact with a variety of pathogens including Gram-positive or
Gram-negative bacteria, viruses and fungi. The interaction of the microorganisms with the collectins leads to agglutination, aggregation and the enhancement of phagocytosis by resident macrophages (Mcneely et al., 1993; Kabha et al., 1997; Ofek et al., 2001). Although both SP-A and SP-D are known to be important mediators in innate immunity, the role of SP-B in alveolar host defense is less clear. A synthetic peptide corresponding to SP-B (residues 1-78) inhibited the growth of *E. coli in vitro* (Kaser et al., 1997); however, bacterial killing of *P. aeruginosa* and group B *streptococcus* was not decreased in the lungs of heterozygous null (SP-B+/−) mice nor was protection conferred by increased expression of SP-B in transgenic mice (Akinbi et al., 1999).

SP-B is a member of the SAPLIP family of proteins, several of which exhibit potent antimicrobial activity (Patthy, 1991) including NK-lysin, granulysin and amoebapore. NK-lysin and granulysin are antimicrobial peptides produced by pig natural killer cells and human cytolytic lymphocytes, respectively. Granulysin lyses a variety of bacteria including *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Stenger et al., 1998) as well as tumor and fungal cells. Increased levels of granulysin in patients diagnosed with leprosy reduce the spread of infection (Ochoa et al., 2001; Stenger, 2001) by increasing membrane permeability (Ernst et al., 2000) of the invading pathogen. Amoebapore is a pore forming peptide from the protozoan parasite and human pathogen *Entamoeba histolytica*. Amoebapore lyses both bacterial and eukaryotic cells by forming pores in target membranes. Antimicrobial peptides (AMPs) are an abundant group of molecules (more than 880 peptides identified to date) (Brogden, 2005) that are produced by many tissues and cell types. They are important mediators of innate host defense and have maintained broad-spectrum antimicrobial activity and resisted most microbial strategies of resistance (Brogden, 2005). These characteristics make AMPs attractive alternative therapeutic agents for microbial infections.
Antimicrobial peptides all kill bacteria by permeabilizing bacterial membranes but the mechanism of membrane permeabilization differs among the peptides. It has been proposed that the charge distribution along the peptide affects the way in which the peptides are oriented on the lipid bilayer and therefore may account for the differences in membrane lysis (Anderson et al., 2003). Positively charged amino acids located on the surface of NK-lysin and granulysin mediate interaction of the peptides with the negatively charged membranes of bacteria resulting in membrane destabilization and/or permeabilization (Ernst et al., 2000; Bruhn et al., 2003). In contrast, amoebapore A lacks a positively charged surface, is much more hydrophobic and permeabilizes bacterial membranes in a pH- and oligomerization-dependent manner (Hecht et al., 2004). SP-B shares features with both types of SAPLIP antimicrobial peptides: it is very hydrophobic and forms oligomers similar to amoebapore but is also cationic (net positive charge of +7) and lysed negatively charged liposomes at neutral pH, similar to NK-lysin and granulysin (Poulain et al., 1992; Ryan et al., 2005). The structural similarity to SAPLIP antimicrobial peptides and its previously reported lytic activity would suggest that SP-B may contribute to bacterial killing at the alveolar surface.

The hypothesis underlying this work is that the fusogenic and/or lytic properties of the 79 residue mature SP-B peptide are critical for (1) SP-C maturation, (2) organization of surfactant phospholipids in the distal secretory pathway, (3) formation and maintenance of a surface film and (4) maintenance of a sterile gas exchange surface. As a first step in determining the functional importance of SP-B mediated fusion and lysis, the fusogenic and lytic domains of SP-B were mapped. Synthetic peptides were generated to the predicted helices of SP-B and tested for liposome fusion and lysis. The molecular basis of these properties was determined by systematically introducing amino acid substitutions into the SP-B sequence to identify specific residues important for membrane fusion and lysis. Finally, the importance of these properties for SP-B function was assessed by analyzing the affect of altered fusion and lysis on
the surface tension reducing properties of surfactant (Chapter II) and determining the importance of the lytic property of SP-B in its ability to kill airway pathogens (Chapter III).
CHAPTER II

Mapping and Analysis of the Lytic and Fusogenic Domains of Surfactant Protein B (SP-B)

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SUMMARY

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

Pulmonary surfactant is a complex mixture of phospholipids and proteins that is synthesized, stored and secreted by alveolar type II cells. The lipid components of surfactant, mainly dipalmitoylphosphatidylcholine (DPPC) and phosphatidylglycerol (PG), reduce surface tension at the air/liquid interface in the lung and prevent alveolar collapse at end expiration. Formation and maintenance of a phospholipid-rich surface film is facilitated by the hydrophobic surfactant proteins SP-B and SP-C. SP-B deficiency results in respiratory failure indicating that this peptide is absolutely required for lung function (Clark et al., 1995; Melton et al., 2003).

Human SP-B is synthesized as a 381 amino acid preproprotein that is processed to the 79 residue mature peptide in the distal secretory pathway of the type II cell. The mature peptide contains 6 cysteine residues that form 3 intramolecular disulfide bridges; a seventh cysteine residue forms an intermolecular disulfide bridge linking two SP-B subunits together to form homodimers. Sequence alignments reveal that the location of the cysteine residues in SP-B is a common feature among a group of proteins referred to as the saposin like family of proteins (SAPLIP) (Patthy, 1991). Members of the SAPLIP family include NK-lysin, granulysin, amoebapore and the sphingolipid activating proteins, saposins A, B, C and D. All SAPLIP proteins interact with lipid membranes; however, SP-B is the only membrane of this family that is always lipid associated.

SP-B interacts with the surface of the lipid bilayer via four or five amphipathic alpha helices (Andersson et al., 1995). Positively charged amino acids, located predominantly in helix 1, facilitate interaction of the mature peptide with the negatively charged head groups of phosphatidylglycerol (Baatz et al., 1990; Vandenbussche et al., 1992). Addition of SP-B to liposomes composed of DPPC/PG (7:3) leads to membrane binding, destabilization (lysis) and fusion ultimately resulting in dramatic rearrangement of membrane structure (Shiffer et al., 1988; Oosterlaken-Dijksterhuis et al., 1992; Poulain
et al., 1992). The fusogenic and lytic properties of SP-B are likely important for the transition of surfactant phospholipid membranes from the intracellular storage form to the functional extracellular surface film.

The lytic property of SP-B may be required for intracellular maturation of the SP-C proprotein. Within type II cells, SP-B and SP-C proproteins are processed to their mature forms by proteolytic enzymes located in the late endosome/multivesicular body (MVB) (Voorhout et al., 1992; Vorbroker et al., 1995b). SP-B resides in the lumen of the MVB whereas the SP-C proprotein is a transmembrane protein that initially resides on the limiting membrane of the MVB (Conkright et al., 2001). Inward vesiculation of the limiting membrane results in relocation of SP-C on internal vesicles, with the N-terminal propeptide in the lumen of the internal vesicle and the C-terminal peptide in the lumen of the MVB. Processing enzymes located in the lumen of the MVB cleave the N- and C-terminal peptides of SP-B (Brasch et al., 2003; Ueno et al., 2004) and the C-terminal peptide of SP-C but cannot access the N-terminus of SP-C. We proposed that the newly processed hydrophobic mature SP-B peptide associates with internal vesicles leading to membrane lysis, entry of processing enzymes and completion of SP-C processing (Weaver et al., 2001). This model of proSP-C processing is supported by the observation that in the absence of SP-B, the propeptide of SP-C is not completely removed (Nogee et al., 1994; Clark et al., 1995; Vorbroker et al., 1995a). Thus the membranolytic property of SP-B may be required for the complete processing of the SP-C proprotein in the MVB.

The fusogenic property of SP-B may be important for organization of surfactant phospholipids in the distal secretory pathway. Mature SP-B and SP-C peptides are stored with surfactant phospholipids as concentric membrane bilayers in specialized secretory granules called lamellar bodies. SP-B is transferred to the lamellar body when a late endosome/multivesicular body fuses with a lamellar body (Stahlman et al., 2000). Fusion of these two organelles also results in the transfer of the SP-C containing
internal vesicles of the MVB to the lumen of the lamellar body where they are incorporated into the surfactant membranes by a process that likely involves membrane fusion and perhaps lysis. Loss of SP-B in type II cells results in the appearance of highly disorganized lamellar bodies containing numerous MVB-derived vesicles that have not been absorbed into the surfactant membranes indicating that vesicle/surfactant membrane fusion is an SP-B dependent process (Clark et al., 1995; Stahlman et al., 2000).

Fusion and lysis may also play a role in restructuring of the surfactant phospholipid membranes in the alveolar spaces. The contents of the lamellar body are secreted into the airspaces where the surfactant membranes unravel and rearrange into a square tubular lattice structure called tubular myelin (Weibel et al., 1968). This form of surfactant may serve as an extracellular surfactant reservoir or intermediate in the transition to the surface film at the air/liquid interface (Perez-Gil et al., 1998). Addition of SP-B and SP-A to liposomes composed of DPPC/PG (7:3) resulted in the formation of tubular myelin-like structures in vitro (Suzuki et al., 1989; Poulain et al., 1992). Tubular myelin was not detected in human infants with hereditary SP-B deficiency or SP-B \(-/-\) mice (deMello et al., 1994; Clark et al., 1995). The formation of tubular myelin is therefore an SP-B dependent process that likely involves lipid membrane fusion and/or lysis.

Membrane fusion and/or lysis likely plays an important role in the formation and maintenance of the alveolar surface film. At birth the initial formation of a surface film involves adsorption of newly secreted phospholipids from an extracellular surfactant pool (consisting of tubular myelin and less organized forms of surfactant, collectively referred to as large aggregate surfactant) to the air-liquid interface of the alveolus. Maintenance of a stable surface film is essential for respiration and requires insertion of phospholipids into the expanding film during inhalation followed by phospholipid packing and exclusion from the contracting surface film during exhalation. Both SP-B and SP-C facilitate the
transfer of phospholipids between the large aggregate surfactant pool and the surface film; however, in mice, only SP-B deficiency results in lethal respiratory distress syndrome indicating that this peptide plays a critical role in surface film dynamics (Clark et al., 1995). It is not known if the fusogenic and lytic properties of SP-B play an important role in the formation and maintenance of the surface film. As a first step toward assessing the functional importance of SP-B mediated fusion and lysis, the fusogenic and lytic domains of human SP-B were mapped and the requirement of these properties for surface activity was assessed.
MATERIALS AND METHODS

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

Peptide Design. Synthetic peptides were designed to the proposed helices and interhelical loops of the mature SP-B peptide (Andersson et al., 1995; Liepinsh et al., 1997)(Fig. 1). Peptides were synthesized by Biosynthesis Inc. (Lewisville, TX) by F-moc chemistry and purified to >95% homogeneity by HPLC. Peptide composition was confirmed by mass spectrometry. Stock solutions (1mg/ml) were prepared in methanol and diluted into assay buffer to achieve the peptide concentrations indicated in the figures. Appropriate solvent controls were used in each experiment.

Preparation of native human surfactant protein B. Human SP-B was isolated from bronchoalveolar lavage fluid of patients with pulmonary alveolar proteinosis, as described by Shen et. al. (Shen et al., 1997). Briefly, surfactant was isolated from bronchoalveolar lavage fluid by centrifugation and dissolved in chloroform/methanol (2:1). The organic phase was recovered, dried, dissolved in chloroform/methanol/0.1 M HCl [1:1:0.1 (v/v)] and loaded onto an LH-60 Sephadex column equilibrated
in the same solvent system. Fractions eluted from the column were screened by SDS-PAGE and silver staining. SP-B-containing fractions were recovered and dialyzed (SnakeSkin dialysis tubing, molecular weight cutoff of 3500, Pierce Chemical Co.) against chloroform/methanol [2:1 (v/v)] overnight at 4 °C to remove HCl, as previously described (Horowitz et al., 1992). The dialysate was dried down and stored at –80 °C.

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

Preparation of phospholipid vesicles. Phospholipids in chloroform were dried under N₂ and resuspended in 50 mM MOPS/140 mM NaCl/0.1 mM EDTA buffer, pH 5.5 or 7.0, to a final concentration of 400 µg/ml. The phospholipid suspension was passed through a mini-extruder (Avanti Lipids, Birmingham, AL) at 45 °C through two stacked 0.1 µm polycarbonate filters. A series of 10 extrusions was performed to generate a population of unilamellar liposomes with diameters of approximately 200 nm.

