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Disease-linked mutations in surfactant protein C (SP-C) cause ER stress and increase susceptibility to viral-induced cell death

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by
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ABSTRACT

Mutations in the gene encoding surfactant protein C (SP-C), *SFTPC*, are associated with both sporadic and familial ILD in children and adults; however, it is unclear if this relationship is causal in nature. The overall goal of this dissertation study was to determine whether a disease-linked mutation in the C-terminal peptide of SP-C causes lung disease. Expression of the index mutation, SP-C\(^{\Delta exon4}\), in type II epithelial cells of transgenic mice resulted in a dose-dependent perturbation of lung development associated with epithelial cell cytotoxicity (Chapter 2). Transient expression of SP-C\(^{\Delta exon4}\) in isolated type II epithelial cells or HEK293 cells resulted in: 1) incomplete processing of the mutant proprotein, 2) a dose-dependent induction of the unfolded protein response, 3) trapping of the mutant proprotein in the ER and 4) rapid degradation via ERAD (Chapters 2 and 3). Further, constitutive expression of SP-C\(^{\Delta exon4}\) resulted in an adaptive, cytoprotective response involving NF-κB and increased susceptibility to RSV-induced cell death associated with accumulation of the mutant proprotein (Chapter 3). Collectively, these results suggest that adaptation to chronic ER stress imposed by misfolded SP-C may promote resistance to ILD while environmental insults, such as viral infection, may trigger the onset of disease in patients with mutations in *SFTPC*.
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ABBREVIATIONS

EOR – endoplasmic reticulum overload response
ER – endoplasmic reticulum
ERAD – endoplasmic reticulum-associated degradation
HEK293 – human embryonic kidney cell line
IIP - idiopathic interstitial pneumonia
ILD- interstitial lung disease
NF-κB – nuclear factor of kappa light chain gene enhancer in B-cells
PI – propidium iodide
RSV – respiratory syncytial virus
SP-C – surfactant protein C
UPR – unfolded protein response
CHAPTER I

INTRODUCTION
Lung development and function

Mammalian lung morphogenesis commences at embryonic day 9-9.5 (E9.5) in the mouse and 5 weeks of gestation in humans when cells from the ventral foregut endoderm (VFE) invade into the surrounding splanchnic mesenchyme, resulting in the formation of a tracheal rudiment (Figure 1.1). This epithelial rudiment separates from the primitive esophagus, further invading the supporting mesenchyme to form two primary bronchi lung buds which, in turn, subsequently branch to form the proximal pulmonary tree consisting of the trachea, primary bronchi, bronchioles and terminal bronchioles that terminate in distal sac-like structures destined to become alveoli in the postnatal period

Figure 1.1 – Diagram of embryonic lung branching morphogenesis in the mouse (adapted from Warburton et al. (Warburton et al. 2000). A) Tracheal rudiment budding from ventral foregut endoderm (VFE) at E9.5. B) Pulmonary tree terminating into alveoli.
(Warburton et al. 2000). Endothelial and smooth muscle cell precursors, present in the mesenchyme as soon as the initial lung buds arise, promote formation of a vascular network that develops in parallel with the conducting airways and eventually enrobes the alveoli (Figure 1.2). The tissue between the mature capillaries of the microvasculature and the alveolar epithelium, referred to as the interstitium, thins markedly towards the end of gestation, resulting in the close juxtaposition of type I epithelial cells and capillary endothelial cells. Formation of this thin, blood-gas barrier facilitates diffusion of the respiratory gases oxygen and carbon dioxide between the alveolar wall and the bloodstream. These elegantly orchestrated developmental processes culminate in a mature, functional lung consisting of

![Diagram of microvasculature enrobing alveoli with conducting vessels](image)

**Figure 1.2 – Diagram of microvasculature enrobing alveoli with conducting vessels** (adapted from Vander, Sherman, Luciano’s Human Physiology text (Vander et al. 1985)).
approximately 300 million alveoli with an estimated gas exchange surface area of 150 m² (Benne et al. 1995).

Differentiation of the fetal respiratory epithelium occurs concomitantly with branching morphogenesis to generate multiple cell types including ciliated, Clara (non-ciliated), goblet, serous, neuro-epithelial bodies and basal cells in the proximal airways and type I and type II cells in the terminal sacs/alveoli. Both epithelial cell differentiation and branching morphogenesis are dependent on numerous developmentally regulated transcription factors, including members of the Nkx e.g. thyroid transcription-1 (TTF-1), GATA and Fox families (for review see (Costa et al. 2001)). Inductive autocrine-paracrine signaling between the mesenchyme and adjacent epithelia is essential for lung morphogenesis and is modulated in part through the fibroblast growth factor (FGF) family, b-catenin/WNT pathway, bone morphogenetic protein-4 (BMP-4) and sonic hedgehog (Shh) (Warburton et al. 2000; Shannon et al. 2004). The complex interplay among these secreted factors and their directed transcriptional responses result in the formation of conducting airways and an extensive gas exchange surface.

Maintenance of the cellular constitution and shape of the alveolus is critical for proper gas exchange to occur. The wall of the alveolus consists of four layers: 1) alveolar epithelial cells (type I and type II); 2) basement membrane of the alveolar epithelial cells; 3) basement membrane of the capillary endothelial cells; 4) capillary endothelial cells (Fig 3). Alveolar type I cells are large epithelial cells that comprise approximately 90% of the alveolar surface (Mason et al. 1997). Type I cells have a very thin profile which allows for the shortest diffusion distance of gas between the alveolar space and the capillaries. Alveolar type II cells are the immediate progenitors of type I cells and rapidly repopulate the alveolar epithelium in response to injury (Evans et al. 1975; Bowden 1981; Uhal et al. 1995). Type II cells are cuboidal, polarized epithelial cells that synthesize and secrete all of the components of pulmonary surfactant. Elastic fibers in the alveolar wall combined with surface tension resulting from a thin liquid layer lining the epithelium generates a high collapsing force at end expiration. Spreading of pulmonary surfactant, a phospholipid-rich film containing the hydrophobic proteins surfactant protein B (SP-B) and surfactant protein C (SP-C), across the alveolar surface dramatically reduces surface tension and prevents collapse of the alveolus at end
expiration. Prevention of alveolar collapse, atelectasis, is critical for proper gas exchange across the air-blood barrier. The space between the basement membranes of the capillaries and alveolar epithelial cells is referred to as the interstitium. The interstitium contains the connective tissue elements of the lung, including collagen (types I and IV), proteoglycans, glycoproteins, fibronectin, laminin and elastin, and small numbers of macrophages, fibroblasts and myofibroblasts. In a healthy lung, the region of the interstitium between the type I cell and the endothelial cell contains a minimal amount of connective tissue elements and cells (Figure 1.3). This anatomical arrangement allows for the closest apposition of these cell types to maximize gas exchange between the alveoli and the bloodstream. Alterations in the architecture of the interstitium can lead to chronic lung disease by impeding gas exchange and decreasing the elasticity of the lung.

Figure 1.3 – Cross sectional diagram of alveolus (adapted from Vander, Sherman, Luciano’s Human Physiology text (Vander et al. 1985)).
Interstitial Lung Disease

Interstitial lung diseases (ILDs) are a heterogenous group of more than 200 disorders in adults and children that cause disruption of the alveolar structures (Green 2002). Individuals diagnosed with ILD usually present with progressive lung disease associated with tachypnea and hypoxemia. Although the exact incidence of ILD is difficult to determine, it is estimated to be 31.5 per 100,000 for men and 26.1 per 100,000 for women worldwide (Coultau et al. 1994). While the pathological manifestations of this group of diseases are diverse and may include alveolar wall denudation and/or collapse, fibroblast proliferation, and the infiltration of inflammatory cells, the common final pathway found in all ILDs is damage/dysfunction of the alveolar epithelium and fibrosis of the lung.

Idiopathic interstitial pneumonias (IIPs) represent a subset of ILD, each with unique histological and clinical classifications, which are characterized by alveolar inflammation and progressive fibrosis of the lung with unknown etiology. The IIPs are comprised of six categories based on histological, clinical, radiographical and pathologic diagnoses: desquamative interstitial pneumonia (DIP), respiratory bronchiolitis (RB)-associated ILD, acute interstitial pneumonia (AIP), non-specific interstitial pneumonia (NSIP), lymphocytic interstitial pneumonia (LIP) and idiopathic pulmonary fibrosis (IPF) (Crystal et al. 2002). Of the IIPs, IPF is the most prevalent and devastating with a mean survival time of 28.2 months following diagnosis (Schwartz et al. 1994). The histological diagnosis that delineates IPF from the other IIPs is usual interstitial pneumonia (UIP) which is characterized by a variegated appearance of fibroblastic foci and honeycombing of the lung, patchy collagen deposition and low levels of interstitial inflammation (Crystal et al. 2002). Honeycombing refers to the honeycomb-like appearance of the lung upon high-resolution radiographic imaging and is characterized by the presence of large, cystic sacs ranging from several millimeters to 1 centimeter in diameter (diameter of normal alveolus is $\sim$200$\mu$m); this process is due to relentless obliteration of the alveolar walls during end-stage disease.

While ILD is historically diagnosed in the adult population, children are also susceptible to ILD. Common forms of ILD found in adults are also found in children, including UIP, DIP and LIP, but the frequency differs from that found in adults. For
instance, in three retrospective studies evaluating 137 children with ILD, only 2 patients were diagnosed with UIP, 4 with DIP and 17 with LIP (Fan et al. 1992). Therefore, while the classic forms of ILD exist in children, distinct disease processes seem to be occurring in this patient population, possibly due to the fact that the pathogenic process occurs in the context of lung growth and cellular differentiation in children (Fan et al. 2004).

Due to the severity of disease and high mortality rate of patients with IPF, this disease has been the focus of much clinical and basic research in the recent past. It is generally assumed that pathogenic sequence in IPF is initiated by injury to the alveolar epithelium associated with an inflammatory insult. Lacking definitive etiology, a large number of environmental agents have been implicated in the initiation of inflammation in IPF including bacteria, fungi, viruses and inhaled antigens. A central paradigm in the field is that the pathogenesis of IPF results from chronic inflammation. Consistent with this concept, transgenic mouse models that overexpressed pro-inflammatory cytokines in the lung, such as IL-1β, TGF-β, and TNF-α, resulted in persistent inflammation and the development of fibrosis (Fujita et al. 2001; Kolb et al. 2001; Hardie et al. 2004). Conversely, ablation of genes encoding pro-inflammatory cytokines or mediators in mice that have been associated with IPF in humans, including ifn-γ, smad3, cytoplasmic phospholipase (cpla2) lipoxygenase (5-LO), decreased the severity of inflammation and lung fibrosis induced by the chemotherapeutic drug bleomycin (Chen et al. 2001a; Nagase et al. 2002; Peters-Golden et al. 2002; Zhao et al. 2002). However, the relative lack of inflammatory infiltrates found in lung biopsy specimens from patients with IPF combined with the failure of anti-inflammatory therapy to significantly improve the outcome in these patients is at odds with the theory that fibrosis results from a incessant inflammatory reaction. Alternatively, Selman and King have proposed that the fibrotic process could be secondary to an inflammation-induced injury to the alveolar epithelium, resulting from a perpetual cycle of injury to and abnormal wound healing of the epithelium following the initial inflammatory insult (Selman et al. 2001). A causal role of inflammation in the pathogenesis of IPF, as well as other IIPs such as DIP and NSIP, remains to be determined.
Several reports exist for the familial transmission of IPF in multiple kindreds, suggesting that genetic mutations may play a causal or influential role in the disease process. In the reported kindreds, IPF is frequently transmitted in an autosomal dominant fashion with incomplete penetrance (for review see (McCormack 2003)). While an exact figure is difficult to calculate, a study in which 29 pulmonary clinics in Finland were screened estimated the frequency of familial IPF to be between 3.3 to 3.7% of all IPF in the country (Hodgson et al. 2002). The selection of candidate genes for the cause of familial IPF has traditionally been focused to cytokines and inflammatory mediators. Indeed, multiple polymorphisms have been identified in the human leukocyte antigen (HLA) locus associated with IPF (Varpela et al. 1979; Libby et al. 1983). Recently, mutations in the gene encoding surfactant protein C, \textit{SFTPC}, have been associated with various forms of familial IIP in adults and children (Nogee et al. 2001; Nogee et al. 2002; Thomas et al. 2002; Brasch et al. 2004; Chibbar et al. 2004; Hamvas et al. 2004; Cameron et al. 2005; Stevens et al. 2005). However, causal relationships between the polymorphisms/mutations and the development of lung disease remained to be determined experimentally.

\textit{Surfactant Protein C}

Surfactant protein C (SP-C) is a single spanning, transmembrane protein that is synthesized and secreted as a component of pulmonary surfactant by alveolar type II cells of the lung. Pulmonary surfactant is a complex mixture of phospholipids and proteins that reduces surface tension along the air-liquid interface of the alveolus, thereby preventing alveolar collapse at end expiration. The importance of surfactant for normal lung function is underscored by the high prevalence of respiratory distress syndrome (RDS) in premature babies whose immature lungs lack surfactant (Farrell et al. 1975). Native surfactant and synthetic phospholipid preparations containing SP-C are highly effective in treating RDS of immaturity in humans (Robertson et al. 1998) and surfactant-depleted animals (Hafner et al. 1995; Hawgood et al. 1996; Davis et al. 1998a).