Fusion assays. The increase in vesicle size associated with protein-promoted vesicle aggregation/fusion was monitored by an N4+ particle size analyzer (Coulter, Miami, FL) as we have previously described (Wang et al., 2003a). Liposomes were added to a 2-ml cuvette (final concentration of 20 ug/ml) and mixed with individual peptides or native human SP-B (1 mg/ml stock solution in methanol) at room
temperature. Light scattering produced as a result of SP-B induced increase in vesicle size (herein referred to as fusion) was acquired at a 90° angle, monitored for 20 minutes and processed using unimodal distribution. Baseline liposome size was recorded prior to the addition of peptide.

Lipid mixing assays. Lipid mixing was measured as previously described (Poulain et al., 1992). Briefly, liposomes (20 µg/ml) were prepared in the presence 0.1 M% of NBD-PE and RH-PE to incorporate the two fluorescent probes into the lipid bilayer. Liposomes containing both fluorescent probes were mixed with unlabeled liposomes (1:9) in MOPSO buffer (50 mM MOPSO, 140 mM NaCl, 0.1 M EDTA), and FRET was monitored in a RatioMaster fluorometer (Photon Technology, South Brunswick, NJ) for 200 seconds as synthetic peptide or native SP-B (2µg/ml) was added. NBD excitation was at 450 nm and emission was set to 520 nm.

Lysis assays. Membrane leakage was measured as previously described (Poulain et al., 1992). Liposomes were prepared in a buffer containing 12.5 mM ANTS and 45 mM DPX. Encapsulated probes were separated from free probes on a Sephadex G-75 column (1.5 cm X 20 cm). Membrane disruption causes the encapsulated ANTS probe to dilute away from the DPX quencher resulting in an increase in fluorescence. The excitation of ANTS was set at 360 nm and its emission was recorded at 520 nm using a RatioMaster fluorometer (Photon Technology, South Brunswick, NJ) for 200 seconds as peptide or native human SP-B was added. Baseline fluorescence was recorded prior to the addition of peptide. Maximum fluorescence was recorded following the addition of 0.2% Triton X-100.

Measurement of Surface Activity. The surface tension reducing properties of individual peptides were assessed by captive bubble analysis (Schurch et al., 1998). Mixtures of synthetic peptide (4%) and
DPPC/POPG (7:3 by weight) were dried and resuspended in saline with brief sonication. The mixture was applied to the air-water interface of a 25 µl air bubble by microsyringe. Surface tension was recorded every 10 sec for 300 sec to establish equilibrium surface tension. The bubble was then pulsated at 10 cycles/min and minimum surface tension recorded at the fifth pulsation when the bubble was reduced to 80% of its original volume.

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

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

Fusogenic activity of SP-B synthetic peptides. Synthetic peptides were used to map the fusogenic and lytic activities of SP-B because there is currently no expression system capable of producing recombinant SP-B. Peptides were synthesized to the predicted helices and interhelical loops of SP-B, based on the 3D structure of NK-lysin (Andersson et al., 1995; Liepinsh et al., 1997) (Fig. 2.1); it is important to note that the exact boundaries of each domain are not known. CD spectra indicate that each peptide has a relatively high helical content in methanol, as does native SP-B as indicated by molar absorptivity at 222 nm (Fig. 2.2). In order to assess the fusogenic properties of the synthetic peptides, DPPC/PG (70:30) liposomes with an average diameter of 200 nm were incubated with increasing concentrations of individual SP-B peptides at room temperature. The increase in vesicle size was monitored by an N4+ sub-µm particle size analyzer, which estimates the sizes of uniform vesicles over the range of 3 to 4000 nm. A peptide encompassing residues 1-37 (N-term helix 1,2) promoted a dose-dependent increase in vesicle size at pH 7.0 (Fig. 2.3) and pH 5.5 (not shown). Helices 3,4,5 also promoted vesicle fusion but to a lesser extent than N-term helix 1,2. Removal of the N-terminal 6 amino acids from N-term helix 1,2 resulted in decreased fusion to values similar to helix 3,4,5; however the 6 amino acid N-term peptide alone was not capable of inducing liposome fusion (not shown). Similarly, helix 2 alone had no fusogenic activity; helix 1 exhibited a small amount of fusion activity at the highest dose of peptide. These results suggest that the entire N-terminal region of SP-B (i.e. residues1-37) is required for liposome fusion.

In order to compare the fusogenic activity of N-term helix 1,2 to native SP-B, DPPC/PG (70:30) liposomes were incubated with increasing concentrations of native human SP-B (Fig. 2.4). Native SP-B promoted a dose-dependent increase in vesicle size at both pH 7.0 and 5.5. Maximum fusion was attained at a concentration of 120 nM native peptide. However, in spite of very similar α-helical structure the N-term helix 1,2 peptide required a 20-fold higher concentration (2.5 µM) to achieve an increase in vesicle
size comparable to native SP-B (Fig. 2.3). The addition of helix 3,4,5 to N-term helix 1,2 did not increase fusogenic activity or decrease the concentration of synthetic peptide needed to achieve maximum fusion (not shown). The fusogenic activity of a full length, synthetic SP-B peptide (residues 1-79) was significantly less than that of N-term helix 1,2 (Fig. 2.3) despite the similar CD spectra of the peptides (Fig. 2.2). These results suggest that while the N-terminal region of SP-B is important for fusogenic activity the tertiary and/or quaternary structure of SP-B is important for maximal activity.

Figure 2.1. **Amino acid sequences of SP-B synthetic peptides.** Bars represent synthetic peptides designed to the proposed helical regions (grey) and interhelical loops (black) of the SP-B mature peptide. The numbers in parentheses represent the corresponding amino acids in human SP-B. A full length synthetic SP-B peptide (residues 1-79) was also synthesized (not shown). It is important to note that the exact boundaries of these domains are not known.
Figure 2.2. **Far U.V. CD Spectra of SP-B synthetic peptides.** CD spectra were generated for native human SP-B (hSP-B), the synthetic full length 79 amino acid protein and the SP-B 1-37 peptide (left panel), and some of the SP-B 1-37 variants (right panel), dissolved in methanol. Spectra for all other peptides listed in figures 1 and 7 but not shown in the figure fell between the spectrum for native SP-B and full length synthetic peptide.
Figure 2.3. **Effect of SP-B synthetic peptides on liposome size.** Liposomes (20 µg/ml) composed of DPPC/PG (7:3 by weight) with an average diameter of 200 nm were suspended in 50 mM MOPS/140 mM NaCl/0.1 mM EDTA buffer, pH 7.0 and incubated with increasing concentrations of individual SP-B synthetic peptides. SP-B mediated increase in vesicle size was determined using an N4 particle size analyzer at room temperature. Results (n=3) are expressed as mean ± SEM; *p< 0.05, for synthetic peptides versus N-term helix 1,2.
Figure 2.4. **Effect of native SP-B on liposome size.** Liposomes (20 µg/ml) composed of DPPC/PG (7:3; wt:wt) with an average diameter of 200 nm were suspended in 50 mM MOPS/140 mM NaCl/0.1 mM EDTA buffer, pH 5.5 or 7.0 and incubated with increasing concentrations of native human SP-B. Increase in vesicle size was determined using an N4 particle size analyzer at room temperature. Results (n=3) are expressed as mean ± SEM. *p<0.05, for liposomes with SP-B versus liposomes alone.

**Lytic activity of SP-B synthetic peptides.** The lytic activity of SP-B was mapped to individual helices by measuring leakage of liposome contents in the presence of synthetic peptides. Disruption of liposome membranes was monitored by recording the release of the fluorescent probe ANTS and its quencher DPX from encapsulated DPPC/PG (70:30) liposomes at baseline and after the addition of synthetic peptide (Fig. 2.5). Native SP-B was very effective in disrupting liposome membranes and caused a rapid increase in fluorescence (comparable to the Triton-X 100 control) at a concentration of 60 nM peptide (Fig. 2.5A). The smallest synthetic peptide capable of inducing liposome lysis was helix 1 (residues 7-22 of SP-B);
maximal lytic activity was achieved at a concentration of 0.5 \( \mu \text{M} \) peptide (Fig. 2.5B, D). Full length synthetic SP-B peptide also exhibited maximal lytic activity at a concentration of 0.5 \( \mu \text{M} \) peptide (Fig. 2.5B). Helix 2 alone had no lytic activity whereas helices 3,4,5 exhibited reduced lytic activity compared to helix 1 (Fig. 2.5C). Collectively these results indicate that native SP-B is significantly more lytic and fusogenic than any of the synthetic peptides.

*Surface activity of SP-B synthetic peptides.* To assess the contribution of individual SP-B helices to surface tension reduction, the surface properties of synthetic peptides were assayed by captive bubble analysis (Fig. 2.6). N-term helix 1,2 (i.e. residues 1-37 of SP-B) and full length SP-B (residues 1-79) were the only synthetic peptides that reduced minimum surface tension to values < 5 mN/m. Helix 1,2 exhibited poor surface tension reducing properties (>15 mN/m) indicating that the non-helical, N-terminal 6 amino acids were essential for surface tension reduction. All other peptides, including the highly lytic helix 1, generated elevated minimum surface tensions (\( \geq 15 \) mN/m) indicating that the lytic activity of SP-B was not sufficient to reduce surface tension. Generation of low minimum surface tension (< 5 mN/m) required at least 1.5% dimeric, native SP-B peptide or 4% monomeric, N-term helix 1,2 synthetic peptide; the minimum surface tension attained with 3% N-term helix 1,2 peptide was > 10 mN/m (not shown).
Figure 2.5. **Leakage of DPPC/PG liposome contents in the presence of native SP-B and synthetic peptides.** Liposomes (20 µg/ml) containing encapsulated fluorescent probes were added to a 2.0 ml cuvette containing MOPS buffer, pH 7.0, and stirred continuously. Fluorescence was recorded at baseline and after the addition of increasing concentrations of native SP-B, synthetic peptide (1mg/ml stock solution in methanol), or vehicle control for 180 seconds. Maximum fluorescence was detected after the addition of triton X-100. Each curve is representative of 3 experiments.
Figure 2.6. **Surface activity of synthetic SP-B peptides.** Surface properties of mixtures of synthetic peptide (4%) and DPPC/POPG (7:3 by weight) were assessed by captive bubble analysis. The bubble was pulsed at 10 cycles/min and minimum surface tension was recorded at the fifth pulsation, when the bubble was reduced to 80% of its original volume. The values shown are the average of three separate experiments \( \pm \) SEM; \(*p<0.05, \) for synthetic peptides versus 1.5% native SP-B.

*Identification of amino acids critical for fusogenic activity.* The domain mapping experiments indicated that helix 1 was absolutely required for the fusion, lytic and surface activities of SP-B. To further examine the molecular basis for these properties, amino acid substitutions were introduced into the N-term helix 1,2 synthetic peptide (i.e. residues 1-37 of SP-B) (Fig. 2.7). Substitution of individual,
positively charged amino acids in helix 1 (R12A or K16A) or the N-terminus of helix 2 (K24) did not affect the fusion activity of N-term helix 1,2 (not shown). Likewise combinatorial substitution of a positively charged residue in helix 2 (K24A) and helix 1 (R12A) did not alter fusogenic activity. However, substitution of two positively charged residues in helix 1 (R12S,K16S or K16S,R17S) partially inhibited liposome fusion (Fig. 2.8A). Fusogenic activity was further inhibited by combinatorial substitution of R12S and K16S in helix 1 and K24S in helix 2. The largest effect occurred when all three positively charged amino acids in helix 1 (R12S,K16S,R17S) were substituted. Paired substitution of serines for hydrophobic residues in the non-polar face of helix 1 (L10S,M21S; I14S,L18S; I15S,I22S) also inhibited liposome fusion (Fig. 2.8B). None of the peptide variants of helix 1 showed significantly different secondary structure compared with the native sequence, as assessed by CD (Fig. 2.2). Thus, the changes in the polarity of either subdomain of helix 1 likely account for the altered fusogenic activity of SP-B peptides.

The N-terminal hydrophobic sequence (residues 1-6) was previously reported to be important for anchoring SP-B to phospholipid bilayers (Perez-Gil et al., 1991). In particular, tryptophan residues are known to play an important role in the interaction of peptides with lipid membranes (Wimley et al., 1996). SP-B has one tryptophan residue at position 9 that has previously been reported to be embedded in the phospholipid bilayer (Cruz et al., 1998; Wang et al., 2003b). Substitution of tryptophan 9 to alanine did not affect the fusogenic activity of SP-B (not shown). The helix-breaking amino acid proline has also been shown to be important for the activity of some fusogenic peptides (Hsu et al., 2002); however, substitution of P2,4,6 to alanine did not affect fusogenic activity; likewise, increasing the polarity of the N-terminal domain, by substituting lysine for I3 and L5, did not alter liposome fusion (not shown). Therefore, although deletion of the N-terminal domain (residues 1-6) significantly decreased fusogenic activity (Fig. 3), the molecular basis for this effect is not clear.
In addition to the N-terminal domain and helix 1, the proline residue between helix 1 and 2 was shown to be important for fusogenic activity (Fig. 2.8B). Substitution of P23 to alanine dramatically inhibited liposome fusion suggesting that a bend between helix 1 and 2 may be required for this activity. Interestingly, the peptide with the substitution P23A had a CD spectrum that was consistent with a significant increase in the proportion of a α-helical conformation.

*Identification of amino acids critical for lytic activity.* Positively charged amino acids have been shown to be critical for liposome lysis and disruption of bacterial membranes (Shai, 1999). Substitution of individual, positively charged amino acids in helix 1 (R12A or K16A) or helix 2 (K24A) did not affect liposome lysis (not shown). In addition, substitution of two positively charged amino acids in helix 1 (R12S,K16S or K16S,R17S) or helix 1 and helix 2 (R12A,K24A) did not alter the lytic activity of SP-B (not shown). However, substitution of three positively charged amino acids (R12S,K16S in helix 1 and K24S in helix 2) partially reduced liposome lysis (Fig. 2.9). Lytic activity was inhibited to an even greater extent by substitution of all three positively charged residues in helix 1 (R12S,K16S,R17S). Compared to N-term helix 1,2, liposome leakage induced by R12S,K16S,K24S or R12S,K16S,R17S peptides was very slow and required higher concentrations of peptide. None of the other substituted SP-B peptides had any affect on membrane lysis (not shown) indicating that positively charged residues were the major determinant of this property.

*Identification of amino acids critical for surface activity.* To determine if amino acids critical for fusion and lysis were also required for surface tension reducing activity, the surface properties of synthetic SP-B peptides were assayed by captive bubble analysis. Substitution of individual, positively charged amino acids in helix 1 (R12A or K16A) or helix 2 (K24A) did not alter the surface properties of SP-B (not
shown). Similarly, substitution of two positively charged amino acids in helix 1 (R12S,K16S and K16S,R17S) or in helix 1 and helix 2 (R12A,K24A) resulted in peptides that retained excellent surface activity (Fig. 2.10A). However, substitution of three positively charged amino acids in helix 1 (R12S,K16S,R17S) or in helix 1 and 2 (R12S,K16S,K24S) significantly increased minimum surface tension (≥20 mN/m) (Fig. 2.10A). These results suggest that the positively charged residues in helix 1 and 2 are important for fusion, lysis and surface activity.

The importance of proline residues (located in N-term and interhelical loop 1) for surface tension reducing activity was also assessed. Substitution of P2,4,6 or P23 to alanine resulted in significantly increased minimum surface tension (≥10 mN/m) (Fig. 2.10B); likewise, substitution of W9, located within the lipid bilayer resulted in elevated minimum surface tension (≥15 mN/m) (Fig. 2.10B). These analyses revealed substitutions (tryptophan 9 and prolines 2,4,6) that selectively inhibited the surface activity without affecting liposome fusion or lysis.

Paired substitution of hydrophobic residues (I15S,I22S or L10S,M21S) located in the non-polar face of helix 1 resulted in elevated minimum surface tension (>10 mN/m) (Fig. 2.10B). Interestingly, low minimum surface tension was attained when L14,I18 was substituted to serine indicating that a decrease in the hydrophobicity of the non-polar face was tolerated.
Figure 2.7. **Helical wheel prediction of SP-B (amino acids 1-25)** showing the relative location of each residue with respect to the phospholipid bilayer (adapted from Wang, Rao and Demchuk (Wang et al., 2003b)). The diagram shows the amphipathic nature of helix 1 with positively charged residues R12, K16, R17 and K24 at the surface of the membrane and hydrophobic residues Y7, C8, W9, M21, L14, I18, C11, I22, and I15 embedded in the lipid bilayer (grey). The horizontal line represents the surface of the phospholipid bilayer. Alanine, serine or lysine substitutions were introduced at the indicated positions (boxes).
Figure 2.8. **Fusogenic activity of the SP-B synthetic peptides (amino acids 1-37) containing polar and non-polar substitutions in Helix 1 and 2.** DPPC/PG (70:30) liposomes (200nM) were incubated with increasing concentrations of 37 amino acid synthetic peptides containing substitutions at positively charged residues (A) or hydrophobic residues (B). Fusion was determined by the increase in vesicle size using an N4 particle size analyzer. Results (n=3) are expressed as means ± SEM; *p<0.05, for synthetic peptide versus N-term helix 1,2.
Figure 2.9. Leakage of DPPC/PG liposome contents in the presence of SP-B synthetic peptides containing polar substitutions in helix 1. Liposome leakage was measured as described in Materials and Methods. Liposomes (20 µg/ml) containing encapsulated fluorescent probes were added to a 1.0 ml cuvette containing MOPS buffer pH 7.0 and stirred continuously. Fluorescence was recorded at baseline and after the addition of substituted synthetic peptides (1.0 µM) or N-term helix 1,2 (0.5 µM) for 180 seconds. Maximum fluorescence was detected after the addition of triton X-100. Data shown is representative of three separate experiments.
Figure 2.10. **Surface activity of synthetic SP-B peptides containing polar and non-polar substitutions.** Surface properties of mixtures of synthetic peptide (4%) and DPPC/POPG (7:3 by weight) were assessed by captive bubble analysis. The bubble was pulsated at 10 cycles/min and minimum surface tension was recorded at the fifth pulsation, when the bubble was reduced to 80% of its original volume. Substituted SP-B peptides were compared to N-term helix 1,2. The values shown are the average of three separate experiments ± SEM; *p<0.05, for synthetic peptide versus N-term helix 1,2.

**Membrane interactions of SP-B synthetic peptides.** To determine if amino acid substitutions altered the interaction of SP-B with membranes, tryptophan fluorescence emission spectra were monitored in the
presence or absence of DPPC/PG liposomes. Changes in the environment of the tryptophan residue (hydrophilic to hydrophobic) were detected by monitoring the maximum emission wavelength and intensity (Qi et al., 2001). The fluorescence emission spectra for N-term Helix 1,2, P23A, R12S,K16S,R17S and R12S,K16S,K24S were obtained and compared to measurements in the presence of lipids. Maximal emission wavelength shifts to the blue direction were recorded for all four peptides suggesting that amino acid substitutions in these peptides did not alter peptide/membrane interaction (Table 2.1).

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<th>SP-B peptides (1-37)</th>
<th>Emission maxima (nm)</th>
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<td>335</td>
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</tbody>
</table>

Table 2.1. **Emission spectra of SP-B synthetic peptides in the absence or presence of DPPC/PG liposomes.** The spectra were acquired at $\lambda_{ex}=280$ nm in 1mM Hepes, 3 mM NaCl pH 7.0 at room temperature. The protein: lipid ratio was 1:20 with 2.0 \( \mu \)M synthetic peptide and 40 \( \mu \)M lipids.
DISCUSSION

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

Native SP-B promoted liposome fusion and lysis at significantly lower concentrations than the most effective synthetic peptide. Maximal liposome fusion and lysis required 20-fold and 8-fold more synthetic peptide than native peptide. The results of a previous study indicated that a 25 residue SP-B peptide (SP-B 1-25) promoted lipid mixing nearly as well as native SP-B (Veldhuizen et al., 2000). In the current study, N-term helix 1 (residues 1-22) promoted lipid mixing, as assessed by FRET (not shown), similar to results reported (Veldhuizen et al., 2000); however, only peptides containing helix 2 produced an increase in vesicle size consistent with liposome fusion. The fusogenic activity of the N-terminal half of SP-B was not enhanced in the presence of the C-terminal peptide; further, the fusogenic activity of full length synthetic SP-B peptide was less than that of the N-terminal domain of SP-B. Synthetic full length SP-B had similar secondary structure to the native protein, as assessed by circular dichroism. The present
results suggest therefore that the tertiary and/or quaternary structure of native SP-B significantly influenced the fusogenic property of the peptide. Native SP-B is a homodimer that is stabilized by an intersubunit disulfide bridge between cysteines at position 48; a salt bridge between glutamic acid 51 of one subunit and arginine 52 of the other subunit may also contribute to dimer stability (Zaltash et al., 2000). Since SP-B interacts with the membrane surface it is possible that each subunit of the homodimer initially resides on separate membranes. Membrane crosslinking by SP-B could promote fusion by bringing opposing membranes into close proximity (crosslinking) prior to lipid mixing as described for the Influenza hemagglutinin fusion protein (Hsu et al., 2002). If this is the case, 37 amino acid N-terminal synthetic peptides would be less effective in promoting fusion because they lack residues involved in subunit association/stabilization. Consistent with this hypothesis, a dimeric form of SP-B 1-25 was shown to be more fusogenic than the monomeric peptide (Veldhuizen et al., 2000).

Both native SP-B and a synthetic peptide encompassing the N-terminal half of SP-B exhibited maximal fusion and lytic activity at concentrations below those required to achieve low surface tension in vitro. Amino acid substitutions that significantly impaired the fusogenic or lytic activities of SP-B also inhibited the surface tension reducing property of the peptide; further, SP-B mediated liposome fusion and lysis proceeded rapidly at pH 7.0. These findings support the hypothesis that SP-B-mediated fusion and lysis play an important role in the formation and maintenance of the alveolar surface film. SP-B mediated fusion and lysis was also active at pH 5.5. Both the late endosome/MVB and lamellar body have an acidic interior (approx. pH 5.5) (Chander et al., 1986) and the estimated concentration of SP-B in the lamellar body approximates that required for liposome fusion and lysis in vitro (Oosterlaken-Dijksterhuis et al., 1991). These observations are consistent with the hypothesis that fusion and/or lysis play a role in the incorporation of internal vesicles of the MVB into the internal membranes of the lamellar body.
Amino acid substitutions were introduced into a synthetic peptide encompassing the N-terminus, helix 1, interhelical loop 1 and helix 2 (i.e. residues 1-37) in order to assess the contribution of individual structural domains to the fusogenic, lytic and surface properties of SP-B (summarized in Table 2.2). Based on the NMR structure of NK-lysin, the N-terminal domain was predicted to consist of 6 amino acids (Liepinsh et al., 1997); however, the results of a recent study by Wang et. al. (Wang et al., 2003b) suggested that this domain may extend to tryptophan at position 9. The 6-9 amino acid N-terminal domain likely forms a highly kinked motif, as it contains 3 regularly spaced proline residues. Substitution of alanines for prolines completely ablated the surface tension reducing property of SP-B; likewise substitution of alanine for tryptophan (W9A) resulted in elevated surface tension in vitro. The tryptophan residue is embedded in the phospholipid bilayer (Cruz et al., 1998; Wang et al., 2003b) and may serve to anchor SP-B to the membrane, as proposed for other lytic peptides (Oh et al., 2000; Anderson et al., 2003). However, the W9A substitution did not alter the ability of SP-B to lyse or fuse liposomes. Similarly, the proline residues in the N-terminal domain were not required for SP-B mediated fusion or lysis. Together, these findings indicate that the N-terminal domain is absolutely required for the surface tension reducing property of SP-B but is completely dispensable for lytic activity. Removal of the N-terminal domain moderately impaired fusogenic activity but the molecular basis for this effect remains unclear. Finally, these results clearly indicate that fusion and lysis are necessary but not sufficient to impart surface tension reducing ability.

Helix 1, extending from residue 7-10 through residue 22, is absolutely required for fusion, lysis and surface activity. Helical wheel projections clearly illustrate the amphipathic nature of this domain (Andersson et al., 1995). The polar face contains 3 positively charged amino acids at positions 12, 16 and 17 that interact with the anionic headgroups at the surface of the membrane. Numerous studies have shown that positively charged residues are important for the lytic properties of antimicrobial peptides
(Shai, 1999) and both charge and hydrophobicity were reported to affect the helical content and lipid association of SP-B 1-25 (Bruni et al., 1991). In the current study, substitution of serine for a single positively charged amino acid in helix 1 had no effect on the fusogenic, lytic or surface properties of SP-B. Substitution of two positively charged residues altered fusogenic activity without any effect on lytic or surface activities. In the absence of any positively charged amino acids in helix 1, the peptide bound to liposomes (Table 2.1) but failed to fuse or lyse membranes; further, the surface tension reducing ability of the peptide was abrogated. These results underscore the importance of the positively charged amino acids in helix 1 for SP-B function.