Four peptide components of surfactant have been identified: surfactant protein (SP)-A (SP-A), SP-B, SP-C and SP-D. The hydrophilic proteins SP-A and SP-D are members of the collectin family that bind to and facilitate the clearance of inhaled
pathogens from the lung, ensuring a sterile alveolar environment. In contrast, SP-B and SP-C are hydrophobic, lipid-associated proteins that are critical for the formation, organization and function of the surfactant film. Due to their hydrophobicity and high affinity for lipids, both SP-B and SP-C are synthesized as proprotein precursors and processed to mature forms in type II cells prior to secretion into the alveolus.

SP-C proprotein is highly conserved across all species for which it has been sequenced, from mouse to man (Weaver et al. 2001). SP-C is synthesized as a 191 or a 197 amino acid proprotein in humans due to alternative splicing of the mRNA transcript (Glasser et al. 1988). The proprotein consists of the mature peptide (residues 24-58) flanked by N-terminal (residues 1-23) and C-terminal (residues 59-191/197) peptides (Figure 1.4). Early in its biogenesis, the proprotein is inserted into the membrane of the endoplasmic reticulum (ER) in a type II orientation such that the N-terminal peptide resides in the cytoplasm and the C-terminal peptide resides in the lumen of the ER (Keller et al. 1991; Russo et al. 1999; Conkright et al. 2001). Trafficking of the proprotein through the regulated secretory pathway to the lamellar body, the major storage site of surfactant, is dependent upon signals encoded by the N-terminal propeptide (Keller et al. 1991; Conkright et al. 2001) and may be facilitated by oligomerization as the SP-C proprotein has been shown to form dimers and oligomers in transiently transfected A549 cells (Wang et al. 2002).

The N- and C-terminal propeptides are cleaved in the late endosomes/multivesicular bodies of the distal secretory pathway to generate the mature, bioactive peptide which is comprised predominantly of a hydrophobic, α-helical transmembrane region and a 12 amino acid, N-terminal extramembrane domain (Beers et al. 1995; Vorbroker et al. 1995b). The hydrophobic nature of the mature peptide stems from the disproportionate number of valine, leucine and isoleucine residues in the transmembrane domain and is further increased, in most species, by the presence of palmitoyl groups attached to cysteines 5 and 6 (Curstedt et al. 1990; Vorbroker et al. 1992; Gustafsson et al. 1997). Palmitoylation has been shown to stabilize the α-helical confirmation of the mature peptide in vitro (Johansson et al. 1995; Johansson et al. 1997; Johansson 1998) and depalmitoylated SP-C transforms into a β-sheet
Figure 1.4 – Illustration depicting primary amino acid structure of SP-C proprotein. N-terminal peptide is shown in green, mature peptide in red and C-terminal peptide in blue (top). Type II orientation of SP-C proprotein in ER membrane; N-terminal peptide in cytosol, C-terminal peptide in ER lumen (bottom, right). Proteolytic cleavage of N- and C-terminal peptides in the late secretory pathway results in liberation of the 35 amino acid mature peptide (bottom, left).

conformation with subsequent amyloid fibril formation in vitro at a higher rate than native SP-C (Veldhuizen et al. 1998; Gustafsson et al. 2001). Furthermore, amyloid fibrils containing aggregated, depalmitoylated SP-C have been detected in surfactant isolated from patients with pulmonary alveolar proteinosis (PAP) (Veldhuizen et al. 1998; Gustafsson et al. 1999). These data are consistent with a role for the palmitoyl groups in stabilizing the native conformation of SP-C. The C-terminal peptide is dispensable for trafficking of the mature peptide through the secretory pathway (Conkright et al. 2001); however, with one exception, all of the mutations in SFTPC associated with IIPs map to this domain.
**SP-C mutations and IIP**

The index mutation in *SFTPC* was first identified in an infant diagnosed with NSIP at six-weeks of age (Nogee et al. 2001). The mutation was present on only one allele and appeared to be familial since the mother also carried the mutation and both the mother and grandfather were afflicted with lifelong lung disease. The mother’s lung disease worsened shortly after childbirth, ultimately leading to her demise. The mutation was a heterozygous base substitution of A for G at the first base of intron 4 (c.460+1 G>A) that led to the internal deletion of 37 amino acids from the C-terminal peptide, generating a truncated proprotein (SP-C<sup>Δexon4</sup>) (Figure 1.5). Low levels of wild-type SP-C (SP-C<sup>wt</sup>) protein were detected in lung tissue and bronchoalveolar lavage fluid of the patient, consistent with a dominant negative effect of the mutant allele leading to a loss of function. The loss of function of wild-type SP-C may indeed be play a role in the pathogenesis of disease as ablation of the SP-C gene in mice, *Sftpc*, caused a pulmonary disorder consistent with interstitial pneumonitis (Glasser et al. 2003); similarly, the lack of SP-C in the airways of human patients, in the absence a *SFTPC* mutation, was associated with familial interstitial lung disease (Amin et al. 2001).

A separate *SFTPC* mutation associated with familial IIP was reported for two separate kindreds each spanning 5 generations (Thomas et al. 2002; Chibbar et al. 2004). The missense mutation was heterozygous, similar to the c.460+1 G>A mutation, and was also in the region encoding the C-terminal peptide of SP-C, resulting in a substitution of glutamate for lysine at codon 188 of the proprotein (SP-C<sup>L188Q</sup>). The mutation was found in children diagnosed with NSIP and adults diagnosed with UIP/IPF in both kindreds. The penetrance of the disease was incomplete and the age of onset of the disease was markedly variable, ranging from 4 months to 57 years in both kindreds. Interestingly, 3/4 children in one kindred (Thomas et al. 2002) and 2/3 children from the other who had the mutation were diagnosed with viral infections prior to the onset of disease, suggesting that inflammatory insults may play a role in the pathogenesis of disease.

A third distinct missense mutation in *SFTPC*, SP-C<sup>I73T</sup>, was detected in association with familial pulmonary fibrosis in a kindred spanning 4 generations (Cameron et al. 2005) and *de novo* in an infant with NSIP and PAP (Brasch et al. 2004).
Subsequent screening of 116 patients with idiopathic IIP revealed 7 additional carriers, making SP-C<sup>I73T</sup> the most common SFTPC mutation to date. Similar to the L188Q kindreds, the penetrance of disease was incomplete as parents of 5/7 affected patients also carried the mutation but were asymptomatic. Single nucleotide polymorphism (SNP) analysis suggested that the SP-C<sup>I73T</sup> mutation occurred in at least 5 distinct genetic backgrounds (Cameron et al. 2005). Abundant staining for the SP-C proprotein in immunohistochemical sections, in contrast to that for the c.460+1 G>A mutation, suggested involvement of mechanisms other than loss of function.

Collectively, a total of 13 mutations have been identified in the SFTPC locus that are associated with familial and sporadic lung disease (Table 1.1). All of the mutations are heterozygous in nature, consistent with a dominant negative effect of the mutant SP-C proprotein. The penetrance and the age of onset of lung disease is variable in the three reported kindreds that are associated with SFTPC mutations, suggesting that environmental and/or genetic factors may modulate pathogenesis. The diagnoses of viral infection prior to the onset of disease in patients from two separate kindreds is consistent with the concept that viral infection may play a role in the pathogenesis of lung disease in patients with SFTPC mutations. Finally, all but one of the reported mutations, P30L, map to the C-terminal peptide of the SP-C proprotein. Although the function of the C-terminal domain is unknown, its orientation in the lumen of the ER subjects SP-C to scrutiny by ER quality control mechanisms.
Protein folding, ER quality control and the unfolded protein response (UPR)

The first destination for proteins that travel through the secretory pathway is the ER where the protein concentration has been estimated to approach ~100mg/ml (Stevens et al. 1999). The ER also serves as the site for phospholipid and sterol biosynthesis and is a major site for intracellular Ca$^{2+}$ storage. The environment of the ER serves to promote protein folding, the process which polypeptides undergo to achieve a mature, functional conformation. Folding occurs in a co-translational fashion as the nascent polypeptide is translocated through the ER membrane. While the primary amino acid sequence of a polypeptide contains all of the necessary information for folding, molecular machinery within the ER, including chaperones and co-chaperones, assist in this process. For example, the ubiquitously expressed chaperone BiP, also known as GRP78, binds to hydrophobic sequences that are intermittently exposed on a nascent protein during the folding process, thereby maintaining the unfolded or misfolded protein in a folding-competent state. Co-chaperones, including members of the ERdj family, assist BiP in this ATP-dependent process. The association and disassociation of chaperones and co-chaperones with unfolded/misfolded proteins continues until a mature conformation is reached, at which time the protein is allowed to exit the ER. If the protein is terminally misfolded, it is cleared from the ER by a process known as ER-associated degradation (ERAD). Quality control machinery in the ER constantly monitor the folding status of proteins, determining which are correctly or terminally misfolded.

ERAD of terminally misfolded proteins consists of 5 interconnected processes: 1) recognition of a protein as terminally misfolded by a quality control receptor, 2) unfolding of the misfolded protein, 3) retrotranslocation out of the ER into the cytosol, 4) attachment of ubiquitin moieties to the protein and 5) degradation of the protein by the 26S proteasome. Recently, identification of the machinery that detects terminally misfolded, glycosylated proteins, such as EDEM (see below), has provided insight into the mechanisms underlying the recognition event of ER quality control. Following recognition, the misfolded protein must be unfolded into its primary structure in order to be retro-translocated out of the ER through the Sec61 translocon or an alternate translocon such as Der1 (Lilley et al. 2004; Ye et al. 2004). The attachment of a polyprotein chain, consisting of multimers of the highly conserved 76 amino acid protein,
Table 1.1 – *SFTPC* mutations associated with IIP

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<th>Nature of mutation</th>
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<td>c.460+1 G&gt;A (SP-C&lt;sup&gt;Δexon4&lt;/sup&gt;)</td>
<td>C-terminal peptide</td>
<td>familial</td>
<td>(Nogee et al. 2001)</td>
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<td>c.460+1 G&gt;T (SP-C&lt;sup&gt;Δexon4&lt;/sup&gt;)</td>
<td>C-terminal peptide</td>
<td>sporadic</td>
<td>(Nogee et al. 2002)</td>
</tr>
<tr>
<td>L188Q</td>
<td>C-terminal peptide</td>
<td>familial</td>
<td>(Thomas et al. 2002; Chibbar et al. 2004)</td>
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<td>I73T</td>
<td>C-terminal peptide</td>
<td>familial and sporadic</td>
<td>(Nogee et al. 2002; Brasch et al. 2004; Cameron et al. 2005)</td>
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<td>G100V</td>
<td>C-terminal peptide</td>
<td>familial</td>
<td>(Nogee et al. 2002)</td>
</tr>
<tr>
<td>Y104H</td>
<td>C-terminal peptide</td>
<td>familial by history</td>
<td>(Nogee et al. 2002)</td>
</tr>
<tr>
<td>P115L</td>
<td>C-terminal peptide</td>
<td>familial</td>
<td>(Nogee et al. 2002)</td>
</tr>
<tr>
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<td>C-terminal peptide</td>
<td>unknown</td>
<td>(Nogee et al. 2002)</td>
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<td>T187N</td>
<td>C-terminal peptide</td>
<td>familial by history</td>
<td>(Nogee et al. 2002)</td>
</tr>
<tr>
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<td>C-terminal peptide</td>
<td>sporadic</td>
<td>(Nogee et al. 2002)</td>
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<tr>
<td>Del codons 91-93</td>
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<td>sporadic</td>
<td>(Hamvas et al. 2004)</td>
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<tr>
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to lysine residues of the misfolded protein occurs while the protein is being retro-translocated into the cytosol. Poly-ubiquitination of the protein by E3 ubiquitin ligases serves as a degradation signal for the 26S proteasome. The culmination of these events results in degradation of the protein by the proteasome, sparing the cell from the undesired affects of a deployed, aberrantly folded protein.

The majority of proteins that enter the ER undergo post-translational modification including disulfide bond formation between two cysteine residues and N-linked glycosylation. Glycosylation of proteins serves three primary functions: 1) to increase the overall solubility of proteins, 2) to serve as a signal for intracellular trafficking (e.g. mannose-6-phosphate targets proteins to lysosomes) and 3) serve as a reporter of the folding status of a protein. Lectin binding proteins, including mannosidase 1 and the chaperones calreticulin and calnexin, transiently interact with and process glycan moieties of immature, glycosylated proteins within the ER. This process, referred to as the calnexin/calreticulin cycle, assists in the maturation process by preventing aggregation of immature proteins and retaining malfolded proteins. EDEM (ER degradation-enhancing α-mannosidase-like protein) is a ER-resident protein that has the ability to recognize terminally misfolded, glycosylated proteins via the composition of their glycan chains and shuttle them for degradation via ERAD (Molinari et al. 2003; Oda et al. 2003). Because of its ability to recognize terminally misfolded, glycosylated proteins, EDEM is referred to as an ER quality control receptor. In contrast to ER quality control for glycosylated proteins, virtually nothing is known about the mechanisms underlying ER quality control for non-glycosylated, transmembrane proteins such as SP-C.

Inhibition of global protein glycosylation by the antibiotic tunicamycin or disruption of Ca^{2+} homeostasis in the ER by the plant-derived, sesquiterpene lactone thapsigargin leads to accumulation of proteins with the ER. Accumulation of misfolded or unfolded proteins in the ER results in a condition known as ER stress and leads to the activation of signaling cascades collectively referred to as the unfolded protein response (UPR). The UPR is highly conserved from yeast to mammals and serves to alleviate the stress imposed upon the ER and return the cell to a normal, physiological state through coordinated translational and transcriptional responses. To date, three proximal sensors
Figure 1.6 – Diagram depicting the three proximal sensors of the UPR. IRE1, ATF6 and PERK are transmembrane proteins that reside in the membrane of the ER. Upon sensation of ER stress, the sensors dimerize leading to activation and subsequent translational/transcriptional responses to restore ER homeostasis.

of the UPR have been identified, ATF6, IRE1 and PERK, all of which are ER-resident, transmembrane proteins (Figure 1.6). The binding of BiP to their ER luminal domains holds these three sensors in an inactive state. When the concentration of misfolded proteins rises in the ER, BiP is competitively titrated from these sensors to the misfolded proteins, resulting in their activation. The structure and function of the three sensors will be briefly described below.

ATF6 (activating transcription factor 6) is a type II transmembrane protein that contains a basic leucine zipper (bZIP) transcription factor in its cytosolic domain (Haze et al. 1999). BiP binding to the luminal domain of ATF6 prevents its translocation to the...
Golgi (Shen et al. 2002a). When BiP is titrated away, ATF6 translocates to the Golgi where it undergoes regulated intramembrane proteolysis by two proteases, S1P and S2P, releasing the active transcription factor (Haze et al. 1999; Ye et al. 2000). ATF6 translocates to the nucleus and, in coordination with nuclear factor Y (NF-Y), binds to two promoter elements known as the ER-stress response element (ERSE-I) and ERSE-II to activate transcription of numerous target genes, including ER chaperones (Kokame et al. 2001; Yoshida et al. 2001b).

IRE1 is a type I transmembrane protein consisting of an ER-luminal dimerization and cytosolic kinase and endonuclease domains. The titration of BiP from the luminal domain of IRE1 induces dimerization and autophosphorylation of IRE1, activating the endoribonuclease function (Bertolotti et al. 2000; Liu et al. 2003). The only identified substrate for the endonuclease function of IRE1 in mammals is the mRNA encoding a bZIP-containing transcription factor, X-box binding protein 1 (XBP-1). XBP-1 mRNA is constitutively transcribed but translated at low levels and rapidly degraded due to the presence of a 26 nucleotide inhibitory intron (Lee et al. 2003a). Activated IRE1 splices the inhibitory intron out of XBP-1 and, in yeast, the resultant 5’ and 3’ ends are joined by a tRNA ligase, RLG1/TRL1 (Sidrauski et al. 1996). Following splicing, a new open reading frame is established which aligns the DNA binding and transactivation domains of XBP-1. Translation of spliced XBP-1 mRNA, XBP-1(s), results in the generation of a transcription factor that induces genes containing ERSE elements, including those involved in the refolding pathway (i.e. chaperones) and ERAD (Lee et al. 2003b; Yoshida et al. 2003). In addition, XBP-1(s) can also bind to a motif known as the UPR element (UPRE), upregulating a distinct set of genes, including EDEM, that are not activated by ATF6 (Yoshida et al. 2001a).

Similar to IRE1, PERK is a type I transmembrane protein that contains an ER-luminal dimerization domain and a cytosolic kinase domain; however, unlike IRE1, PERK lacks an endoribonuclease domain. The ER-luminal domains of IRE1 and PERK show a small degree of homology and genetic studies in yeast demonstrate that the domains are functionally interchangeable (Liu et al. 2000). In an inactive state, the luminal domain of PERK is occupied by BiP and activated upon its removal (Bertolotti et al. 2000). The kinase domain of activated PERK phosphorylates eIF2α, a subunit of the
methionyl-initiator tRNA complex, decreasing protein translation and the subsequent protein load within ER. This translational block is transient and, if homeostasis is restored, reversed by dephosphorylation of eIF2α.

Based on the observations that the activation of ATF6 precedes XBP-1 splicing and that IRE1 activation is necessary for EDEM induction, a model for the time-dependent induction of the UPR has been proposed (Yoshida et al. 2003) (Figure 1.7). Initially, when proteins accumulate within the cell, ATF6 is activated, resulting in the transcription of genes involved in the refolding process. Next, IRE1 is activated, generating XBP-1(s) and the subsequent upregulation of genes involved in refolding and degradation (e.g. EDEM) of the misfolded proteins. Exactly where PERK activation temporally fits into this model is unclear although it is thought to be part of the acute response. If these actions are unable to restore ER homeostasis and chronic ER stress occurs, apoptosis ensues to avoid the undesired effects of necrosis.

Disruption of ER homeostasis imposed by chronic ER stress results in an efflux of Ca²⁺ from the ER. The increase in cytosolic Ca²⁺ levels leads to the apoptosis by two primary mechanisms: 1) release of cytochrome C from the mitochondria, resulting in activation of the caspase cascade and 2) activation of ER stress-specific caspase-12. Both the release of cytochrome C and activation of caspase-12 result in activation of the executioner caspase, caspase-3. Ablation of caspase-12 in the mouse results in decreased apoptosis induced by ER stress-inducing agents (Nakagawa et al. 2000) and also protects against cytotoxicity induced by the accumulation of amyloid beta (Aβ) protein (Nakagawa et al. 2000) or prion protein (Hetz et al. 2003). Similarly, knock-down of caspase-4, the human homolog of caspase-12, by RNAi in cultured human cells protects against Aβ-induced apoptosis (Hitomi et al. 2004). However, ER stress-induced apoptosis has been reported to occur in a caspase-3 dependent manner independent of caspase-12 and caspase-4 (Obeng et al. 2005). Chronic ER stress also results in the transcriptional induction of the pro-apoptotic gene CHOP via PERK and ATF6 activation (Ma et al. 2002) and c-Jun N-terminal inhibitory kinase (JNK) via activation and association of IRE1/apoptosis signal-regulating kinase 1(ASK1)/TRAF2 (Urano et al. 2000; Nishitoh et al. 2002).
Figure 1.7 – A model of the time-dependent induction of the UPR. In ER stress situations, the UPR is activated in a time-dependent manner, beginning with PERK, followed by the ATF6 and IRE1/XBP1 pathways. Collectively, these translational and transcriptional responses serve to restore homeostasis. In the event homeostasis is not restored, apoptosis ensues, mediated in part by all three pathways.

The role of NF-κB in the regulation of cytokine-stimulated pro-inflammatory gene expression is well established (Li et al. 2002). However, NF-κB activity is also increased in response to the accumulation of transmembrane proteins in the ER, including the E3/19k protein of adenovirus (Pahl et al. 1996), MHBS of hepatitis B virus (Meyer et al. 1992), expression of MHC class 1 in the absence of β2-microglobin protein (Pahl et al. 1996) and p450 (Szczesna-Skorupa et al. 2004). Activation of NF-κB in response to accumulation of newly synthesized membrane proteins is referred to as the ER overload
response (EOR). Constitutive activation of NF-κB promotes survival of a wide range of cells, including B-cells, hepatic cells and cancer cells, and is a major target for cancer therapy (Kucharczak et al. 2003). NF-κB promotes cell survival, in part, by activating anti-apoptotic genes including members of the IAP family, TRAF1 and TRAF2, and the Bcl2 homologues Bfl-1/A1 and Bcl-Xl (Kucharczak et al. 2003). Recent studies, using tunicamycin and thapsigargin as stressor agents, demonstrated an association between ER stress-induced PERK activation, eIF2α phosphorylation and NF-κB activation (Jiang et al. 2003; Deng et al. 2004), effectively linking the UPR and the EOR. Although a definitive role for NF-κB activation in response to ER-stress has not been established, it is thought to promote cell survival in this context. Consistent with this concept, expression of a transmembrane protein, p450, in transiently transfected HepG2 cells resulted in early activation of the UPR followed by NF-κB activation and transcriptional increases in anti-apoptotic gene expression; in contrast, expression of p450 in COS1 cells, resulted in UPR induction without NF-κB activation. Failure to activate NF-κB in COS1 cells was associated with apoptosis including an increase in both JNK activation and CHOP transcription (Szczesna-Skorupa et al. 2004). Collectively, these data support an anti-apoptotic role for NF-κB activation in response to ER stress imposed by misfolded transmembrane protein in the ER.

The overall goal of this dissertation study is to determine whether a disease-linked mutation in the C-terminal peptide of SP-C causes lung disease. The hypotheses underlying this work are: 1) mutations in SFTPC lead to misfolding and accumulation of the mutant proprotein and subsequent activation of ER stress pathways, 2) chronic ER stress imposed by misfolded SP-C promotes adaptation and cell survival and 3) adaptation increases susceptibility to environmental stress.
CHAPTER II

Expression of a human SP-C mutation associated with interstitial lung disease disrupts lung development in transgenic mice

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Abstract

Surfactant Protein C (SP-C) is a secreted transmembrane protein that is exclusively expressed by alveolar type II epithelial cells of the lung. SP-C associates with surfactant lipids to reduce surface tension within the alveolus, maintaining lung volume at end expiration. Mutations in the gene encoding SP-C (SFTPC) have recently been linked to chronic lung disease in children and adults. The goal of this study was to determine if a disease-linked mutation in SFTPC causes lung disease in transgenic mice. The SFTPC mutation, designated g.1728 G>A, results in the deletion of exon4, generating a truncated form of SP-C (SP-CΔexon4). cDNA encoding SP-CΔexon4 was constitutively expressed in type II epithelial cells of transgenic mice. Viable F0 transgene-positive mice were not generated after two separate rounds of pronuclear injections. Histological analysis of lung tissue harvested from E17.5 F0 transgene-positive fetuses revealed that SP-CΔexon4 caused a dose-dependent disruption in branching morphogenesis of the lung associated with epithelial cell cytotoxicity. Transient expression of SP-CΔexon4 in isolated type II epithelial cells or HEK293 cells resulted in incomplete processing of the mutant proprotein, a dose-dependent increase in BiP transcription, trapping of the proprotein in the endoplasmic reticulum and rapid degradation via a proteasome-dependent pathway. Taken together, these data suggest that the g.1728 G>A mutation causes misfolding of the SP-C proprotein with subsequent induction of UPR and ERAD pathways ultimately resulting in disrupted lung morphogenesis.
Introduction

Type II epithelial cells synthesize and secrete pulmonary surfactant, a complex mixture of phospholipids and proteins that coats the alveolar surface. Surfactant forms a bioactive film that effectively reduces the amount of work required during inspiration and prevents alveolar collapse at end expiration. The protein components of surfactant, in particular the lipophilic proteins surfactant protein B (SP-B) and surfactant protein C (SP-C), facilitate the adsorption and spreading of lipids during the respiratory cycle and are critical for the formation and maintenance of the surfactant film. The importance of SP-C in mediating this process is underscored by the efficacy of exogenous surfactant preparations containing SP-C as the sole protein component (Hafner et al. 1995; Hawgood et al. 1996; Davis et al. 1998a).

SP-C is exclusively expressed in type II epithelial cells of the lung and is highly conserved among all species studied to date (Glasser et al. 1991; Wert et al. 1993; Weaver et al. 2001). Human SP-C is synthesized as a 197-amino acid proprotein (MW = 21kDa) consisting of a 35 amino acid mature peptide flanked by an N-terminal propeptide (residues 1-23) and a C-terminal domain (residues 59-197). The intact proprotein traverses the regulated secretory pathway of the type II cell from the endoplasmic reticulum (ER) to the late endosome/multivesicular body (MVB) where cleavage of the flanking amino and carboxyl-terminal domains liberates the extremely hydrophobic, bioactive mature peptide (Beers et al. 1995; Vorbroker et al. 1995b). Fusion of the MVB with a lamellar body results in delivery of the mature peptide to the intracellular compartment in which the fully assembled surfactant complex is stored until it is secreted into the airspace.

SP-C is an integral membrane protein that contains a single membrane-spanning domain located within the mature peptide (Johansson et al. 1994). The newly synthesized proprotein is inserted into the ER membrane in a type II orientation with the N-terminal propeptide located in the cytosol and the C-terminal domain residing in the lumen of the ER (Russo et al. 1999; Conkright et al. 2001). Trafficking of the proprotein through the regulated secretory pathway of the type II cell is dependent upon signals encoded in the N-terminal propeptide (Keller et al. 1991; Conkright et al. 2001) and may
be facilitated by oligomerization since the SP-C proprotein has been shown to form dimers and oligomers in transfected A549 cells (Wang et al. 2002). The C-terminal peptide is dispensable for trafficking and secretion of the mature peptide (Conkright et al. 2001); however, the orientation of the C-terminal domain in the lumen of the ER subjects SP-C to scrutiny by ER quality control mechanisms.