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

The loop between helix 1 and 2 contains a proline residue which may serve as a molecular hinge. The helix-hinge-helix motif was reported to be important for the lytic activity of several antimicrobial peptides including melittin, cecropin and brevinins (Bazzo et al., 1988; Holak et al., 1988; Ikura et al., 1991; Sipos et al., 1992). A recent study of the topographical organization of the N-terminal segment of SP-B (residues 1-25) in phospholipid bilayers indicated that the formation of a sulfydryl-dependent dimer (via C8) would require rotation of helix 1, consistent with a flexible hinge between helix 1 and helix 2.
(Wang et al., 2003b). In the current study, substitution of alanine for proline (P23) impaired both the fusogenic and surface tension lowering properties of SP-B implying that a flexible hinge may also be important for these properties. The ellipticity of the P23A peptide was actually greater than that of the original fully synthetic sequence or of native SP-B (Fig. 2.2). Estimations of the contribution of α-helical conformation from the CD spectra are consistent with the substitution P23A producing an extension of helix 1 that continues through helix 2. Surprisingly, the lytic activity of SP-B was not affected by the P23A substitution.

Helix 2 is predicted to extend from residue 26 through residue 37 and is more hydrophobic than helix 1. The importance of helix 2 for SP-B function was evident from the results of domain mapping experiments, which indicated that a 22 amino acid synthetic peptide lacking this domain (i.e. N-term helix 1) was deficient in membrane fusion and surface tension reducing activity. This result was unexpected because a peptide consisting of the first 25 amino acids of SP-B (SP-B 1-25) was reported to promote surface tension reduction in vitro and improve lung compliance in two animal models of surfactant deficiency (Bruni et al., 1991; Bruni et al., 1998). This discrepancy may be due in part to the fact that SP-B 1-25 includes the putative molecular hinge (P23) and the first 2 residues of helix 2 (K24 and G25). However, the SP-B 1-25 peptide also required the presence of palmitic acid in the lipid mixture to achieve low \( \gamma_{\text{min}} \) values (Veldhuizen et al., 2000). In the current study, 37 amino acid peptides that included helix 2 were able to achieve \( \gamma_{\text{min}} \) values of less than 5 mN/m (Fig. 2.10) in the absence of palmitic acid consistent with an important role for this domain in surface tension reduction. Collectively, these results support the hypothesis that helix 2 is required for membrane fusion and surface tension reduction but do not indicate if part or all of the helix is required for SP-B function.

In summary, this study demonstrates that a synthetic peptide corresponding to the N-terminal 37 amino acids of human SP-B promotes liposome lysis and fusion as well as rapid surface tension reduction
in vitro. Association of helix 1 alone (residues 7-22) with the membrane surface through ionic and hydrophobic interactions, results in membrane permeabilization but not aggregation (Fig. 2.11). We propose that helix 2 (residues 24-37) mediates membrane crosslinking (aggregation) which, in turn, facilitates lipid mixing, membrane fusion and interfacial adsorption/surface tension reduction. Helix 2 may promote membrane crosslinking via peptide-peptide interaction (i.e. peptides anchored to separate membranes associate via helix 2, see figure 2.11) or via peptide-lipid interactions (i.e. helix 1 interacts with one membrane and helix 2 interacts with a separate membrane, not shown). In the presence of helix 2, the membrane perturbing properties of helix 1 might promote lipid mixing between bilayers or between bilayers and a monolayer, preceding either fusion or interfacial adsorption, respectively. In the absence of the membrane cross-linking activity of helix 2, perturbation of membranes by helix 1 produces lysis but not fusion or surface activity. This model is consistent with the finding that a flexible hinge between helix 1 and 2 is important for lipid mixing/fusion and surface activities but not membrane lysis. The N-terminal domain (residues 1-9) is critical for the surface tension reducing property of SP-B but plays little or no role in membrane fusion or lysis. We speculate that the N-terminal structural motif, including prolines 2,4,6 and tryptophan 9, might be required to sustain deep association of SP-B peptides with surface films supporting higher lateral pressures than those existing in membrane bilayers. This would explain why membrane fusion and lysis alone are not sufficient to impart surface tension reducing activity. Overall, decreased lytic or fusogenic activities of SP-B were associated with altered surface properties of the peptide.
Figure 2.11. **Model of interactions of SP-B helix 1 and 2 with phospholipid membranes.** Association of helix 1 with the membrane surface induces deep perturbation in the phospholipid bilayer and monolayer. Helix 2 promotes membrane fusion or surface activity but not liposome lysis, suggesting that this domain may be a membrane aggregation motif. In the absence of membrane cross-linking, perturbations induced by helix 1 at the lipid surface leads to content leakage. In the context of aggregated liposomes, membrane perturbation leads to lipid mixing and eventual fusion. Membrane-to-monolayer association followed by lipid perturbation would be the basis for surface activity, which seems to require additional specific motifs at the N-terminal end of SP-B.
Table 2.2. **Summary of the fusogenic, lytic and surface properties of the SP-B synthetic peptides.**

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

<table>
<thead>
<tr>
<th>Domain</th>
<th>Peptide</th>
<th>Fusion</th>
<th>Lysis</th>
<th>Surface activity</th>
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<tr>
<td>N-term</td>
<td>I3,L5K</td>
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<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>P2,4,6A</td>
<td>NC</td>
<td>NC</td>
<td>↓</td>
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<tr>
<td></td>
<td>W9A</td>
<td>NC</td>
<td>NC</td>
<td>↓↓</td>
</tr>
<tr>
<td>Helix 1</td>
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<td>NC</td>
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<tr>
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<td>NC</td>
</tr>
<tr>
<td></td>
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<td>↓↓</td>
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</tr>
<tr>
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ACKNOWLEDGEMENTS

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CHAPTER III

ANTIMICROBIAL ACTIVITY OF NATIVE AND SYNTHETIC SURFACTANT PROTEIN B (SP-B) PEPTIDES

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SUMMARY

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 *E. 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 red blood cells 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; further, 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.
INTRODUCTION

The respiratory tree terminates in small sac-like structures (alveoli) that provide an extensive gas exchange surface composed of type I epithelial cells. Hydration of the gas exchange surface leads to elevated surface tension at the air/liquid interface generating a high collapsing force at end expiration. Type II epithelial cells synthesize and secrete pulmonary surfactant which forms a stable phospholipid-rich film at the air-liquid interface and prevents alveolar collapse and impaired gas exchange. Dipalmitoylphosphatidylcholine (DPPC), the main lipid component of surfactant, reduces surface tension to near zero as the surfactant film is compressed during exhalation. During inhalation, surfactant phospholipids are inserted into the expanding surface film, a process facilitated by the hydrophobic peptides surfactant protein (SP)-B and SP-C. The importance of SP-B for surfactant function is underscored by the fact that deficiency of SP-B in both mice and humans results in lethal neonatal respiratory distress syndrome (Clark et al., 1995; Melton et al., 2003).

In addition to its biophysical function, surfactant plays an important role in maintaining the sterility of the gas exchange surface. The surface film serves as a physical barrier to inhaled pathogens and the hydrophilic surfactant proteins SP-A and SP-D, associated with the large and small aggregate fractions of surfactant respectively (Crouch, 1998; Ikegami et al., 2005), promote clearance of microorganisms from the distal airspaces. SP-A and SP-D opsonize, aggregate and enhance phagocytosis of microbes by resident macrophages (Mcneely et al., 1993; Kabha et al., 1997; Ofek et al., 2001). SP-A and SP-D may also directly kill bacteria by permeabilizing the bacterial membrane (Wu et al., 2003). The role of SP-B in alveolar host defense is less clear. A synthetic peptide corresponding to SP-B was reported to inhibit the growth of *E. coli* in vitro (Kaser et al., 1997); however, bacterial burden was not increased in the lungs of SP-B heterozygous null (SP-B*) mice nor was protection conferred by increased expression of SP-B in transgenic mice (Akinbi et al., 1999).
SP-B is a member of the saposin-like family of proteins (SAPLIP), several members of which exhibit potent antimicrobial activity (Patthy, 1991). SAPLIP family members NK-lys, granulysin and amoebapore all kill bacteria by permeabilizing bacterial membranes but the mechanism of membrane permeabilization differs among the peptides. Positively charged amino acids located on the surface of NK-lys and granulysin mediate interaction of the peptides with the negatively charged membranes of bacteria resulting in membrane destabilization and/or permeabilization (Ernst et al., 2000; Bruhn et al., 2003). In contrast, amoebapore A is much more hydrophobic and permeabilizes bacterial membranes in a pH- and oligomerization-dependent manner (Hecht et al., 2004). SP-B shares features with both types of SAPLIP antimicrobial peptides: it is very hydrophobic and forms oligomers similar to amoebapore but is also cationic (net positive charge of +7) and lyses negatively charged liposomes at neutral pH, similar to NK-lys and granulysin (Poulain et al., 1992; Ryan et al., 2005). We have previously mapped the lytic domain of SP-B to helix 1, an α-helical, amphipathic region containing a net charge of +3 (Ryan et al., 2005). 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. Dipalmitoylphosphatidylcholine and phosphatidylglycerol were purchased from Avanti Lipids (Birmingham, AL). HEPES buffer was purchased from Cambrex Bioscience (Walkersville, MD) and melittin peptide from bee venom was purchased from Sigma Aldrich, St. Louis, MO.

Peptide Design. Synthetic peptides were designed to the proposed helices and interhelical loops of the mature SP-B peptide (Ryan et al., 2005). Peptides were synthesized by Biosynthesis Inc. (Lewisville, TX) by F-moc chemistry and purified to >95% homogeneity by HPLC. Peptide composition was confirmed by mass spectrometry. Stock solutions (1 mg/ml) were prepared in methanol and diluted into assay buffer to achieve the peptide concentrations indicated in the figures. Appropriate solvent controls were used in each experiment.

Preparation of native human surfactant protein B. Human SP-B was isolated from bronchoalveolar lavage fluid of patients with pulmonary alveolar proteinosis, as described by Shen et. al. (Shen et al., 1997). Briefly, surfactant was isolated from bronchoalveolar lavage fluid by centrifugation and dissolved in chloroform/methanol (2:1). The organic phase was recovered, dried, dissolved in chloroform/methanol/0.1 M HCl [1:1:0.1 (v/v)] and loaded onto an LH-60 Sephadex column equilibrated in the same solvent system. Fractions eluted from the column were screened by SDS-PAGE and silver staining. SP-B-containing fractions were recovered and dialyzed (SnakeSkin dialysis tubing, molecular weight cutoff = 3500, Pierce Chemical Co.) against chloroform/methanol [2:1 (v/v)] overnight at 4°C to remove HCl and was quantitated by amino acid composition analysis (Horowitz et al., 1992).
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 JoRae Wright, Duke University Medical Center) 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 x g for 10 min, washed and resuspended in sterile PBS at a concentration of $10^3$ colony forming units (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 polystyrene microtiter plate (Becton Dickenson, Franklin Lakes, NJ) and serial dilutions of each peptide in methanol were added to individual wells in triplicate and incubated for 6 hours at 37°C with rocking. Bacteria were subsequently dispersed and aliquots were plated on blood agar plates to obtain colony counts. Viable pathogen counts after peptide treatment were determined from the number of colonies obtained on the methanol treated control plates compared to the number of colonies from peptide treated samples. Bacterial killing results are expressed as Percent killing = $100 \times \left( \frac{\text{CFU from control wells (without SP-B or peptide)} - \text{CFU from experimental wells}}{\text{CFU from control wells (without SP-B or peptide)}} \right)$.

Bacterial membrane permeabilization assay. *K. pneumoniae* was grown to mid-log phase in LB at 37°C, washed twice with 5 mM Tris, 150 mM NaCl and diluted to OD$_{600}$1.0. Bacteria were aliquoted into a 96-well polystyrene microtiter plate to a final concentration of OD$_{600}$0.5. Increasing concentrations of native SP-B (0.1-5.0 μg/ml final concentration) were added to each well and incubated for 15 minutes at 37°C with shaking. Alkaline phosphatase enzyme levels were quantitated over a 90 minute period in the presence of the fluorescently labeled enzyme substrate ELF-97 (Molecular probes Inc., OR) at excitation and emission wavelengths of 355 nm and 535 nm respectively.
Preparation of phospholipid vesicles. Phospholipids in chloroform were dried under N₂ and resuspended in 50 mM HEPES/140 mM NaCl/0.1 mM EDTA buffer, pH 7.0, to a final concentration of 1 mg/ml. The phospholipid suspension was passed through a mini-extruder (Avanti Lipids, Birmingham, AL) at 45°C through two stacked 0.1 µm polycarbonate filters. A series of 10 extrusions was performed to generate a population of unilamellar liposomes with diameters of approximately 100 nm. Peptides were incubated with liposomes at 20:1 or 10:1 lipid:peptide ratios for 5 minutes before adding to bacteria. Methanol controls were included in all experiments.