Mutations in the human SP-C gene (SFTPC) have recently been linked to familial interstitial lung disease (ILD). Although numerous histopathological classifications exist for this diverse group of diseases, including non-specific interstitial pneumonitis (NSIP) and usual interstitial pneumonitis (UIP), pulmonary fibrosis is generally regarded as the final common outcome. The index case was a patient diagnosed at one year of age with NSIP (Nogee et al. 2001). Sequence analysis of genomic DNA from the patient revealed a point mutation on one allele of SFTPC. The mother of the infant also harbored the mutation and was afflicted with lifelong lung disease. The mutation substitutes an adenosine for a guanosine at the first base pair of intron 4 (genomic DNA base 1728 [g.1728 G>A]), resulting in ablation of the normal donor splice site at the exon4/intron4 boundary (Fig. 1a). Splicing of the mutant mRNA results in deletion of exon4, which encodes thirty-seven amino acids in the C-terminal peptide of SP-C, ultimately resulting in the generation of a truncated proprotein of 17kDa (SP-C\textsuperscript{Δexon4}). The mutant proprotein was associated with a decrease in wild-type SP-C and a complete absence of mature SP-C in the BALF of the affected patient, suggesting that both SP-C\textsuperscript{Δexon4} and wild-type SP-C might be retained in the ER by the quality control apparatus.

A separate point mutation in the SFTPC locus (exon 5 + 128 T>A) was also identified in an extended kindred with a history of ILD, including adults presenting with UIP and children with cellular NSIP (Thomas et al. 2002). The mutation results in the substitution of a glutamine for a conserved leucine residue (L188Q) in the C-terminal domain of SP-C. Similar to patients with the g.1728 G>A mutation, affected individuals in this kindred carried the mutation on only one allele. Pedigree analysis demonstrated variability in the age of disease onset and phenotypic presentation, suggesting that multiple genetic and/or environmental factors were involved in the pathogenic process. To date, eleven mutations in the SFTPC locus have been linked to ILD and all except one map to the C-terminal peptide of SP-C (Nogee et al. 2002). This study was designed to
test the hypothesis that a disease-linked mutation in the C-terminal peptide of SP-C causes lung disease.
Results

**Generation of SP-C\(^{\Delta}\text{exon4}\) transgenic mice**

To test the hypothesis that mutations in the carboxyl terminal peptide of SP-C cause lung disease, SP-C\(^{\Delta}\text{exon4}\) was expressed in Type II epithelial cells of transgenic mice. The SP-C\(^{\Delta}\text{exon4}\) construct was synthesized by deleting the nucleotides encoding the amino acids of exon4 (residues109-145) via overlapping PCR mutagenesis using human SP-C cDNA as a template. Expression of SP-C\(^{\Delta}\text{exon4}\) was specifically targeted to type II epithelial cells of the lung using the 13kb mouse SP-C promoter (Figure 2.1B). Despite two rounds of pronuclear injections into FVB/N eggs, no transgene-positive progeny were recovered suggesting that expression of SP-C\(^{\Delta}\text{exon4}\) was associated with neonatal lethality. The construct was therefore injected again and lungs harvested from potential founder mice (F\(_0\)) at E17.5 for histochemical and biochemical analyses. Thirty embryos were recovered from this injection, five of which had integrated the transgene into the genome.

**SP-C\(^{\Delta}\text{exon4}\) disrupts lung organogenesis in transgenic mice**

Two of five transgene-positive F\(_0\) fetuses displayed disrupted lung organogenesis, as assessed by H&E staining of fetal lung tissue (Figure 2.2), while the remaining three F\(_0\) mice were morphologically indistinguishable from weight–matched, wild-type fetuses. Lung tissue from the most severely affected animal (TG#1) was extremely hypoplastic and characterized by large cystic saccules, little branching morphogenesis and loss of typical distal acinar structures (Figure 2.2A). Although the lungs of the less affected animal (TG#2) were comparable in size to wild-type fetuses, branching morphogenesis was also disrupted, albeit to a lesser extent than that observed in TG#1 (Figure 2.2B). Lung structure of the other three F\(_0\) mice (one of which is depicted in Figure 2.2C as TG#3) was completely normal.

Analysis of the H&E-stained sections at higher magnification revealed the presence of vacuolated (arrows Figure 2.3A) and hypertrophic (arrowheads Figure 2.3A) epithelial cells in TG#1. The epithelium of TG#2 appeared largely intact and contained
few vacuolated cells ([Figure 2.3B]). Both TG#1 and TG#2 contained a significant amount of debris in the proximal and distal airways. The airway debris was more prominent in TG#2 than in TG#1 and consisted of sloughed epithelial cells and macrophages recruited into the airspaces. In contrast, the epithelium in TG#3 and WT were intact, devoid of airway debris and supported by condensed mesenchymal tissue indicative of the canicular stage of murine lung development ([Figure 2.3, C&D]). Collectively these data demonstrate that expression of SP-C$^{\Delta}$exon4 in Type II epithelial cells of transgenic mice disrupted normal lung organogenesis, ultimately leading to neonatal lethality. The prominent abnormalities associated with this disruption included cytotoxicity, hypoplasticity and perturbation of branching morphogenesis.

To determine if variability in phenotype was associated with the level of SP-$C^{\Delta}$exon4 expression, *in situ* hybridization was performed with a radiolabeled riboprobe specific for transgenic mRNA. A gradient of SP-$C^{\Delta}$exon4 mRNA expression was detected among F0 mice with the highest expression observed in TG#1 and lowest expression in TG#3 ([Figure 2.4 A,B,C]). The anti-sense signal in WT ([Figure 2.4D]) was indistinguishable from sense controls (data not shown). These results demonstrate that the expression level of SP-$C^{\Delta}$exon4 was correlated with the severity of the lung phenotype. The expression pattern of SP-$C^{\Delta}$exon4 was assessed by immunohistochemistry using an antibody specific for the amino-terminal propeptide of SP-C (proSP-C) ([Figure 2.4 E,F,G,H]). Since this antibody detects both endogenous SP-C and SP-$C^{\Delta}$exon4, the primary antibody was titered to a concentration at which only SP-$C^{\Delta}$exon4 was detected (1:44K); endogenous SP-C staining was detected at a primary antibody dilution of 1:1K but not 1:4K. Intense SP-C immunoreactivity was observed in the epithelial cells lining the cystic saccules of TG#1 ([Figure 2.4E]), confirming successful targeting of SP-$C^{\Delta}$exon4 to the distal epithelium. In addition, high expression of SP-$C^{\Delta}$exon4 in TG#1 was associated with sloughing of the epithelium, leading to an accumulation of SP-C-positive cellular debris in the airspaces ([Figure 2.4E, inset]). Interestingly, SP-C immunoreactivity was only detected in the airspaces of TG#2 and not in the epithelium at the 1:44K dilution, suggesting that intracellular SP-$C^{\Delta}$exon4 protein was turned over rapidly in this animal ([Figure 2.4F, and inset]). Lung tissue from TG#3 was completely devoid of SP-C staining at the 1:44K dilution ([Figure 2.4G]) even though SP-$C^{\Delta}$exon4 mRNA was detected
in this mouse (Figure 2.4C). For comparison, the endogenous SP-C staining pattern of distal epithelial cells is shown on wild-type tissue at a 1:1K dilution of primary antibody (Figure 2.4H). The staining pattern for a proximal epithelial cell marker, Clara cell secretory protein (CCSP), appeared normal suggesting that cell specification was not perturbed even in the most severely affected lungs (Figure 2.4 I,J,K,L). Taken together, these data are consistent with the hypothesis that SP-C∆exon4 exerts a dose-dependent, cytotoxic effect in the respiratory epithelium of transgenic mice.

In order to determine the relative expression levels of SP-C∆exon4 protein in transgenic F0 mice, Western blot analysis was performed on lung tissue using the proSP-C antibody. SP-C proprotein is not normally detected by Western blotting due to rapid processing to the mature peptide in the biosynthetic pathway of the type II cell (WT, Figure 2.5A). Immunoreactive SP-C was readily detected in TG#1 (Figure 2.5A) and the size of the proSP-C positive band (M_r=17k) corresponded to the predicted molecular weight of SP-C∆exon4. Immunoreactive proSP-C also co-migrated with newly synthesized SP-C∆exon4 transcribed and translated in vitro from a mammalian expression vector (Figure 2.5B). These data demonstrate that the expression of SP-C∆exon4 in TG#1 detected by Western analysis correlate with the expression levels observed by immunohistochemistry. Interestingly, SP-C∆exon4 protein was undetectable in the lung homogenate of TG#2, despite abnormal lung morphogenesis, suggesting that the mutant protein was rapidly degraded in this animal.

**SP-C∆Exon4 is rapidly degraded in vitro**

Newly synthesized SP-C is a type II integral membrane protein in which the carboxyl terminal peptide domain resides in the lumen of the ER (Russo et al. 1999; Conkright et al. 2001). Although the function of the carboxyl terminal peptide is unknown, mutations in this region may result in misfolding of the protein, resulting in retention in the ER and incomplete processing of the proprotein. To determine if deletion of exon4 prevented processing of the proprotein, type II epithelial cells were isolated from SP-C-/- mice, infected with adenoviral particles encoding SP-C1-197 or SP-C∆exon4 and cell lysates immunoprecipitated with an antibody directed against the mature
SP-C peptide. In type II cells infected with SP-C<sub>1-197</sub>, the full length proprotein (M<sub>r</sub>=21kDa), two processing intermediates and mature SP-C (M<sub>r</sub>=4kDa) were detected (Figure 2.6, lanes1&2). An identical banding pattern was observed when endogenous SP-C was immunoprecipitated from metabolically-labeled Type II cells isolated from a wild-type mouse (data not shown). In contrast, only the mutant proprotein (M<sub>r</sub>=17kDa) and a smaller immunoreactive form were detected in cells expressing SP-C<sup>Δexon4</sup> (Figure 2.6, lanes3&4). These results show that SP-C<sup>Δexon4</sup> was not completely processed to the mature peptide suggesting that the mutant proprotein was not sorted to the distal compartments of the secretory pathway in SP-C<sup>-/-</sup> type II cells. To determine if SP-C<sup>Δexon4</sup> was degraded early in the biosynthetic pathway, SP-C<sub>1-197</sub> or SP-C<sup>Δexon4</sup> was transiently transfected into HEK 293 cells in the presence or absence of the proteasome inhibitor MG-132. Cell lysates were harvested four hours following the addition of MG-132 and Western analysis was performed with the proSP-C antibody. Robust expression of SP-C<sub>1-197</sub> was detected in the absence of proteasome inhibitor (Figure 2.7, lanes3&4); in contrast, only faint immunoreactive bands, corresponding to the mutant proprotein, were detected in cells expressing SP-C<sup>Δexon4</sup>, demonstrating that the mutant protein was rapidly degraded in the absence of the proteasome inhibitor (Figure 2.7, lanes 7&8). SP-C<sup>Δexon4</sup> mutant proprotein was readily detected following MG-132 treatment and approached levels observed in cells expressing SP-C<sub>1-197</sub> (Figure 2.7, lanes 5&6). Similarly, expression of SP-C<sup>Δexon4</sup> in transiently transfected HeLa or 3T3 cells was only detectable in the presence of MG-132, demonstrating that ERAD-dependent turnover of the mutant proprotein was not cell type specific (data not shown). MG-132 had no effect on the level of SP-C<sub>1-197</sub> indicating that little or no wild-type proprotein was degraded via the proteasome pathway (Figure 2.7, lanes1&2).

To confirm that SP-C<sup>Δexon4</sup> was not exported from the ER, immunolocalization of SP-C was performed on transfected HEK293 cells expressing either SP-C<sub>1-197</sub> or SP-C<sup>Δexon4</sup>. SP-C<sub>1-197</sub> exhibited a punctate staining pattern that was unaltered by proteasome inhibition (Figure 2.8, top panels). This staining pattern is consistent with trafficking of the wild-type protein to the lysosome, the compartment to which regulated secretory proteins, such as SP-C, traffic in a cell that lacks such a pathway (DellAngelica et al. 2000). In contrast, low levels of protein were detected in cells that expressed SP-C<sup>Δexon4</sup>
in the absence of a proteasome inhibitor, consistent with results obtained by Western analysis (Figure 2.8, bottom panels). Treatment with MG-132 prevented degradation of the SP-C^{Δexon4}, revealing a diffuse staining pattern indicative of ER localization (Figure 2.8, bottom panels). These results support the hypothesis that SP-C^{Δexon4} is recognized as a misfolded peptide, retained in the ER and rapidly degraded via the ER-associated degradation (ERAD) pathway in a non-type II epithelial cell line.

**SP-C^{Δexon4} induces ER stress**

BiP is an abundant chaperone protein whose primary function is to facilitate the folding of proteins in the ER. Transcription of BiP is increased in response to the accumulation of unfolded or misfolded proteins in the ER and thus serves as a classical marker for the induction of ER stress pathways (Kaufman 1999). To determine if SP-C^{Δexon4} induced ER stress, mammalian expression vectors encoding SP-C^{Δexon4}, SP-C^{1-197} or an empty vector (pcDNA3) were individually transfected into HEK293 cells with a reporter vector consisting of a minimal BiP promoter driving luciferase (BiP/Luc) (Figure 2.9). Cell lysates were harvested forty-eight hours post-transfection and analyzed for luciferase activity and SP-C levels via Western analysis. Cells transfected with BiP/Luc and subjected to a six-hour exposure of 10µg/ml tunicamycin showed a 2.4-fold increase in luciferase activity over the pcDNA3 control, indicating that the BiP promoter was indeed responsive to a known ER stress-inducing agent (Figure 2.9A). 75ng of SP-C^{Δexon4} co-transfected with the BiP/Luc reporter resulted in a 2-fold increase in luciferase activity compared to cells transfected with the empty vector control. Co-transfection of an equivalent amount of SP-C^{1-197} and BiP/Luc caused a modest increase in luciferase activity compared to pcDNA3 but failed to reach significance (Figure 2.9A). When the amount of input cDNA was increased to 250ng, SP-C^{Δexon4} augmented luciferase activity 3.5-fold while SP-C^{1-197} increased luciferase activity 2.4-fold over that observed for pcDNA3 (Figure 2.9A). The increases in luciferase activity observed with SP-C^{Δexon4} were statistically significant compared to both SP-C^{1-197} and pcDNA3 for the two input quantities of cDNA tested. Western analyses on cell lysates from the 250ng input group showed high expression of SP-C^{1-197} (Figure 2.9B, lanes1-3). SP-C^{Δexon4} was
undetectable in the cell lysates, despite the increase in BiP promoter activity (Figure 2.9B, lanes 4-6) consistent with rapid degradation of the mutant proprotein. Collectively, these results indicate that the expression of SP-C\textsuperscript{Δexon4} in HEK293 cells elicits an ER stress response in a dose-dependent manner.
Discussion

Mutations in the gene encoding human surfactant protein C are associated with chronic lung disease in both children and adults. The goal of this study was to determine if the g.1728 G>A (SP-C\(^{\Delta\text{exon4}}\)) point mutation in the SFTPC locus was directly linked to the pathogenesis of lung disease. This hypothesis was tested by generating transgenic mice that expressed SP-C\(^{\Delta\text{exon4}}\) in type II cells of the respiratory epithelium. SP-C\(^{\Delta\text{exon4}}\) caused a dose-dependent perturbation of lung development associated with epithelial cell cytotoxicity. Transient expression of SP-C\(^{\Delta\text{exon4}}\) in isolated type II epithelial cells or HEK293 cells resulted in incomplete processing of the proprotein, a dose-dependent increase in BiP transcription, trapping of the proprotein in the ER and rapid degradation via a proteasome-dependent pathway. Taken together these data suggest that the g.1728 G>A mutation leads to misfolding of the SP-C proprotein with subsequent induction of UPR and ERAD pathways.

Lung development was profoundly disrupted despite the fact that expression of the SP-C\(^{\Delta\text{exon4}}\) protein was restricted to one cell type and occurred in the presence of two wild-type alleles. Three lines of evidence implicate SP-C-mediated cytotoxicity as the basis for altered lung structure. Firstly, epithelial cells expressing high levels of the transgene exhibited cell swelling consistent with necrosis. Secondly, the sloughed respiratory epithelium and the cellular debris detected in the airways of two independent F\(_0\) animals stained intensely for proSP-C at an antibody dilution that detected only the transgene-derived protein. Thirdly, macrophage infiltrates were present in the lungs of both affected animals. Since macrophages are never observed in the fetal lung in the absence of inflammation it is likely that these cells were recruited to the lung following cell injury.

To determine if SP-C\(^{\Delta\text{exon4}}\)-induced dysmorphogenesis was linked to altered epithelial cell specification, markers of the proximal and distal respiratory epithelium were analyzed in lung tissues from three F\(_0\) animals. The staining patterns of the proximal epithelial cell marker CCSP and the distal epithelial cell marker proSP-C were normal suggesting that cell specification was not altered. Therefore, it is likely that inappropriate epithelial cell death resulted in disruption of branching morphogenesis.
rather than a defect in cell specification. It is well established that epithelial-mesenchymal interactions are absolutely required for proper branching morphogenesis in numerous organs including the lung (Demayo et al. 2002). Ablation of type II epithelial cells would effectively terminate signaling between the two cell compartments, resulting in altered morphogenetic signaling. It is unlikely that the dysmorphogenesis was solely due to overexpression of transgene protein in type II epithelial cells since mice expressing SP-B\textsuperscript{αC} or lysozyme transgenes were viable with no lung abnormalities (Akinbi et al. 1997; Akinbi et al. 2000). A phenotype similar to that seen in the SP-C\textsuperscript{Δexon4} mice was observed in mice expressing high levels of the SP-C mature peptide, SP-C\textsuperscript{24-58}, or diptheria toxin in type II cells (Korfhagen et al. 1990; Conkright et al. 2002). Collectively, these data suggest that demise of fetal type II epithelial cells, irrespective of the causal insult, leads to altered lung morphogenesis.

Deletion of exon4 from SP-C resulted in incomplete processing of the mutant proprotein in isolated type II epithelial cells. Processing of the amino-terminal propeptide and the carboxyl-terminal peptide occurs in the MVB of the type II cell leading to the generation of the 4kDa active peptide (Beers et al. 1995; Vorbroker et al. 1995b). Lack of SP-C\textsuperscript{Δexon4} processing suggested that the mutant proprotein did not traffic to the MVB and was retained in a proximal compartment of the secretory pathway; confocal microscopy identified this compartment as the ER. Quality control mechanisms within the ER ensure the correct folding and assembly of polypeptides prior to export from this compartment. Accumulation of unfolded or misfolded protein triggers an ER-to-nucleus signal transduction pathway, the unfolded protein response (UPR), which upregulates the production of chaperone proteins, such as BiP, within the ER. Failure to fold under these conditions results in induction of ERAD leading to retrotranslocation of the terminally misfolded protein and degradation by the ubiquitin-proteasome pathway (for review see (Kaufman 1999; Harding et al. 2002)). Expression of SP-C\textsuperscript{Δexon4} in HEK293 cells induced a dose-dependent increase in BiP transcription and rapid degradation of SP-C\textsuperscript{Δexon4} via proteasome-dependent mechanisms. Proteasome-dependent degradation of SP-C\textsuperscript{Δexon4} in HEK293 cells together with the inability to detect SP-C\textsuperscript{Δexon4} protein in TG#2 and incomplete processing of the mutant proprotein in type II cells support the hypothesis that SP-C\textsuperscript{Δexon4} is recognized as a misfolded protein within the
ER and rapidly degraded via ERAD. Wild-type SP-C <sup>1-197</sup> also caused an increase in BiP transcription at the higher input dose of cDNA; however, unlike SP-C<sup>Δ exon4</sup>, SP-C<sup>1-197</sup> was successfully exported from the ER. The increase in BiP transcription was therefore most likely due to robust expression of wild-type SP-C resulting in an increase in unfolded substrate that triggered the UPR. Collectively these results indicate that SP-C<sup>Δ exon4</sup> induces ERAD in HEK293 cells resulting in selective degradation of mutant but not wild-type SP-C.

The high level of SP-C<sup>Δ exon4</sup> expression in transgenic mice may have been sufficient to saturate ERAD leading to epithelial cell death and disruption of lung morphogenesis. Lower levels of mutant SP-C proprotein may cause a milder phenotype leading to postnatal ILD observed in human patients – this hypothesis remains to be tested. Attenuation of translation, which accompanies induction of UPR, may also have contributed to dysmorphogenesis by inhibiting new protein synthesis during a critical stage of lung growth and differentiation. We also cannot dismiss the possibility that the loss of SP-C in the airspaces contributed to the severity of the disease in humans and transgenic mice. ILD was detected in a family with no detectable SP-C in BALF as well as in SP-C<sup>+/−</sup> mice (Amin et al. 2001; Glasser et al. 2003). However, SP-C deficiency cannot be the sole cause of disease in transgenic mice since lung structure and function is normal in newborn SP-C<sup>+/−</sup> mice (Glasser et al. 2001).

A previous study in transiently transfected A549 cells demonstrated that deletion of exon4 in the context of SP-C/GFP fusion proteins resulted in ubiquitination and aggresome formation (Wang et al. 2003). The formation of aggresomes suggested that mutant SP-C fusion protein had a prolonged half-life and was resistant to degradation. The proteasome-dependent turnover of SP-C<sup>Δ exon4</sup> in HEK293 cells is consistent with ubiquitination of the SP-C cysteine mutants in A549 cells. However, rapid turnover of SP-C<sup>Δ exon4</sup> both in vitro and in vivo is inconsistent with aggresome formation. Although ultrastructural analysis was not performed on SP-C<sup>Δ exon4</sup> F<sub>0</sub> mice due to the limited amount of lung tissue, aggresomes were not detected in transgenic mice expressing SP-C<sup>24-58</sup>, which displayed a similar phenotype to SP-C<sup>Δ exon4</sup> transgenic mice (Conkright et al. 2002). Perhaps very high expression of the SP-C/GFP mutant in a few isolated A549 cells overwhelmed the degradative capacity of the proteasome, leading to aggresome
formation in a subset of cells. The frequency of aggresome formation was not reported in the study by Wang et. al.; however, in HEK293 cells transiently expressing high levels of the folding mutant CFTR$^{AF508}$, only 5-15% of cells contained aggresomes (Bence et al. 2001). Taken together, we postulate that the constitutive expression of misfolded SP-C$^{\Delta exon4}$ overwhelmed the degradative capacity of ERAD machinery resulting in chronic induction of ER stress pathways, type II cell injury and disrupted lung morphogenesis.
Materials and Methods

DNA constructs, generation of transgenic mice and adenovirus production

Full-length human SP-C (SP-C\textsuperscript{1-197}) cDNA was cloned into pcDNA3 (Invitrogen, San Diego, CA) to generate SP-C\textsuperscript{1-197}/pcDNA3. SP-C\textsuperscript{Δexon4} was generated by deleting nucleotides 325-435 (adenosine of start ATG is basepair #1) via overlapping polymerase chain reaction mutagenesis using SP-C\textsuperscript{1-197}/pcDNA3 as a template and cloned into pcDNA3. A transgene construct was generated by subcloning SP-C\textsuperscript{Δexon4} into a puc19-based vector containing the 13kb mouse SP-C promoter (Glasser et al. 2000), rabbit globin intronic/exonic sequences and a bovine growth hormone polyadenylation signal (Figure 2.1A) as previously described (Beck et al. 2000). All completed constructs were sequenced bidirectionally to verify the integrity of the SP-C coding sequence. To generate transgenic mice, the transgene was excised from the vector DNA, purified and microinjected into the male pronuclei of fertilized FVB/N oocytes by the University of Cincinnati Transgenic Core facility. Potential founder mice were identified by transgene-specific PCR analysis of tail DNA. Adenoviral constructs were generated by subcloning the SP-C\textsuperscript{1-197} or SP-C\textsuperscript{Δexon4} construct from the pcDNA3 vector into the Adv2 shuttle vector (Davis et al. 1998b). Recombination and adenovirus production were performed as described previously (Davis et al. 1998b).

Histochemical and Western analysis of lung tissue

Potential founder mice (F\textsubscript{0}) were harvested by Caesarian section at E17.5. Left lung tissues were removed for Western blot analyses and right lung tissues were fixed en bloc for light microscopy, immunohistochemistry and in situ hybridization as previously described (Zhou et al. 1996). For Western analysis, lung tissues were homogenized in PBS containing 1% per volume protease inhibitor cocktail (Sigma, St. Louis, MO). Total protein concentration of the lung homogenate was determined by BCA assay (Pierce, Rockford, IL) and equal amounts of protein were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were electrophoretically transferred to nitrocellulose membranes and probed with a polyclonal antibody directed against the N-terminal peptide of SP-C (Vorbroker et al. 1995a) or
actin (a kind gift from Dr. James Lessard, Cincinnati Children’s Hospital, Cincinnati, OH). Stripping of the antibody complexes was performed using Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL). Immunohistochemistry was performed using rabbit polyclonal antibodies directed against the N-terminal propeptide of SP-C and CCSP (the latter antibody kindly provided by Dr. Barry Stripp, University of Pittsburgh) at the indicated dilutions. Biotinylated secondary antibodies and a streptavidin-biotin-peroxidase detection system (Vector Laboratories, Inc.) were used to localize the antibody-antigen complexes in the tissues, as previously described (Zhou et al. 1996). In situ hybridization was performed as previously described (Wert et al. 1993) using a 35S-UTP-labeled, transgene-specific riboprobe directed against the bovine growth hormone polyadenylation signal (283bp fragment).