Bacterial clearance in transgenic mice. Wildtype mice and transgenic mice, in which SP-B concentrations in the airspaces were increased 2- to 3-fold (Akinbi et al., 1999) were anesthetized with isofluorane and a dose of 10⁴ CFU K. pneumoniae suspended in 100 µl of sterile PBS was delivered intratracheally just beneath the cricoid cartilage as previously described (Akinbi et al., 1999). To assess bacterial clearance, mice were anesthetized 24 hours postinfection with intraperitoneal pentobarbital, exsanguinated by transecting the abdominal aorta and lung and splenic tissues were harvested and subsequently homogenized in sterile PBS. Serial dilutions of homogenates were plated on blood agar plates and incubated overnight at 37°C. Colony counts in the lung and spleen (data not shown) were obtained and expressed as CFU/g tissue.

Hemolytic assay. Fresh human red blood cells (hRBC) were rinsed in PBS and centrifuged for 10 min at 800 × g three times and resuspended in PBS to a final erythrocyte concentration of 4% v/v. The hRBC suspension (100 µl) was added to 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 hRBC suspended in PBS and 1% Triton-X 100, respectively; additional controls included hRBC suspended in 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 monitored 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}$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 hours. Samples were stained using the permeant fluorescent probe Syto 9 and impermeant fluorescent probe propidium iodide (BacLight Bacterial Viability kit, Molecular Probes Inc.). Bacteria were examined by fluorescence microscopy to assess bacterial aggregation and changes in propidium iodide or Syto 9 staining compared to untreated or methanol treated controls.

*Isolation of pulmonary surfactant.* Surfactant was isolated by high speed centrifugation of cell free bronchoalveolar lavage fluid (BALF) (2 mls in sterile PBS/mouse) obtained from 25 g FVBN mice (6-8 week old). Phosphorous in total BAL was measured by the Bartlett assay (Bartlett,1959).

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

Antimicrobial activity of human SP-B against Klebsiella 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. To determine if SP-B was also bacteriolytic, a clinical isolate of K. pneumoniae (10⁴ CFU) was incubated with increasing concentrations of purified human SP-B for 6 hours at 37°C. Mature SP-B peptide exhibited potent, dose-dependent antimicrobial activity (Fig. 3.1A) 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).

Since SP-B is always associated with membranes, experiments were designed to determine if SP-B-mediated bacterial killing was altered in the presence of surfactant phospholipids. Surfactant-like liposomes, composed of DPPC/PG (9:1, wt:wt) 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 approximately 70% (Fig. 3.1B); 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 P. aeruginosa and group B streptococcus was not enhanced in transgenic mice in which the concentration of SP-B in BALF was increased 2-3 fold (Akinbi et al., 1999). To determine if this outcome was related to lipid inhibition or pathogen-specific effects of SP-B, K. pneumoniae (10⁴ CFU) were intratracheally instilled into transgenic mice and bacterial burden assessed 24 hours post-infection (Fig. 3.1C). Killing of K. pneumoniae was not enhanced in transgenic mice supporting the hypothesis that surfactant phospholipids inhibit the bactericidal activity of native SP-B in vivo.
Fig. 3.1. **Effect of SP-B on *K. pneumoniae* viability.** (A) Increasing amounts of native hSP-B dissolved in methanol were added to *K. pneumoniae* (10³ CFU) in 100 µl of sterile PBS and incubated for 6 hours at 37°C. Results (n=4) are expressed as mean ± SEM; *p<0.05 vs methanol control (B) SP-B was preincubated with liposomes (20:1 lipid:peptide ratio) composed of DPPC or DPPC/PG (9:1) and then incubated with *K. pneumoniae*. Bacteria were dispersed, plated on blood agar plates and incubated overnight at 37°C. Solvent (methanol) controls were included in all experiments. Results (n=6) are expressed as mean ± SEM; *p<0.05 vs SP-B lipid free; +p < 0.05 vs SP-B/DPPC/PG. (C) Wildtype and transgenic mice expressing elevated levels of human SP-B were intratracheally instilled with 10⁴ CFU *K. pneumoniae* and lungs were harvested 24 h post infection. CFU were counted from plated lung homogenates and data are expressed as CFU/g lung tissue ± SEM; WT vs. transgenic SP-B overexpressors, p= 0.7363. n = 8 mice/group.
**Effect of SP-B on bacterial aggregation.** The hydrophilic surfactant proteins SP-A and SP-D play important roles in lung host defense by inducing bacterial aggregation. To determine if SP-B could also induce bacterial aggregation, *K. pneumoniae* (OD<sub>600</sub>0.1) was incubated with SP-B for 90 minutes 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 to untreated or methanol treated controls. Addition of SP-B (1-3 μM) to *K. pneumoniae* induced significant bacterial aggregation compared to controls (Fig. 3.2). The mean area of bacterial aggregates was 540 \( \mu \text{m}^2 \pm 80 \mu \text{m}^2 \) and aggregates as large as 5,000 \( \mu \text{m}^2 \) were detected. Similar results were obtained with other strains of bacteria including *P. aeruginosa*, *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 if surfactant phospholipids altered the ability of SP-B to aggregate bacteria, SP-B was added to DPPC or DPPC/PG liposomes prior to incubation with bacteria (OD<sub>600</sub>0.1) for 90 minutes. 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 colony forming units in the bacterial killing assays. Thus, surfactant phospholipids, in particular PG, inhibited both bacterial killing and aggregation.
Fig. 3.2. **SP-B mediated bacterial aggregation and membrane permeabilization.** *K. pneumoniae* (OD₆₀₀ 0.1) were incubated with human SP-B (1-3 µM) or solvent (methanol) controls for 90 minutes at 37°C. Bacteria were stained with the vital dyes Syto 9 (stains live bacteria, panel A, D, G, J) and propidium iodide (stains dead/dying bacteria, panel B, E, H, K) and analyzed by fluorescence microscopy to assess aggregation and viability. Results are representative of four separate experiments and depict small (panels G-I) and large aggregates (panels J-L). Bar = 20 µm.
Hemolytic activity of human SP-B. To determine the specificity of the membranolytic activity of SP-B, native peptide was incubated with human red blood cells in the presence or absence of DPPC or DPPC/PG liposomes (Fig. 3.3). SP-B induced a dose-dependent release of hemoglobin from red blood cells at a concentration of 1.0-7.5 µM. Membrane lysis was significantly reduced in the presence of DPPC and was virtually ablated in the presence of DPPC/PG liposomes.

Antimicrobial activity of SP-B synthetic peptides against Klebsiella 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 3D structure of NK-lysin, as previously described (Ryan et al., 2005) (Fig. 3.4A). Antimicrobial activity was assessed by incubating individual synthetic peptides with a clinical isolate of *K. pneumoniae* (10³ CFU) for 6 hours at 37°C. Bacteria were subsequently plated on blood agar plates and the number of colonies counted after 18 hours. SP-B peptides containing helix 1 exhibited potent antimicrobial activity against *K. pneumoniae* (Fig. 3.4B). A peptide encompassing residues 1-37 (N-term helix 1,2) killed more than 60% of the bacteria at a concentration of 2.5 µM. Removal of the N-terminal 6 amino acids from N-term helix 1,2 resulted in significantly higher levels of bacterial killing (>80%). Helix 1 (residues 7-22) killed more than 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.
Fig. 3.3. **Hemolytic activity of native hSP-B.** 4% human red blood cells were incubated for 1 hour with SP-B or SP-B preincubated with DPPC or DPPC/PG (9:1) liposomes (20:1 ratio of lipid:peptide) 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.
Fig. 3.4. **SP-B synthetic peptides containing helix 1 kill Klebsiella pneumoniae.** (A) Bars represent synthetic peptides designed to the proposed helical regions (grey) and interhelical loops (black) of the human SP-B mature peptide. The numbers in parentheses represent the corresponding amino acids in the native human SP-B peptide. (B) SP-B peptides (2.5 µM) or solvent controls were added to K. pneumoniae (10³ CFU) in 100 µl of sterile PBS and incubated for 6 hours at 37°C. Bacteria were dispersed, plated on blood agar plates and incubated overnight at 37°C to obtain colony counts. Results (n=8) are expressed as mean ± SEM; *p<0.05 vs methanol control.
**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. 3.5A). Maximal killing was attained at a 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* (OD₆00 0.05) for 90 minutes 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 if SP-B mediated bacterial killing was altered in the presence of surfactant phospholipids. Liposomes, composed of DPPC/PG (9:1, wt:wt) at lipid:peptide ratios of 20:1 or 10:1, were first mixed with synthetic peptide followed by incubation with bacteria. Preincubation of liposomes (DPPC/PG) with the SP-B peptides at 20:1 or 10:1 markedly impaired the ability of helix 1, helix 1,2 (Fig. 3.5B) and N-term helix 1 (data not shown) to kill bacteria, similar to results obtained for native SP-B (Fig. 3.1B). Removal of PG from the liposomes significantly improved SP-B mediated bacterial killing (Fig. 3.5B). PG-mediated inhibition was observed even when the peptides were incubated with bacteria prior to the addition of liposomes, although the extent of inhibition was much reduced (data not shown).
Fig. 3.5. **Dose-dependent and surfactant inhibition of Klebsiella pneumoniae killing by SP-B.** (A) Increasing amounts of synthetic peptide (helix 1 (residues 7-22), N-term helix 1, or helix 1,2) or solvent controls were added to *K. pneumoniae* (10^3 CFU) in 100 µl of sterile PBS and incubated for 6 hours at 37°C. Results (n=6) are expressed as mean ± SEM; *p<0.05 vs Helix 1; +p<0.05 vs N-term Helix 1 (B) SP-B peptides (5.0 µM) Helix 1 (residues 7-22) or helix 1,2 were preincubated with DPPC/PG (9:1) liposomes or DPPC liposomes (lipid:protein ratios 20:1 or 10:1) followed by incubation with bacteria. Bacteria were dispersed, plated on blood agar plates and incubated overnight at 37°C. Results (n=6) are expressed as mean ± SEM; *p<0.05 vs synthetic peptide lipid-free; +p < 0.05 vs DPPC/PG.
Antimicrobial activity of SP-B peptides against S. aureus. To determine if 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 Staphylococcus aureus (Fig. 3.6A). 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 to >95% killing for residues 7-22) (Fig. 3.6B). 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. 3.6C); 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.
Fig. 3.6. **Dose-dependent and surfactant inhibition of Staphylococcus aureus killing by SP-B.** (A) Increasing concentrations of the SP-B peptides (helix 1, N-term helix 1, or helix 1,2) were added to S. aureus (10^3 CFU) in 100 µl of sterile PBS and incubated for 6 hours at 37°C. Results (n=6) are expressed as mean ± SEM; *p<0.001 vs. methanol control; +p<0.001 vs Helix 1,2. (B) Dose-dependent killing curves were compared for Helix 1 peptides, residues 7-22 and 10-22. Results (n=3) are expressed as mean ± SEM; *p<0.05 vs methanol control. (C) The SP-B peptides helix 1 or helix 1,2 (5.0 µM) were preincubated with DPPC/PG (9:1) liposomes or DPPC liposomes (lipid:protein ratios 20:1 or 10:1) followed by incubation with bacteria. Bacteria were dispersed, plated on blood agar plates and incubated overnight at 37°C. Results (n=6) are expressed as mean ± SEM; *p<0.05 vs. lipid-free peptide; +p<0.001 vs DPPC/PG.
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. 3.7). N-term helix 1,2 was previously shown to be the smallest SP-B peptide that promoted surface tension reduction (Ryan et al., 2005). Positively charged amino acids have been shown to be important for the bacteriolytic activity of several antimicrobial peptides (Shai, 1999). To determine if 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. Substitution of a single positively charged amino acid (R12, K16 or K24) for 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. 3.7) 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 (OD$_{600}$ 0.1) and incubated for 90 minutes. Bacteria were stained with the vital dyes Syto 9 and propidium iodide and analyzed by fluorescence microscopy (Fig. 3.8). Helix 1 (residues 7-22) did not induce bacterial aggregation but caused a significant increase in propidium iodide staining (Fig. 3.8D-F) compared to control (Fig. 3.8A-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 µm$^2$ ± 10 µm$^2$; p<0.001) (Fig. 3.8G-I). In a few fields, larger bacterial aggregates were observed with sizes
similar to those induced by native SP-B (Fig. 3.8J-L). Virtually all of the bacteria within the aggregates were positive for propidium iodide staining consistent with dead/dying bacteria (Fig. 3.8K). Helix 1,2 exhibited similar activity toward other strains of bacteria including *P. aeruginosa* and group B *streptococcus* (data not shown).