Type II cell isolation, adenoviral infection, metabolic labeling, immunoprecipitation and In Vitro Transcription/Translation

Type II epithelial cells were isolated from SP-C⁻/⁻ mice (Glasser et al. 2001; Glasser et al. 2003) using the method described by Rice et. al. (Rice et al. 2002). 1X10⁶ cells per well were plated on 100% EHS matrix (BD Pharmingen, San Diego, CA) in growth media consisting of BEGM containing all accompanying additives except hydrocortisone (Clonetics, Walkersville, MD). The media also included 10% charcoal-stripped FBS (Sigma, St. Louis, MS) and 10ng/ml KGF (Peprotech, Rocky Hill, NJ). Cells were cultured at 37°C in a humidified incubator containing 5% CO₂. Seventy-two hours post-isolation, the cells were infected with 50 MOI of purified adenoviral particles encoding SP-C¹⁻¹⁹⁷ or SP-CΔexon4 in infection media containing 2% FBS; infection media was replaced with complete media 90min following infection. Forty-eight hours post-infection, cells were metabolically labeled with 0.5mCi/ml of 35S-methionine/cysteine (ICN, Aurora, OH) for 4hr. Cell lysates were immunoprecipitated exactly as described previously (Lin et al. 1996) with 5ul of an antibody directed against the mature SP-C peptide (Ross et al. 1999). SDS-PAGE and autoradiography was performed as previously described (Lin et al. 1996). SP-C¹⁻¹⁹⁷/pcDNA3 and SP-CΔexon4/pcDNA3 were transcribed and translated in vitro in the presence of 35S-Methionine/Cysteine (ICN, Aurora, OH)
using the TNT® Coupled Reticulocyte Lysate System (Promega, Madison, WI). Completed reactions were analyzed by SDS-PAGE/autoradiography.

**HEK293 cell culture and transfection**

HEK293 cells were purchased from ATCC (Manassas, VA). Growth media consisted of Richter’s media (Biowhittaker, Walkersville, MD) containing 10% FBS (Sigma, St. Louis, MO), 2mM L-glutamine and 1µ/ml penicillin/streptomycin (Sigma, St. Louis, MO). Cells were cultured at 37°C in a humidified incubator containing 5% CO₂. For proteasome inhibitor experiments, 2X10⁵ cells were plated into a 12-well plate 24hrs prior to transfection. The cells were transiently transfected with 1µg/well SP-C¹⁻¹⁹⁷/pcDNA3 or SP-CΔexon⁴/pcDNA3 using LT-1 reagent (Mirus, Madison, WI). Four-hours prior to harvest, the cells were treated with 5µM MG-132 (Calbiochem, La Jolla, CA) or DMSO as a vehicle control. Cells were harvested in PBS, sonicated immediately, total protein content assessed by BCA assay and equal amounts of protein were analyzed by SDS-PAGE/Western blotting with proSP-C or actin antisera as described above.

**Immunofluorescence**

HEK293 cells transfected with SP-CΔexon⁴ or SP-C¹⁻¹⁹⁷ were plated on poly-D-lysine coverslips. 4hrs prior to fixation, cells were treated with 5µM MG-132 or DMSO vehicle control (Calbiochem, La Jolla, CA) as indicated. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 and stained with a polyclonal antibody directed against the N-terminal propeptide of SP-C for 2hrs at 37°C. Cells were washed and incubated with anti-rabbit, FITC-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) for 2hrs at 37°C. Cells were washed 3X with PBS, 1X with dH₂O and mounted on slides with Vectashield mounting medium (Vector Laboratories, Inc.). Fluorescence was imaged with a Nikon microscope using a UV lamp and a FITC filter. Images were captured using an Optronics MagnaFire digital color camera.
BiP/Luciferase assays

The BiP/Luciferase reporter (a kind gift from Dr. Randal Kaufman, University of Michigan) consists of a minimal BiP promoter (nucleotides −457 to +33) containing the ER stress element placed immediately upstream of the luciferase gene in the pGL3-basic plasmid (Promega, Madison, WI) (Tirasophon et al. 1998). The β-galactosidase plasmid, pSV-β-galactosidase, was purchased from Promega. 2x10⁵ HEK293 cells were plated in a 12-well dish 24hrs prior to transfection. The cells were co-transfected with three plasmids including: 1) 75ng or 250ng of one of the test plasmids (SP-C¹⁻¹⁹⁷, SP-C∆exon⁴ or pcDNA3 as an empty vector control) 2) 250ng BiP/Luciferase reporter plasmid and 3) 75ng β-galactosidase plasmid. The cells were harvested 48hrs post-transfection in Glo Lysis Buffer (Promega). Luciferase activity was measured with the Bright Glo™ Luciferase Assay system (Promega) and β-galactosidase activity was measured using the Luminescent β-galactosidase Detection Kit II (BD Biosciences, Palo Alto, CA) in a luminometer. Data is plotted as luciferase activity/ β-galactosidase activity to correct for transfection efficiency among samples. The presented data represents one of three independent transfection experiments with each group performed in triplicate. Statistical differences between groups were assessed by one-way ANOVA.
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Figure Legends

Figure 2.1 - Diagram of mutation and transgene construct. A) The g.1728+1 G>A point mutation at position 1728+1 corresponds to the first base of intron4. This basepair substitution ablates a normal donor splice site, resulting in deletion of exon4 upon splicing of the mRNA. Translation of the mutated mRNA results in a truncated proprotein, designated SP-C\(\Delta\)exon4. Open boxes represent exons while lines represent introns. The black box in exon2 represents the mature peptide. B) Human SP-C\(\Delta\)exon4 cDNA was placed downstream of the 13kb mSP-C promoter to restrict expression to type II epithelial cells.

Figure 2.2 - Disrupted lung morphogenesis in mice expressing SP-C\(\Delta\)exon4. Hematoxylin and eosin stained fetal lung sections from mice expressing SP-C\(\Delta\)exon4 (E17.5). TG#1 represents a section through all right lobes of the lung compared to a section through one right lobe for the remaining animals. Note severe hypoplasia in TG#1 and presence of large, cystic saccules and a loss of mesenchymal tissue in TG#1 and TG#2 as compared to the wild-type control (WT). TG#3 was morphologically indistinguishable from the wild-type control. Scale bar: 500\(\mu\)M.

Figure 2.3 - Vacuolated epithelium and cellular airway debris in transgenic lungs. Hematoxylin and eosin stained fetal lung sections from mice expressing SP-C\(\Delta\)exon4 (E17.5). Note cellular debris and infiltrating macrophages lining the airways of TG#1 and TG#2 as well as vacuolated (arrows) and hypertrophic (arrowheads) epithelial cells in TG#1 as compared to a wild-type control. Epithelial cells are highlighted in WT (arrowheads) for comparison to those shown in TG#1. Scale bar: 100\(\mu\)M.

Figure 2.4 - Level of SP-C\(\Delta\)exon4 expression correlates with the severity of phenotype. *In situ* hybridization (ISH) and immunohistochemistry (IHC) were performed on serial lung sections from E17.5 F\(_0\) mice. *In situ* hybridization (panels A-D) was performed using a transgene-specific radiolabeled riboprobe directed against the polyA tail of the
transgenic construct (see Fig. 1B). Immunohistochemistry was performed with a polyclonal antibody directed against the N-terminal propeptide of SP-C (panels E-H) or against the proximal epithelial marker CCSP (panels I-L). Primary antibody dilutions for proSP-C are indicated at the top right of panels E-H. Primary antibody dilution for CCSP was 1:5K for panels I through L. Scale bar: 500µM for A-L, 50µM for insets.

Figure 2.5 - Western analysis of fetal lung homogenate. A) 30µg of total protein from fetal lung homogenate was separated by SDS-PAGE, transferred to nitrocellulose and blotted for SP-C using a polyclonal antibody specific to the N-terminus of the propeptide. The blot was stripped and reprobed with an anti-actin antibody. B) Plasmids encoding SP-C1-197 and SP-CΔexon4 were transcribed and translated in vitro in the presence of 35S-Cysteine/Methionine, separated by SDS-PAGE and subjected to autoradiography. Molecular weight markers are indicated on the right.

Figure 2.6 - Inappropriate processing of SP-CΔexon4 in SP-C-/- type II cells. Type II cells were isolated from SP-C-/- mice and cultured on 100% Matrigel for 48hrs. Cells were infected with adenoviral particles encoding SP-C1-197 (duplicates, lanes 1 and 2) or SP-CΔexon4 (duplicates, lanes 3 and 4). Forty-eight hours post-infection, cells were metabolically labeled with 35S Cysteine/Methionine and cell lysates were immunoprecipitated with a polyclonal antibody directed against the mature SP-C peptide. Immunoprecipitates were separated by SDS-PAGE and subjected to autoradiography. Top portion of gel containing proproteins was exposed to film for 24 hours, bottom portion exposed for 72 hours. Molecular weight markers are indicated on the right.

Figure 2.7 - ER-associated degradation (ERAD) of SP-CΔexon4 in HEK293 cells. Mammalian expression plasmids encoding SP-C1-197 (lanes 1-4) or SP-CΔexon4 (lanes 5-8) were transiently transfected into HEK293 cells. Twenty-four hours post-transfection, the proteasome inhibitor MG-132 was added (lanes 1, 2, 5 and 6) for 4hr prior to harvest. Cell lysates were separated by SDS-PAGE and immunoblotted with an antibody specific for the N-terminal propeptide of SP-C. The blot was stripped and reprobed with an anti-
Figure 2.8 - Distinct trafficking patterns for SP-C^{1-197} and SP-C^{Δexon4} in HEK293 cells. Stably transfected HEK293 cells expressing SP-C^{1-197} (top panels) or SP-C^{Δexon4} (bottom panels) were plated on coverslips in the presence (left panels) or absence (right panels) of the proteasome inhibitor MG-132. Cells were stained with an antibody directed against the N-terminal propeptide of SP-C and examined by fluorescence microscopy. Note the punctate staining pattern of SP-C^{1-197} in contrast to the diffuse staining of SP-C^{Δexon4}. SP-C^{Δexon4} was virtually undetectable in the absence of MG-132, consistent with rapid turnover of the mutant proprotein. Presented data represents an n=3 experiments.

Figure 2.9 - SP-C^{Δexon4} induces ER stress in HEK293 cells. A) Mammalian expression vectors encoding SP-C^{1-197}, SP-C^{Δexon4} or an empty vector control (pcDNA3) were individually transfected into HEK293 cells with a reporter vector consisting of a minimal BiP promoter driving the firefly luciferase gene (BiP/Luc). A third plasmid encoding β-galactosidase was co-transfected to standardize the samples for transfection efficiency. The amount of input cDNA for the tested plasmids was either 75ng (top panel) or 250ng (bottom panel) while the inputs of the BiP/Luc and β-galactosidase plasmids were constant. Cells were harvested forty-eight hours post-transfection and cell lysates were analyzed for luciferase and β-galactosidase activity using a luminometer. Cells transfected with the empty vector control were subjected to a six-hour exposure of 10µg/ml tunicamycin (pcDNA3 + TM) prior to harvest to demonstrate the responsiveness of the BiP/Luc reporter to a known ER stress-inducing agent. *, p<0.001 versus pcDNA3; #, p<0.001 versus SP-C^{1-197}. B) Cell lysates from panel B were separated by SDS-PAGE and immunoblotted with an antibody directed against the N-terminal propeptide of SP-C. Lanes 1-3 represent triplicates for SP-C^{1-197} and lanes 4-6 represent triplicates for SP-C^{Δexon4}. Data are expressed as the mean ± SD.
Figure 2.1 – Diagram of mutation and transgene construct

A.

1. g.1728+1 G>A
   - hSP-C mutant allele
   - hSP-C mutant mRNA
   - 17kDa
   - hSP-C mutant proprotein (SP-CΔexon4)

B.

- Dklb mSP-C promoter
- Rabbit β-globin in/ex
- SP-CΔexon4
- BGH polyA

In Situ probe

Figure 2.1 – Diagram of mutation and transgene construct
Figure 2.2 – Disrupted lung morphogenesis in mice expressing SP-C^{∆exon4}
Figure 2.3 – Vacuolated epithelium and cellular airway debris in transgenic lungs
Figure 2.4 – Level of $\text{SP-C}^{\Delta\text{exon4}}$ expression correlates with the severity of phenotype
Figure 2.5 – Western analysis of fetal lung homogenate
Figure 2.6 – Inappropriate processing of SP-C^{Δexon4} in SP-C^{+/−} type II cells
Figure 2.7 – ERAD of SP-C\textsuperscript{Δexon4} in HEK293 cells
Figure 2.8 – Distinct trafficking patterns of SP-C^{1-197} and SP-C^{\Delta exon4} in HEK293 cells
Figure 2.9 – SP-C<sup>Δexon4</sup> induces ER stress in HEK293 cells
CHAPTER III

Adaptation and increased susceptibility to infection associated with constitutive expression of misfolded SP-C

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Abstract

Mutations in the gene encoding surfactant protein C (SFTPC) have been linked to interstitial lung disease in children and adults. Expression of the index mutation, SP-C\textsuperscript{Δexon4}, in transiently transfected cells and type II cells of transgenic mice resulted in misfolding of the proprotein, activation of ER stress pathways and cytotoxicity. In the current study we show that stably transfected cells adapted to chronic ER stress imposed by constitutive expression of SP-C\textsuperscript{Δexon4} via an NF-κB-dependent pathway. However, infection of cells expressing SP-C\textsuperscript{Δexon4} with respiratory syncytial virus resulted in significantly enhanced cytotoxicity associated with accumulation of the mutant proprotein, pronounced activation of the unfolded protein response and cell death. Adaptation to chronic ER stress imposed by misfolded SP-C was associated with increased susceptibility to viral-induced cell death. The wide variability in the age of onset of ILD in patients with SFTPC mutations may be related to exposure to an environmental insult that ultimately overwhelms the homeostatic, cytoprotective response.
Introduction

Surfactant protein C (SP-C) is a transmembrane protein that is synthesized as a 191 or 197 amino acid proprotein by type II epithelial cells of the lung. Processing of the proprotein in the distal secretory pathway liberates a 35 amino acid that is secreted into the airspaces as a component of pulmonary surfactant. Mutations in the gene encoding SP-C (SFTPC) have been associated with development of sporadic and familial interstitial lung disease (ILD). In all cases to date the mutation was present on only one allele consistent with a dominant negative effect. The index mutation, a heterozygous base substitution of A for G at the first base of intron 4 (c.460+1 G>A), led to the internal deletion of 37 amino acids from the ER luminal domain, generating a truncated proprotein (SP-C\textsuperscript{Δexon4}). Two separate SFTPC mutations associated with ILD, SP-C\textsuperscript{L188Q} and SP-C\textsuperscript{I73T}, were detected in three kindreds (Thomas et al. 2002; Chibbar et al. 2004; Cameron et al. 2005). The age of onset and penetrance of ILD varied markedly in all three kindreds.