Fig. 3.7. **Effect of cationic amino acid substitutions on SP-B peptide-mediated killing of Klebsiella pneumoniae.** Individual synthetic SP-B peptides (2.5 μM) containing positively charged amino acid substitutions were added to *K. pneumoniae* (10^3 CFU) in 100 μl of sterile PBS and incubated for 6 hours at 37°C. Bacteria were dispersed, plated on blood agar plates and incubated overnight at 37°C. Results (n=6) are expressed as mean ± SEM; *p<0.05 vs methanol control.
Fig. 3.8. **Effect of synthetic SP-B peptides on bacterial aggregation and membrane permeabilization.** *K. pneumoniae* (OD<sub>600</sub>0.1) were incubated with the SP-B peptides (10 μM) helix 1 (residues 7-22) or helix 1,2 or solvent (methanol) controls for 90 minutes at 37°C. Bacteria were stained with the vital dyes Syto 9 and propidium iodide and analyzed by fluorescence microscopy to assess bacterial aggregation and viability. Results are representative of four separate experiments. Helix 1,2 (residues 7-37) induced very small aggregates (panels H and I); however, in a few fields, larger bacterial aggregates were detected (panels K and L). Bar = 20 μm.
**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 human red blood cells for 1 hour. All of the SP-B peptides tested exhibited very low levels of hemolytic activity compared to melittin (<15% hemolysis at the highest concentration) (Fig. 3.9). Incubation of A549 cells with 5 µM helix 1 (residues 7-22) for 1 hour 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 to helix 1,2 or N-term helix 1 (Fig. 3.5B and Fig. 3.6C); 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 to <5% killing for helix 1,2) (Fig. 3.6C). We next determined if helix 1 could augment the ability of native surfactant to kill bacteria. Bronchoalveolar lavage was performed on wildtype mice, cells were removed using low speed centrifugation and surfactant phospholipids and associated proteins were pelleted at 18,000 x g for 15 minutes. Increasing amounts of helix 1 peptide were added to 0.75 µg total surfactant lipid followed by incubation with *K. pneumoniae* or *S. aureus* (10³ CFU) for 6 hours at 37°C. Bacteria were plated on blood agar plates and colonies 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. 3.10).
Fig. 3.9. **Hemolytic activity of SP-B peptides.** Synthetic peptides N-term helix 1,2, helix 1,2, N-term helix 1, and helix 1 (residues 7-22) were incubated with 4% human red blood cells for 1 hour 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 Melittin.
Fig. 3.10. **SP-B helix 1 kills bacteria in the presence of native surfactant.** Surfactant was isolated by high speed centrifugation of cell free BALF obtained from FVBN mice (6-8 weeks old). Increasing concentrations of SP-B helix 1 (residues 7-22) were added to 0.75 µg total surfactant phospholipid and incubated with bacteria (10³ CFU *K. pneumoniae* or *S. aureus*). Samples were incubated for 6 hours at 37°C, dispersed, plated on blood agar plates and incubated overnight to obtain colony counts. Results (n=3) are expressed as mean ± SEM; *p<0.05 vs. methanol control.
DISCUSSION

SP-B is a member of the saposin-like family of proteins that includes the potent antimicrobial peptides NK-lysin, granulysin and amoebapore. SAPLIP’s are characterized by a conserved disulfide bond pattern and likely share a similar tertiary structure. All SAPLIP’s interact with lipids and several, including the antimicrobial peptides, have membranolytic 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 alpha helices (Andersson et al., 1995). 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 phosphatidylglycerol (Baatz et al., 1990; Vandenbussche et al., 1992; Perez-Gil et al., 1995). Domain mapping experiments demonstrated that a cationic peptide corresponding to helix 1 (residues 7-22) was sufficient to lyse negatively charged vesicles (Ryan et al., 2005). 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 0.06-1.0 μM. 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 (Kaser et al., 1997) 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 (Crouch, 1998). 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 while helix 1 was sufficient for bacterial killing, aggregation required both helix 1 and helix 2. This finding agrees well with a previous study (Ryan et al., 2005) 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 crosslink 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. 3.2) while virtually all the bacteria aggregated by peptide helix 1,2 were stained by propidium iodide (Fig. 3.8). This suggests that the synthetic peptide aggregates bacteria through “lethal” domains, presumably helix 1, while 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 (Melton et al., 2003) 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 weeks 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. Further, lipid-free SP-B exhibited hemolytic activity comparable to melittin. 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 antibiotic 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 previously been reported. 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 protein histone H2A (Kim et al., 2000). 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 airspaces. 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 Pseudomonas aeruginosa, group B streptococcus (Akinbi et al., 1999) 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% (Akinbi et al., 1999). Thus, while 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 et. al. (Shai, 2002), 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/depolarization 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 lipopolysaccharide 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 sidechains of Tyr7 and Trp9. This model would explain why 1) lipid vesicles are less able to inhibit the antibiotic activities of SP-B peptides towards 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. (unpublished data) 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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CHAPTER IV

DISCUSSION/FUTURE DIRECTIONS
Role of SP-B mediated fusion and lysis in surface film dynamics

Although it is clear that SP-B is absolutely required for postnatal lung function and survival, the molecular mechanism(s) underlying SP-B action remains unknown. The results of in vitro studies indicate that SP-B can dramatically remodel membranes through fusion and lysis. The goal of this work was to map the fusogenic and lytic domains of SP-B and assess the importance of these properties for SP-B lung function. Synthetic peptides were generated to predicted helices and/or interhelical loops of SP-B and tested for fusion and lytic activities. The N-terminal half of SP-B (residues 1-37) promoted rapid liposome fusion whereas shorter SP-B peptides were significantly less effective. Liposome lysis required only helix 1 (residues 7-22). The structural domains required for optimal surface tension reduction were similar to those for fusion (residues 1-37). The C-terminal half of SP-B (helices 3,4,5) exhibited significantly lower levels of fusogenic, lytic and surface tension reduction activities compared to the N-terminal half. Taken together, these results indicated that SP-B fusion, lytic and surface activities map predominantly to the N-terminal half of SP-B. Amino acid substitutions were introduced into a synthetic peptide encompassing the N-terminal domain of SP-B (residues 1-37) to assess the contribution of individual amino acids to the fusogenic, lytic and surface properties of SP-B. In general, residues that were important for fusion and lysis were also important for surface activity supporting the hypothesis that these properties are necessary for surface film dynamics.

The role of the N-terminal domain of SP-B in surface activity

The N-terminal sequence of SP-B (residues 1-9) may constitute an essential structural domain required for optimal surface tension reduction. Substitution of the proline residues or tryptophan 9 for alanines (P2,4,6 and W9A) in the N-terminal domain (residues 1-9) of SP-B completely ablated the surface tension reducing property of SP-B in vitro (Figure 2.10); however, these substitutions did not alter
the fusogenic or lytic properties of the peptides. To better understand the role of W9 and P2,4,6 in surface film dynamics, the surface properties of the individual peptides were further characterized (Alicia G. Serrano, Marnie A. Ryan, Timothy E. Weaver and Jesus Perez-Gil; submitted manuscript).

Native SP-B adsorbs rapidly to a membrane interface during compression/expansion cycling and is maintained in the interface at high surface pressures. The surface inactive synthetic peptides W9A and P2,4,6A were tested for their ability to adsorb to an interface under static and dynamic conditions and compared to the surface active peptide N-term helix 1,2. Interfacial adsorption of lipid/protein mixtures was assayed using a specially designed surface balance (Wilhelmy balance) containing a 1.5 ml subphase maintained at 25°C. The rate of adsorption to an air-liquid interface was measured after injection of the mixtures into the subphase. A rapid increase in surface pressure after peptide injection indicates spontaneous adsorption of the peptide into the interface. Injection of the W9A peptide into the subphase resulted in a significant decrease in the rate of adsorption (Figure 4.1) compared to N-term helix 1,2 and P2,4,6A indicating that the tryptophan residue in the N-terminal domain is critical for rapid adsorption to an air-liquid interface.

We next determined whether the synthetic SP-B peptides were able to readily insert into a preformed phospholipid monolayer. The peptides were injected into a subphase underneath an existing phospholipid monolayer. Insertion kinetics of the peptides was measured at different initial surface pressures (π). The SP-B peptides (N-term helix 1,2, P2,4,6A and W9A) were able to perturb and insert into a prepared monolayer at different initial surface pressures (π); however, W9A adsorbed to and inserted into the film at a significantly slower rate than the native sequence or P2,4,6A (Figure 4.2).
Figure 4.1. **Interfacial adsorption of SP-B peptides.** Interfacial adsorption kinetics of SP-B synthetic peptides (N-term helix 1,2, W9A and P2,4,6A) to an air-liquid interface from a buffered subphase (5 mM Tris pH 7.0, containing 150 mM NaCl) (Panels a-c). After 5 minutes of equilibrium, 10 µl of individual peptides in methanol were injected into the subphase at the indicated amounts (µg) and changes in surface pressure were monitored. Control experiments included no peptide and vehicle alone.
Figure 4.2. **Insertion of SP-B peptides into phospholipid monolayers.** Insertion kinetic experiments of SP-B synthetic peptides (N-term helix 1,2-solid line, W9A-dotted line, P2,4,6-dashed line) injected underneath phospholipid monolayers were performed at different initial surface pressures ($\pi_i$). The trough was thermostated at 25°C and the subphase was composed of 5 mM Tris buffer pH 7.0 containing 150 mM NaCl.

Figure 4.3. **Effect of SP-B peptides on the compression dynamics of a surface film.** Compression isotherms of DPPC:POPG (7:3, w/w) monolayers in the presence or absence of the SP-B peptides at the indicated amounts.
Further, compression/expansion cycling of the phospholipid monolayer resulted in the peptide being progressively displaced from the film at high surface pressures (Figure 4.3, note isotherms converge at high surface pressures indicating that the peptide is completely excluded from the phospholipid film). These results suggest that W9 increases the affinity of the peptide for the interface and promotes rapid insertion between phospholipid acyl chains.

A previous study determined the topographical location of residues 1-25 of SP-B and demonstrated that only residues Y7-W9 were embedded in the lipid bilayer (Wang et al., 2003b). Hydropathy plots of the synthetic SP-B peptides (W9A, P2,4,6A and N-term helix 1,2) were calculated using the hydrophobic-at-interface scale proposed by Wimley and White (Wimley et al., 1996) which identifies the putative membrane-interacting domains of peptides. This scale assigns free energy values to individual amino acids based on the energy it takes for a particular residue to transfer from water to a membrane interface. Residues 1-37 of SP-B were evaluated using the hydropathy index and results indicated that residues 7-9 exhibited the highest affinity for phospholipid interfaces of any motif in SP-B (figure 4.4).

Although the peptide P2,4,6A was able to rapidly insert into an expanding interface, most likely because of the hydrophobic domain (Y7-W9) (Figure 4.1), this peptide failed to penetrate into the interface efficiently and produced a much more limited expansion isotherm (Figure 4.3, note area between the curves is reduced). These results suggest that the proline residues (P2,4,6) form a structure in the N-terminal domain of SP-B that is important for orienting the peptide on the lipid monolayer/bilayer and allowing for lipid attachment and insertion. Substitution of the proline residues for alanines resulted in an extension of helix 1 which likely changed the disposition of the aromatic residues on the membrane ultimately affecting how well they penetrated into the interface. Taken together (Chapter II and current studies), these data demonstrate that fusion and lysis alone are not sufficient for surface tension reduction.
The N-terminal domain is required for this property and likely ensures rapid, stable association of the peptide with the surface film.

Figure 4.4  **Hydropathy-at-interface profile using the SP-B peptides (residues 1-37).** Hydropathy plot of the sequences of the SP-B derived peptides calculated using the hydrophobic-at-interface scale proposed by Wimley and White. The assigned free energy value of the individual amino acids in each of the SP-B peptides (N-term helix 1,2-solid line, W9A-dotted line and P2,4,6A-dashed line) is plotted against its position in the sequence.

**Role of SP-B dimerization in lung function**

Although the SP-B synthetic peptide N-term helix 1,2 was effective at lowering minimum surface tension *in vitro*, higher concentrations of peptide were required to achieve optimal surface tension reduction compared to native peptide (Figure 2.6). In addition, native SP-B was fusogenic and lytic at much lower concentrations than the shorter SP-B derivatives (Figures 2.3-2.5). These findings suggested that the tertiary or quaternary structure of SP-B is also important for its function. To test the hypothesis that formation of homodimers is critical for SP-B function, the cysteine residue reported to be involved in SP-B disulfide bond formation was substituted for serine (hSP-B$^{C248S}$) and the protein was targeted to the
distal respiratory epithelium of transgenic mice (Beck et al., 2000). Lung function was not significantly altered in the presence of the transgene even when the mice were bred into the SP-B+/− background. However, further studies demonstrated that SP-B isolated from the transgenic mice was still able to form dimers through a salt bridge between glutamic acid 51 of one SP-B subunit and arginine 52 of another subunit (Zaltash et al., 2000) (Figure 4.5).

Figure 4.5. **Model of two SP-B subunits.** A disulfide bridge between cysteine residues in helix 3 joins two SP-B subunits. Stabilization of the disulfide bridge likely occurs by a salt bridge formed by glutamic acid 51 of one SP-B subunit and arginine 52 of an opposing SP-B subunit.

**Generation and Characterization of hSP-B<sup>C248S/R252A</sup> Transgenic Mice**

To test whether the salt bridge promoted stability of the homodimer, the cysteine residue involved in dimerization (C248) and the arginine residue involved in salt bridge formation (R252) were substituted for serine and alanine, respectively. This protein (hSP-B<sup>C248S/R252A</sup>) was targeted to the distal respiratory epithelium of transgenic mice using the 3.7-kb human SP-C promoter. To determine if the transgene (hSP-B<sup>C248S/R252A</sup>) could reverse the neonatal lethal phenotype in the SP-B<sup>−/−</sup> mice, fertilized oocytes were isolated from SP-B<sup>+/−</sup> females mated with SP-B<sup>+/−</sup> males for pronuclear injection. This strategy would
establish transgenic mouse lines that expressed the transgene (hSP-B\(^{C248S/R252A}\)) in the SP-B\(^{+/+}\) or SP-B\(^{-/-}\) background. Approximately 400 fertilized oocytes were injected but founder lines expressing hSP-B\(^{C248S/R252A}\) were never identified. This outcome suggested that the monomer transgene could not functionally replace endogenous SP-B when placed in the SP-B\(^{-/-}\) background. Unexpectedly, transgene positive mice were not identified in the SP-B\(^{+/+}\) or SP-B\(^{+/+}\) background either (unpublished results). Closer examination of surrogate dams revealed that all transgene positive pups, regardless of their genetic background (SP-B\(^{+/+}\), SP-B\(^{+/+}\) or SP-B\(^{-/-}\)), suffered from neonatal respiratory distress within the first 30 minutes of birth suggesting that the expression of the transgene was incompatible with life.

**Intracellular Processing of hSP-B\(^{C248S/R252A}\)**

To determine if the lethality observed in hSP-B\(^{C248S/R252A}\) mice was related to an inability to process endogenous SP-B or hSP-B\(^{C248S/R252A}\) proprotein to mature SP-B peptide *in vivo*, SP-B processing was analyzed in tissue homogenates prepared from fetal lung of transgene positive pups and wildtype littermates. Western blot analysis revealed that both the monomeric and dimeric forms of proSP-B were processed correctly to their mature forms (Figure 4.6). Unprocessed SP-B proprotein, Mr = approx. 45 kDa, was detected in transgene positive pups (Figure 4.6, lanes 4-6) but not in WT pups; in addition, transgene positive pups contained another processing intermediate Mr = approx. 23 kDa, that was not detected in WT pups. These forms of SP-B were also immunoreactive to an SP-B antibody that only reacts to the N- and C-terminal propeptides (data not shown) further demonstrating that these fragments are unprocessed (Mr = 45 kDa) or incompletely processed (Mr = 25 kDa) forms of the proprotein. It is not clear whether these propeptide forms represent endogenous SP-B or the transgenic monomeric protein. However, the fact that the monomer protein is processed to the mature peptide (Mr = 8 kDa) suggests that at least some of the peptide is being routed out of the ER to the MVB where processing
occurs. Taken together, targeted expression of hSP-B<sup>C248S/R252A</sup> in type II cells of transgenic mice results in an SP-B processing defect that results in the accumulation of unprocessed and partially processed forms of SP-B.

Figure 4.6. **Western blot analysis of fetal lung homogenates from wildtype and transgenic positive pups.** Fetal lungs were harvested on day 18.5 and SP-B proprotein and SP-B mature peptide expression were assessed. Lanes 1-3 represent SP-B protein samples (non-reduced) from wildtype animals and lanes 4-6 represent protein samples from transgenic positive animals (SP-B<sup>+/−</sup> background). SP-B protein was detected using a rabbit polyclonal antibody to the mature form of the peptide.
**Lung structure of hSP-B^{C248S/R252A}**

To determine if the lethality observed in the hSP-B^{C248S/R252A} transgenic mice was due to altered lung structure, H&E staining and ultrastructural analysis was performed on lung sections from transgenic positive pups and wildtype littermate controls. Lung sections of transgenic mice stained with H&E were histologically indistinguishable from wildtype mice indicating that the lethality observed at birth was not due to gross abnormalities in lung structure (data not shown). EM analysis of hSP-B^{C248S/R252A} transgenic mice revealed some areas within type II cells that were characterized by a dilated ER (Figure 4.7 A), abnormal lamellar bodies (Figure 4.7 B) and disorganized or absent tubular myelin (Figure 4.8). These characteristics are similar to those observed in SP-B^−/− mice; however, since mature dimeric SP-B was detected by western blotting it is unlikely that the lethal phenotype is related to SP-B deficiency.

**Role of dimerization in membrane cross-linking**

It is possible that the hSP-B^{C248S/R252A} protein has reduced fusogenic and lytic activities necessary for lung function. The model of SP-B mediated membrane fusion proposed in this work suggested that the cross-linking activity of SP-B is important in bringing two opposing membranes together. SP-B dimerization may be important for this property. An SP-B subunit located on one membrane interacts with another SP-B subunit located on a separate membrane. This type of orientation on lipid bilayers may facilitate membrane cross-linking and fusion. Trafficking of the mutant protein through the distal secretory pathway in the type II cell may result in the hSP-B^{C248S/R252A} protein occupying membrane binding sites that would ordinarily be occupied by endogenous SP-B thereby interfering with peptide-peptide interactions. Reduced fusogenic activity could account for the disorganized lamellar bodies and absence of tubular myelin detected in transgene positive animals.
Figure 4.7. **Ultrastructural analysis of alveolar type II cells from wildtype and transgenic positive pups.**  (A) Dilated endoplasmic reticulum (ER) and (B) deformed and disorganized lamellar bodies were detected within type II cells in transgenic positive animals but not in wildtype littermate controls.
Figure 4.8. **Airway ultrastructure in wildtype and transgenic positive fetal mice.** Tubular myelin (TM) was present in wildtype animals at the alveolar interface but in some type II cells of hSP-B$^{C248S/R252A}$ transgenic mice this form of surfactant was absent or highly disorganized.
**Generation of conditional hSP-B\textsuperscript{C248S/R252A} mice**

To better understand the role of dimerization in lung function and to obtain viable animals at birth, conditional compound transgenic hSP-B\textsuperscript{C248S/R252A} mice were generated. Two transgenic mouse lines were utilized to confer tissue specific conditional gene expression of hSP-B\textsuperscript{C248S/R252A}. A transgenic activator line expressing the reverse tetracycline responsive transactivator (rtTA) under the control of the lung specific promoter clara cell secretory protein (CCSP) directs rtTA expression to the distal lung epithelium. The second transgenic line carries the hSP-B\textsuperscript{C248S/R252A} transgene under control of the tetracycline-operator (tetO). The transgene is induced by binding of doxycycline (dox) to rtTA, which activates the (tetO)\textsubscript{7}CMV target promoter. Potential F\textsubscript{0} (tetO)\textsubscript{7} hSP-B\textsuperscript{C248S/R252A} mice were identified by PCR using transgene specific primers and subsequently bred with transgenic homozygous activator mice (CCSP rtTA). Compound transgenic [CCSP rtTA/(tetO)\textsubscript{7} hSP-B\textsuperscript{C248S/R252A}] offspring were identified by PCR using transgene specific primers and ten independent transgenic lines were identified. SP-B transgene expression was assessed by western blot analysis of BALF after doxycycline treatment (0.5 mg/ml in drinking water) for 72 hours.

**Characterization of CCSP rtTA/tetO\textsubscript{7} hSP-B\textsuperscript{C248S/R252A} mice**

Multiple founder lines expressed the hSP-B\textsuperscript{C248S/R252A} transgene after dox treatment; however, protein levels in BALF were significantly lower than endogenous SP-B (Figure 4.9). Animals expressing hSP-B\textsuperscript{C248S/R252A} were phenotypically indistinguishable from wildtype littersmates and did not suffer from neonatal lethal respiratory distress as did the non-conditional transgenic mice. Detection of the mature form of proSP-B\textsuperscript{C248S/R252A} indicated that the protein was being routed to the MVB and likely being secreted. It is not clear why only low levels of transgene protein were secreted into the airways. To
further characterize the transgenic mice, a more detailed examination of the expression of both mRNA and protein levels in whole lung tissue of hSP-B$^{C248S/R252A}$ mice should be determined.

In order to increase the ratio of mutant to wildtype SP-B in the compound transgenic animals two strategies were employed: (1) pregnant dams were given doxycycline in the drinking water from day 0 of gestation to stimulate transcription of the SP-B transgene during fetal lung development and (2) compound transgenic mice were bred into the SP-B$^{+/\text{null}}$ background to reduce levels of endogenous SP-B by half. Despite both of these strategies, levels of the transgene protein were still not high enough to induce a phenotype. Another possible strategy would be to breed the (tetO)$_7$ hSP-B$^{C248S/R252A}$ transgenic mice into the SP-B$^{+/\text{null}}$ background or with transgenic mice expressing rtTA under control of the 3.7-kb human SP-C promoter.

Transgenic mice expressing SP-C-rtTA/tetO-Luc were compared to CCSP-rtTA/tetO-Luc to determine levels of expression after dox treatment during fetal development (Perl et al., 2002). SP-C-rtTA/tetO-Luc mice expressed luciferase at detectable levels in the absence of dox and early in gestation (E10.5) while luciferase expression in CCSP-rtTA/tetO-Luc mice was strictly inducible and detected later in gestation (E12.5). Low expression of the hSP-B$^{C248S/R252A}$ transgene in addition to the later induction may allow for enough endogenous SP-B to be secreted into the airspaces to form a surface active monolayer. The monomeric form of SP-B may be excluded from incorporating into the surface film and this may explain why low levels of transgene protein were detected in the BALF. Further, exclusion of the peptide from the airspaces may also account for the lack of respiratory distress at birth. It is possible that constitutive expression of hSP-B$^{C248S/R252A}$ in transgenic mice resulted in both the monomeric and dimeric SP-B forms being synthesized and secreted at similar timepoints allowing for enough monomeric peptide to reach the surface film and interfere with the surface tension reducing properties of surfactant.
Figure 4.9. **Expression of hSP-B$^{C248S/R252A}$ mice in the presence of doxycycline.** Compound transgenic mice were maintained on doxycycline from fetal development through 6 weeks of age. BALF was performed using three 1-ml aliquots of sterile PBS and large aggregate surfactant was isolated. Equal amounts of surfactant pellet were subjected to SDS-PAGE under non-reducing conditions for analysis of SP-B mature peptide. The monomeric form of SP-B migrates at approx. 8 kDa and was detected in compound transgenic mice but not in WT mice.

**Surfactant replacement therapies using synthetic SP-B peptides**

N-term helix 1,2 (residues 1-37) was the smallest peptide capable of optimal surface activity in vitro (Figure 2.6). Surfactant therapies based on residues 1-25 (N-term helix 1 plus the interhelical loop between helix 1 and 2) of SP-B have proven successful in two animal models of surfactant insufficiency but require the presence of palmitic acid (Bruni et al., 1991; Bruni et al., 1998). Studies presented here argue that longer peptides are more effective at reducing minimum surface tension, even in the absence of palmitic acid. The model of surface film dynamics proposed in this work (Figure 2.11) suggests that helix 2 is required for membrane cross-linking, a process that is likely involved in membrane fusion and surface activity. A potential drawback to including helix 2 in a synthetic analog is that this domain is extremely hydrophobic and will dramatically increase the overall hydrophobicity of the peptide making it difficult to express in bacteria. It would be advantageous to identify the shortest domain within helix 2 that still
permits membrane cross-linking. Although N-term helix 1,2 was capable of reducing minimum surface tension, higher concentrations of the peptide were required to achieve this effect compared to the native peptide. Although not conclusive, the results obtained from the monomeric transgenic mice indicated that dimerization is also important for SP-B function. Taken together, synthetic peptides that encompass both helices 1 and 2 and are dimerized may provide an excellent surfactant replacement peptide.