Studies in transiently transfected cells demonstrated that the c.460+1 G>A mutation led to misfolding of the mutant proprotein, retention of SP-C\textsuperscript{wt} in the ER, activation of the unfolded protein response (UPR) and apoptosis (Bridges et al. 2003; Wang et al. 2003; Mulugeta et al. 2005). SP-C\textsuperscript{Δexon4} was also associated with cytotoxicity and lung dysmorphogenesis when expressed in type II cells of transgenic mice (Bridges et al. 2003). The UPR is activated by conditions that perturb ER homeostasis, including the accumulation of misfolded proteins (Schroder et al. 2005). This response encompasses translational and transcriptional changes within the cell to alleviate the stress and to promote restoration of ER homeostasis. A model for the time-dependent induction of the UPR has been proposed, suggesting that translational repression via PERK activation/eIF2α phosphorylation occurs first, followed by the cleavage of ATF6, activation of IRE1/XBP-1 and expression of ATF6 and XBP-1 target genes (Yoshida et al. 2003). If ER homeostasis cannot be restored by these pathways or by induction of adaptive responses, apoptosis may occur as a means of avoiding the untoward effects of cell necrosis. ER stress-induced apoptosis has been associated with induction of the transcription factor CHOP, activation of JNK via IRE1, and activation of the ER stress-
specific caspases 4 (human) and 12 (mouse) (Nakagawa et al. 2000; Urano et al. 2000; Hetz et al. 2003; Hitomi et al. 2004; Oyadomari et al. 2004). While the effects of acute ER stress, imposed by xenotoxic agents such as thapsigargin and tunicamycin, are well established, little is known about the molecular pathways involved in adaptation to chronic ER stress imposed by a misfolded protein.

The variability in the age of onset and penetrance of disease in the SP-C<sup>L188Q</sup> and SP-C<sup>I73T</sup> pedigrees suggests that both genetic and environmental factors may influence the manifestation of lung disease. Based on the results of studies in human patients and transiently transfected cells, experiments were designed to test the hypotheses that: 1) chronic ER stress imposed by misfolded SP-C promotes adaptation and cell survival and 2) adaptation increases susceptibility to environmental stress. Clonal cell lines stably expressing SP-C<sup>Δexon4</sup> or SP-C<sup>wt</sup> were generated to identify cytoprotective pathways associated with adaptation to constitutive expression of misfolded SP-C and to assess the cytotoxic effects of environmental stress on adapted cells.
Results

Generation and characterization of stably transfected cell lines

To determine the molecular mechanisms underlying SP-C\(^{\Delta}_{\text{exon4}}\)-induced cytotoxicity, HEK293 cell lines stably expressing SP-C\(^{\text{wt}}\) or SP-C\(^{\Delta}_{\text{exon4}}\) were generated. Multiple clonal lines were obtained for each construct and two lines were chosen for subsequent experimentation based on equivalent expression of SP-C mRNA, initially assessed by RT-PCR (data not shown) and subsequently confirmed by microarray analysis (Figure 3.1b). These cell lines were morphologically indistinguishable by light microscopy (Figure 3.1a) or electron microscopy (Figure 3.8a and b) and exhibited similar doubling rates (data not shown). Basal SP-C protein levels were assessed by Western blot analysis of cell lysates with an antibody directed against the N-terminal peptide of the proprotein (proSP-C), a region which is unaffected by the \(\Delta_{\text{exon4}}\) mutation. Despite equivalent mRNA levels, expression of the SP-C\(^{\Delta}_{\text{exon4}}\) protein was barely detectable compared to SP-C\(^{\text{wt}}\), consistent with rapid turnover of the mutant proprotein (Figure 3.1c).

SP-C\(^{\Delta}_{\text{exon4}}\) protein was previously shown to be rapidly degraded in a proteasome-dependent manner and failed to be exported from the endoplasmic reticulum when transiently expressed in HEK293 cells (Bridges et al. 2003). To determine the subcellular localization of the SP-C variants in stably transfected cell lines, cells were stained with antibodies directed against proSP-C and LAMP-1 and analyzed by confocal microscopy. A bright, punctuate staining pattern was observed for proSP-C in SP-C\(^{\text{wt}}\) cells while cells expressing SP-C\(^{\Delta}_{\text{exon4}}\) showed faint, diffuse staining that was detected only in the presence of proteasome inhibitor (Figure 3.1d, panels a and d, respectively). SP-C\(^{\text{wt}}\) traffics to the lamellar body in type II cells but is redirected to the lysosome in cells which lack lamellar bodies (DellAngelica et al. 2000; Weaver et al. 2002). Co-localization of SP-C\(^{\text{wt}}\) with the lysosomal marker LAMP-1 demonstrated efficient export of wild type protein from the ER (Figure 3.1d, panel c). In contrast, SP-C\(^{\Delta}_{\text{exon4}}\) failed to co-localize with LAMP-1 and exhibited a faint and diffuse staining pattern consistent with ER localization (Figure 3.1d, panel f). These data in clonal cell lines, coupled with previous results in transiently transfected cells, suggest that SP-C\(^{\text{wt}}\) is correctly folded and
exported from the ER while SP-C\textsuperscript{Δexon4} fails quality control and is rapidly degraded by the proteasome.

Transcriptional profiling reveals differential expression of genes associated with apoptosis in SP-C\textsuperscript{Δexon4} cells.

Molecular pathways induced by chronic expression of SP-C\textsuperscript{Δexon4} were identified by transcriptional profiling of SP-C\textsuperscript{wt} and SP-C\textsuperscript{Δexon4} clonal cell lines (the complete dataset can be found at http://www.ncbi.nlm.nih.gov/geo/, accession number GSE2980). Unexpectedly, known components of the UPR/ERAD pathways were not increased in cells that constitutively expressed SP-C\textsuperscript{Δexon4}, in contrast to results in transiently transfected cells (Bridges et al. 2003). However, several genes associated with anti and pro-apoptosis pathways were differentially expressed in the SP-C\textsuperscript{Δexon4} clonal cell line, including BAX and Bcl-2 (Tables 3.1 and 3.2, Figure 3.2). Interestingly, two transcripts linked to the NF-κB pathway, interleukin-1 receptor associated 1 (IRAK1) and the gamma subunit of the Iκ-B kinase complex (IKBKG), were modestly increased in the SP-C\textsuperscript{Δexon4} cells, consistent with activation of NF-κB. Analyses of the 5’ flanking sequences of apoptosis-associated genes revealed that ~44% (15/34) contained putative NF-κB binding sites suggesting that they may be direct targets of NF-κB.

To determine if NF-κB activity was increased in SP-C\textsuperscript{Δexon4} cells, the NF-κB luciferase reporter construct, pELAM-Luc, was transiently transfected into the clonal cell lines. SP-C\textsuperscript{Δexon4} cells exhibited an 8.6-fold increase in basal NF-κB activity compared to cells stably transfected with empty vector or SP-C\textsuperscript{wt} (Figure 3.3a). Co-transfection of a stabilized, non-phosphorylatable form of I-κBα (SR) with the pELAM-Luc resulted in a dose-dependent decrease in luciferase activity in SP-C\textsuperscript{Δexon4} cells that was completely suppressed to levels detected in untreated SP-C\textsuperscript{wt} cells (Figure 3.3b). Treatment of SP-C\textsuperscript{Δexon4} cells with SN50 to inhibit nuclear translocation of NF-κB resulted in cellular retraction and a significant increase in cell death, as indicated by PI staining (Figure 3.4a) and MTS-reduction assay (Figure 3.4c). Treatment of SP-C\textsuperscript{Δexon4} cells with a control peptide, SN50\textsuperscript{mut}, at equivalent doses and duration had no effect (Figure 3.4b). Cells expressing SP-C\textsuperscript{wt} were unaffected by SN50 or SN50\textsuperscript{mut} treatment (data not shown).
Taken together, these results suggest that NF-κB plays an important cytoprotective role in adaptation constitutive expression of SP-CΔexon4.

SP-CΔexon4 expression increases susceptibility to viral-induced death

The increased expression of pro-apoptotic transcripts in SP-CΔexon4 cells suggested that adapted cells may be more susceptible to secondary stress. Infection with respiratory syncytial virus (RSV) preceded the onset of ILD in several patients carrying the SP-CΔL188Q mutation. To determine if the expression of SP-CΔexon4 increased susceptibility to RSV the clonal cells lines were infected at 10 MOI and cell viability was assessed 24hrs post-infection. While cell viability was minimally affected in SP-Cwt cells, as indicated by the low level of PI staining (Figure 3.5a, panels b and f), infection of cells expressing SP-CΔexon4 resulted in significant cell death (Figure 3.5a, panels d and h). Western blot analysis demonstrated that SP-CΔexon4 protein accumulated in a manner directly correlated with the viral titer while SP-Cwt protein levels were unaffected (Figure 3.5b). Several viruses, including adenovirus, influenza and hepatitis B and C viruses, have been reported to induce ER stress/UPR pathways (Meyer et al. 1992; Pahl et al. 1995; Pahl et al. 1996; Tardif et al. 2004). To determine if RSV activated the UPR in cells stably expressing SP-C, an UPRE-luciferase reporter construct was transiently transfected into cells expressing SP-CΔexon4 or SP-Cwt prior to infection with RSV. Baseline levels of luciferase activity were similar between the cell lines, consistent with the microarray data, indicating that the UPR was not activated in cells stably expressing SP-CΔexon4. However, luciferase activity was increased in both cell lines following RSV infection and the effect was exacerbated in SP-CΔexon4 cells (2.5-fold increase in SP-Cwt vs. 4.4-fold in SP-CΔexon4) (Figure 3.5c). Collectively, these data indicate that RSV infection induces cell death associated with an accumulation of the mutant proprotein and pronounced activation of the UPR.

Proteasome function is decreased in RSV-infected SP-CΔexon4 cells

Accumulation of mutant SP-C proprotein in RSV-infected SP-CΔexon4 cells suggested that proteasome function was inhibited. To assess the impact of RSV infection
on proteasome activity, a proteasome reporter construct, pZsProSensor-1, was transiently transfected into the clonal cell lines. Cells were infected with 1-10 MOI of RSV and analyzed 24hrs later by fluorescence microscopy and FACS analysis. Fluorescence in untreated SP-C^Δexon4 cells was higher than that observed in untreated SP-C^wt cells, indicating a modest inhibition of basal proteasome activity in the presence of the mutant proprotein (**Figure 3.6a, panel e vs Figure 3.6b, panel e**). Infection of SP-C^Δexon4 cells with RSV resulted in a dose-dependent increase in fluorescence detected by microscopy and FACS (**Figure 3.6a, panels e-h**). In contrast, fluorescence was not altered in SP-C^wt cells infected with RSV (**Figure 3.6b, panels e-h**). These results demonstrate that basal proteasome function is decreased in SP-C^Δexon4 cells and that this perturbation is exacerbated during RSV infection.

**SP-C^Δexon4 sensitizes cells to proteasome inhibition and is associated with accumulation of mutant proprotein and activation of XBP-1**

To determine if accumulation of SP-C^Δexon4 induces ER stress and activation of apoptosis independently of RSV infection, the clonal cell lines were treated with the proteasome inhibitor MG-132. The SP-C^Δexon4 cell line was exquisitely sensitive to proteasome inhibition. The majority of cells retracted and detached from the plate following treatment with 250nM MG-132 for 18 hours and a more pronounced effect was detected at the 500nM concentration (**Figure 3.7a, panels g and h**). SP-C^wt cells were minimally affected by similar treatment (**Figure 3.7a, panels a through d**). Cell viability was decreased approximately 10% in SP-C^wt cells and 50% in SP-C^Δexon4 cells treated with 500nM MG-132 (**Figure 3.7b**).