**Role of SP-B mediated fusion and lysis in SP-C processing and lamellar body structure**

After identifying the domains required for SP-B mediated fusion and lysis, amino acids substitutions were systematically introduced into the SP-B peptide N-term helix 1,2 to identify residues important for fusion and lysis. In general, residues that were important for SP-B mediated fusion were also important for membrane lysis; however, the proline residue between helices 1 and 2 (P23) was shown to be absolutely critical for fusion but not required for membrane lysis. This substitution will allow us to test the hypothesis that SP-B mediated fusion and/or lysis is important for lamellar body structure whereas only SP-B mediated lysis is required for SP-C processing. A construct containing the SP-B<sup>P23A</sup> substitution was cloned into a bicistronic vector expressing GFP. Transfection of this construct into conditional SP-B knockout type II cells will allow us to determine if the SP-B<sup>P23A</sup> protein can functionally replace wildtype SP-B. Based on the model of SP-C processing previously proposed (Weaver et al., 2001), it is predicted that a fusogen deficient SP-B peptide will still correct the misprocessing of SP-C that is detected in SP-B deficient type II cells (Figure 1.4). Membrane lysis of the internal vesicles of the MVB may be sufficient to allow entry of the proteolytic enzymes for cleavage of the N-terminal propeptide of SP-C. Unlike SP-C processing, organization of phospholipids in lamellar bodies likely requires both SP-B mediated membrane fusion and lysis. Phospholipid membranes from the MVB must
reorganize and incorporate into the membrane sheets of the lamellar body. A fusogen deficient SP-B protein would at best partially restore the disorganized lipids in lamellar bodies of SP-B<sup>−/−</sup> type II cells.

A major limitation to the in vitro approach is that an efficient transfection protocol for type II cells has not been established. Limited success has been obtained with liposome based protocols as well as electroporation techniques. An alternative approach is to generate transgenic mice that express SP-B<sup>P23A</sup> in the SP-B<sup>−/−</sup> background to assess the role of SP-B mediated membrane fusion in vivo. It is possible that these animals will suffer from lethal respiratory distress at birth due to the requirement of membrane fusion for surface film dynamics; however, western blot analysis of fetal lung tissue from day 18.5 would identify whether SP-C was being processed correctly. In addition, ultrastructural analysis of type II cells from day 18.5 fetal lung tissue would reveal the structure of lamellar bodies in SP-B<sup>P23A</sup> expressing mice.

**Role of SP-B lysis in alveolar sterility**

The results of the mapping experiments in Chapter II identified a 13 residue domain (helix 1) in SP-B that contained potent membranolytic activity. To test the hypothesis that the lytic property of SP-B is important for lung host defense, native SP-B and synthetic peptides derived from SP-B were tested for antimicrobial activity. Chapter III described two important findings: (1) Native SP-B exhibited potent antimicrobial activity against clinical isolates of Gram-positive and Gram-negative bacteria in vitro; however, in its native environment, SP-B probably does not act as an antimicrobial peptide due to the fact that bacterial killing was significantly inhibited in the presence of surfactant phospholipid mixtures. These findings settle a long standing debate regarding the role of SP-B in host defense. (2) Synthetic peptides derived from native SP-B also have potent antimicrobial activity in vitro; however, unlike the native peptide, synthetic peptide derivatives may be very useful for treatment of bacterial pneumonias.
Strategies to improve bacterial killing by the SP-B derivatives

The SP-B derivatives (helix 1 and helix 1,2) were effective at killing bacteria at low peptide concentrations and exhibited antimicrobial activity in the presence of surfactant phospholipid mixtures. In addition, the peptides were selective towards bacterial membranes and did not lyse red blood cells. Although these properties make the SP-B peptides attractive candidates for treatment of bacterial infections, slight changes to the structure of the peptides could improve effectiveness in vivo. The positively charged residues in helix 1 facilitate the interaction of the peptide with negatively charged membranes (Baatz et al., 1990; Vandenbussche et al., 1992). (1) Reducing the net positive charge of helix 1 may reduce the lipid inhibition effect while maintaining the membranolytic activity of the peptide. Experiments in chapter III demonstrated that substitution of a single positively charged amino acid in helix 1 in the context of the synthetic peptide N-term helix 1,2 had no affect on bacterial killing indicating that a slight reduction in charge was tolerated. (2) SP-B is very hydrophobic and is always associated with lipids. Decreasing the overall hydrophobicity of the peptides may also reduce sensitivity to lipid inhibition. Systematic substitution of two hydrophobic residues on the hydrophobic face of helix 1 in the context of N-term helix 1,2 were made (Chapter II) and the peptides were tested for bacterial killing. Slight changes to the hydrophobic face of SP-B did not alter the ability of the SP-B peptides to kill bacteria (Figure 4.10). SP-B helix 1 (residues 7-22) or helix 1,2 (residues 7-37) peptides containing single positively charged amino acid substitutions or hydrophobic substitutions could be designed and tested for antimicrobial activity and lipid sensitivity.
Figure 4.10. **SP-B mediated *Klebsiella pneumoniae* killing.** Double amino acid substitutions were introduced into the SP-B synthetic peptide N-term helix 1,2 (residues 1-37) to assess the contribution of the hydrophobic residues for bacterial killing. Individual SP-B peptides (2.5 µM) were added to $10^3$ CFU/100 µl of *K. pneumoniae* and incubated for 6 h at 37°C. Bacteria were dispersed, plated on blood agar plates and incubated overnight at 37°C to quantitate colony counts. n=3

**Strategies for testing SP-B synthetic peptides in vivo**

There are two significant obstacles that need to be overcome in order to test the SP-B synthetic peptides in vivo. (1) Preparation of large scale quantities of the peptide(s) would be necessary to achieve sufficient amounts required for animal model studies. Bacterial expression of SP-B has been largely unsuccessful probably due to the fusogenic and lytic properties of the peptide as well as its overall high hydrophobicity. These characteristics cause SP-B to be sequestered in inclusion bodies of bacteria resulting in a denatured recombinant protein that must be refolded to its native state. Strategies to circumvent this problem have included the generation of smaller SP-B fragments with lower overall hydrophobicity but the peptides were still retained in inclusion bodies.

Alternatively, recombinant proteins can be expressed in insect cells. This type of expression system allows for the expression of more hydrophobic proteins that would ordinarily be problematic for
bacteria. Constructs were generated that expressed the N-terminal propeptide in addition to the mature peptide to ensure proper routing/secretion and to “hide” hydrophobic domains of the mature peptide. A factor X cleavage site was introduced between the N-terminal propeptide and the mature peptide of SP-B. This strategy would allow for removal of the N-terminal propeptide after secretion of the peptide into the media. High expression levels of proSP-B were achieved in insect cells and the protein was efficiently secreted (data not shown); unfortunately, attempts at removing the N-terminal propeptide from the mature peptide were unsuccessful. Other types of cleavage sites were introduced at the N-terminal propeptide/mature peptide junction including a hydroxylamine cleavage site and an acid cleavage site. Because both of these strategies were also unsuccessful it was determined that the junction between these two domains was inaccessible for cleavage and that the removal of the N-terminal domain is likely a multi-step process with cleavage occurring first at sites proximal to the one that was inserted (Ueno et al., 2004).

The most attractive and technically attainable approach is to synthesize the peptide(s) in large scale amounts by chemical methods. Synthesis of the peptide(s) in the quantities needed for animal model studies could be accomplished in a relatively short period of time. One drawback would be the expense associated with the production of large scale quantities of synthetic peptides; however, the advantage of this approach is that the peptides have already been tested in vitro (Chapter III) and based on CD analysis (Chapter II) the peptides retained a significant amount of helical structure compared to the native peptide indicating that they are folded correctly.

The second challenge would be to design an effective delivery system for administration of the SP-B peptides in vivo. Since the peptides are only soluble in methanol, delivery into the airways would need to occur using a different vehicle. Studies in Chapter III revealed that the SP-B peptides retained significant antimicrobial activity in the presence of DPPC liposomes. Liposomes have been used
successfully for the delivery of aerosolized asthma medications (Konduri et al., 2005), proteins associated with inhibiting tumor growth (Dutour et al., 2005) and gene transfer (Luton et al., 2004). It is likely that the SP-B peptides could be delivered into the airways of mice using similar methods. To determine whether the SP-B peptides could kill bacteria in vivo, bacterial clearance and survival studies would be performed after intratracheal (i.t.) administration of *Klebsiella pneumoniae* followed by i.t. administration of increasing concentrations of the SP-B peptide/DPPC complexes. It is expected that the SP-B peptides will significantly improve bacterial clearance in mice compared to liposome-only control groups.

**Role of SP-B peptides in anti-cancer therapy**

There is an emerging field of research describing the role of host defense peptides in cancer treatment. Several antimicrobial peptides that are highly potent against a broad spectrum of bacteria are also effective at lysing cancer cells while having no effect on normal mammalian cells (cecropins and magainins) (Steiner et al., 1981; Zasloff, 1987; Cruciani et al., 1991; Baker et al., 1993; Chen et al., 1997). The mechanism underlying the membrane specificity exhibited by these antimicrobial peptides is not clear. Like bacterial membranes, cancer cells also have a more negatively charged surface compared to normal eukaryotic cells. Slight increases in the amount of the negatively charged phospholipid, phosphatidylserine (PS), have been found on the surface of tumor cells (Verkleij et al., 1973). In addition, the membranes of cancer cells also contain mucins which creates an additional negative charge on the surface of the membrane. Despite these subtle differences in cancer cells and non-cancer cells, some cationic antimicrobial peptides selectively disrupt the membranes of tumor cells making these peptides potential therapies for treatment of cancer.

Drug resistance is a clinical problem for cancer patients (Fojo et al., 2003). The drug compounds presently used for cancer treatment generally have intracellular targets and require membrane penetration in order to function. Several mechanisms by which tumor cells can avoid cell death have been described.
For example, drugs can be prevented from entering the cells, drugs can be pumped out of the cell or drugs can be enzymatically inactivated within the cell (Komarova et al., 2005). Peptide based therapies may provide significant benefits because killing is mediated by membrane lysis and does not require an internal target. Identification of antimicrobial peptides that display a wide spectrum of anti-tumor activity, kill cancer cells rapidly and do not affect non-cancerous mammalian cells would be highly desirable. Based on the membrane selectivity exhibited by the SP-B peptides, it is possible that these peptides may also be active against cancer cells.

As a first step in determining whether the SP-B peptides might be useful for cancer therapy, the peptides will be evaluated for their ability to disrupt membranes of cancer cells without affecting normal cells. Increasing concentrations of the SP-B peptide(s) will be added to individual wells seeded with a carcinoma cell line such as SAS-H1 (derived from a human oral squamous cell carcinoma cell line) or a normal cell line (293, RAW). The number of dead cells will be evaluated microscopically by counting cells that exclude trypan blue. Alternatively, the cells will be stained with Syto9 and propidium iodide (LIVE/DEAD Viability/Cytotoxicity kit; Molecular Probes Inc., Eugene, OR) to verify dead/dying cells. Percent cytotoxicity will be compared to controls. It is expected that the SP-B peptides will selectively kill tumor cells without affecting normal cells (as demonstrated by the lack of hemolytic activity).

**Summary/Conclusions**

The overall theme of the studies presented here is that SP-B has a dramatic effect on membrane structure by disrupting and/or remodeling membranes. Surfactant phospholipid reorganization occurs throughout the distal secretory pathway of the type II cell as well as extracellularly at the air-liquid interface. Experiments in chapter II mapped the fusogenic and lytic domains of SP-B. In addition, individual amino acids were identified that were critical for membrane fusion and lysis and the
importance of these residues for surface activity was assessed. The results clearly demonstrated that the fusogenic and lytic properties of SP-B were required for optimal surface activity. Chapter III further characterized the lytic property of SP-B and assessed the role of membrane lysis in bacterial killing of lung pathogens. Native SP-B killed clinical isolates of both Gram-positive and Gram-negative bacteria in vitro; however, SP-B also lysed human red blood cells indicating that the membranolytic activity of the peptide was not selective for bacteria. Both the antimicrobial and hemolytic activities were inhibited by surfactant phospholipids suggesting that endogenous SP-B may not play a significant role in host defense. SP-B derivatives were identified that selectively killed bacteria and were more resistant to inhibition by phospholipids indicating that the SP-B peptides may be useful in the treatment of bacterial pneumonias. Taken together, these data demonstrate a critical role for membrane remodeling in SP-B lung function.
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