To determine if SP-C^Δexon4 accumulation was associated with cytotoxicity, immunoblot analysis was performed with a proSP-C antibody following MG-132 treatment. There was a dose-dependent increase in SP-C^Δexon4 protein while levels of the SP-C^wt protein were unaffected by MG-132 treatment (**Figure 3.7c**). Accumulation of unfolded proteins in the ER results in the activation of the IRE1/XBP-1 pathway (Harding et al. 2002). Active XBP-1 was not detected in SP-C^wt cells following MG-132 treatment; in contrast, robust activation of XBP-1 was detected in SP-C^Δexon4 cells following proteasome inhibition (**Figure 3.7d**). Collectively, these results demonstrate
that proteasome inhibition resulted in dose-dependent cytotoxicity associated with accumulation of SP-CΔexon4 and activation of XBP-1.

Accumulation of SP-CΔexon4 is associated with apoptosis

To determine if accumulation of SP-CΔexon4 following proteasome inhibition was associated with apoptosis, cell lines were treated with MG-132 and stained with the fluorescent DNA-intercalating dye H33342 to assess nuclear architecture. Cells expressing SP-Cwt showed a diffuse, uniform staining of the nucleus typical of viable, healthy cells (Figure 3.8a, left panel and inset). Although the majority of cells expressing SP-CΔexon4 detached from the plate following MG-132 treatment and were excluded from this analysis, the cells that remained attached exhibited punctuate staining and nuclear condensation consistent with apoptosis (Figure 3.8a, right panel and inset). Further, treatment of SP-CΔexon4-expressing HEK293 cells with MG-132 for 18 hours resulted in activation of caspase 3 (Figure 3.8b). Caspase 3 activation was not detected in SP-Cwt cells treated with MG-132, indicating that this event was specific for cells expressing mutant SP-CΔexon4 (Figure 3.8c). Expression of SP-CΔexon4 in the distal lung epithelium of transgenic mice resulted in cytotoxicity associated with lung dysmorphogenesis (Chapter 2). Immunohistochemistry was performed on fetal lung sections from a transgene positive animal (TG#2) and a wild-type littermate control (WT) with an antibody that detects the cleaved isoform of caspase 3. Robust staining for active caspase 3 was detected in sloughed epithelial cells in the distal airway and isolated intact epithelial cells in the transgenic animal while lung epithelial cells in the wild-type animal were negative (Figure 3.8d). Taken together, these results demonstrate that the accumulation of SP-CΔexon4, induced by proteasome inhibition in vitro or overexpression in vivo, is associated with the activation of apoptotic pathways.

Dilation of pre-Golgi intermediate (pGI) compartment and detection of inclusion bodies in MG-132-treated SP-CΔexon4 cells

SP-CΔexon4 cells were examined by transmission electron microscopy following MG-132-treatment to detect ultrastructural changes associated with cytotoxicity. Cells expressing SP-Cwt showed prototypical ER ultrastructure (Figure 3.9, panel a) which
was not affected by treatment with MG-132 (data not shown). Untreated SP-C\textsuperscript{Δexon4} expressing cells also exhibited normal ER ultrastructure that was indistinguishable from that in SP-C\textsuperscript{wt} cells (Figure 3.9, panel b). Following treatment with MG-132, approximately 20% of SP-C\textsuperscript{Δexon4}-expressing cells exhibited profound dilation of the pre-Golgi intermediate compartment (Figure 3.9, panel c). A similar proportion of MG-132-treated SP-C\textsuperscript{Δexon4}-expressing cells also contained large, membrane-bound inclusion bodies, resembling autophagosomes (Figure 3.9, panel d). The ER of SP-C\textsuperscript{Δexon4} cells appeared to be unaffected by proteasome inhibition (Figure 3.9, panel c). Immunogold labeling detected mutant SP-C\textsuperscript{Δexon4} in ER, Golgi complex, vesicles, lysosomes, and membrane bound inclusions (Figure 3.9, panel e). Contingency table analysis demonstrated that MG-132-treated SP-C\textsuperscript{Δexon4} expressing cells had greater than expected SP-C\textsuperscript{Δexon4} accumulated in membrane-bound inclusions (p<0.001, degrees of freedom=4), while label distribution in ER and Golgi complex was not affected by MG-132 treatment (Tables 3.3, 3.4 and 3.5). These data indicate that the accumulation of mutant SP-C\textsuperscript{Δexon4} proprotein is associated with dilation of the pre-Golgi intermediate compartment and formation of large cytoplasmic inclusion bodies prior to cell death.
Discussion

The SP-C\textsuperscript{Δexon4} mutation was chosen for these experiments because of its link to ILD in human patients (Nogee et al. 2001; Nogee et al. 2002), the severity of this mutation on the structure of the proprotein, and its association with cytotoxicity and lung dysmorphogenesis when expressed in transgenic mice (Bridges et al. 2003). Results of studies in stably transfected cells indicated that the SP-C\textsuperscript{Δexon4} cell line adapted to constitutive expression of misfolded SP-C and that NF-κB played a pivotal role in the adaptive response. Infection of cells expressing SP-C\textsuperscript{Δexon4} with RSV resulted in enhanced cytotoxicity associated with accumulation of the mutant proprotein, pronounced UPR activation and cell death. Collectively, these results suggest that adaptation to chronic ER stress imposed by misfolded SP-C may promote resistance to ILD while environmental insults, such as viral infection, may trigger the onset of disease in patients with mutations in \textit{SFTPC}.

While the effects of acute ER stress, imposed by xenotoxic agents such as thapsigargin and tunicamycin, are well established, little is known about the molecular pathways involved in adaptation to chronic ER stress imposed by a misfolded protein. The lack of a cytotoxic response coupled with the absence of ER stress induction in SP-C\textsuperscript{Δexon4} clonal cells suggested that these cells successfully adapted to constitutive expression of misfolded SP-C. Several lines of evidence support this hypothesis. Firstly, the level of SP-C mRNA in the SP-C\textsuperscript{Δexon4} cell line was comparable to that in the SP-C\textsuperscript{wt} cell line, thus excluding low expression of the mutant protein as a reason for the survival of SP-C\textsuperscript{Δexon4} cells. Secondly, in contrast to the wild-type protein, SP-C\textsuperscript{Δexon4} protein was barely detectable indicating that the misfolded proprotein was recognized as terminally misfolded and rapidly degraded via the ERAD pathway; further, chronic expression of SP-C\textsuperscript{Δexon4} was associated with modestly reduced proteasome function consistent with near saturation of this degradative pathway. Thirdly, components of the ER stress/UPR pathways were not transcriptionally upregulated in the clonal SP-C\textsuperscript{Δexon4} cell line, in marked contrast to results in transiently transfected HEK293 cells (Bridges et al. 2003; Mulugeta et al. 2005). Fourthly, chronic expression of SP-C\textsuperscript{Δexon4} was associated with
differential expression of both pro- and anti-apoptotic genes, approximately 50% of which contained putative NF-κB binding sites in their promoters. Lastly, inhibition of NF-κB, which is known to promote cell survival in response to a variety of stresses, resulted in death of SP-CΔexon4 cells. Collectively, these results are consistent with an NF-κB-dependent adaptive response to chronic ER stress imposed by the constitutive expression of SP-CΔexon4. Although induction of other molecular pathways involved in cell survival likely occur, activation of NF-κB appears to be critical for survival in HEK293 cells that constitutively express SP-CΔexon4.

The role of NF-κB in the regulation of cytokine-stimulated pro-inflammatory gene expression is well established (Li et al. 2002). However, NF-κB activity is also increased in response to the accumulation of transmembrane proteins in the ER, including the E3/19k protein of adenovirus (Pahl et al. 1996), MHBS' of hepatitis B virus (Meyer et al. 1992), expression of MHC class 1 in the absence of β2-microglobin protein (Pahl et al. 1996) and p450 (Szczesna-Skorupa et al. 2004). Activation of NF-κB in response to accumulation of newly synthesized membrane proteins is referred to as the ER overload response (EOR). Constitutive activation of NF-κB promotes survival of a wide range of cells, including B-cells, hepatic cells and cancer cells, and is a major target for cancer therapy (Kucharczak et al. 2003). NF-κB promotes cell survival, in part, by activating anti-apoptotic genes including members of the IAP family, TRAF1 and TRAF2, and the Bcl2 homologues Bfl-1/A1 and Bcl-Xl (Kucharczak et al. 2003). Recent studies, using tunicamycin and thapsigargin as stressor agents, demonstrated an association between ER stress-induced PERK activation, eIF2α phosphorylation and NF-κB activation (Jiang et al. 2003; Deng et al. 2004), effectively linking the UPR and the EOR. Although a definitive role for NF-κB activation in response to ER-stress has not been established, it is thought to promote cell survival in this context.

When cells were transiently exposed to SP-CΔexon4, the UPR and ERAD pathways were activated, promoting clearance of the misfolded protein and alleviation of ER stress. Transient transfection of HEK293 cells with SP-CΔexon4 resulted in specific upregulation of BiP, XBP-1 and HedJ1, consistent with induction or UPR and ERAD (Bridges et al. 2003; Mulugeta et al. 2005). In contrast, constitutive expression of SP-CΔexon4 resulted in differential expression of apoptosis-related genes with an apparent balance shifted toward
NF-κB-dependent anti-apoptotic responses. Identification of environmental triggers that tip the balance toward an apoptotic fate are clearly important for understanding disease pathogenesis in humans with SFTPC mutations. ILD is often associated with infection and/or inflammation (Vassallo 2003; Noble et al. 2004). The marked variability in severity and age of onset of lung disease in the SP-C^{L188Q} pedigrees suggested that environmental factors might be involved in triggering the onset of ILD (Thomas et al. 2002; Chibbar et al. 2004). Consistent with this hypothesis, five individuals in the SP-C^{L188Q} kindreds were diagnosed with viral infection prior to manifestation of lung disease. Similarly, the survival of cells constitutively expressing SP-C^{Δexon4} was dramatically decreased in the presence of RSV infection. RSV-induced cytotoxicity was associated with accumulation of the mutant proprotein SP-C^{Δexon4}, pronounced activation of the UPR and proteasome dysfunction.

Although inhibition of proteasome function has not been previously reported for RSV, RSV infection did induce ER stress in the SP-C clonal cell lines used in this study. Proteasome inhibition may be related in part to forced synthesis of viral membrane proteins including the F, G and SH proteins. The level of UPR activation was higher in SP-C^{Δexon4} cells than SP-C^{wt} cells suggesting that production of RSV membrane proteins superimposed on the expression of misfolded SP-C^{Δexon4} protein may overwhelm the degradative capacity of the proteasome, leading to accumulation of cytotoxic forms of SP-C^{Δexon4} and subsequent cell death. Alternatively, RSV-mediated inhibition of proteasome function may lead to stabilization of IkB and subsequent inactivation of NF-κB. However, proteasome inhibition had a minimal effect on NF-κB translocation induced by RSV infection in A549 cells (Fiedler et al. 1999) or thapsigargin treatment in mouse embryonic fibroblasts (Jiang et al. 2003).

Accumulation of SP-C^{Δexon4} in HEK293 cells was associated with a marked increase in the efficiency of XBP-1 splicing. Activation of the IRE1/XBP-1 pathway has been shown to increase the transcription of gene products associated with the refolding and degradation of unfolded/misfolded proteins, including members of the HSP-40 family and EDEM (Yoshida et al. 2003). Active IRE1/XBP-1 did not appear to play a protective role in stably transfected HEK293 cells as overexpression of the active form of XBP-1 (XBP-1(s)) failed to protect against RSV-mediated accumulation of SP-C^{Δexon4}.
(data not shown). The inability of XBP-1 to protect in the current study may be due to saturation of the degradative capacity of the proteasome following RSV infection. Expression of SP-C\textit{Δ}exon4 \textit{in vivo} was associated with accumulation of SP-C\textit{Δ}exon4, caspase 3 activation, cytotoxicity and lung dysmorphogenesis ((Bridges et al. 2003) and \textbf{Figure 3.8d} of this study). Since degradation of SP-C\textit{Δ}exon4 is proteasome-dependent, it is possible that elevated expression of SP-C\textit{Δ}exon4 in transgenic mice saturated the proteasome, leading to apoptosis of distal epithelial cells and lung dysmorphogenesis. It is also likely that apoptosis due to the accumulation of SP-C\textit{Δ}exon4 contributed to the phenotype in SP-C\textit{Δ}exon4 transgenic mice. Consistent with this concept, apoptosis of alveolar epithelial cells was associated with idiopathic pulmonary fibrosis in humans (Kuwano et al. 1996) and bleomycin-induced pulmonary fibrosis in mice (Hagimoto et al. 1997; Kuwano et al. 1999) while inhibition of apoptosis prevented bleomycin-induced fibrosis in mice (Kuwano et al. 2001).

Accumulation of SP-C\textit{Δ}exon4 proprotein was associated with ultrastructural changes of subcellular compartments including dilation of the pre-Golgi intermediate (pGI) complex, also known as ER-Golgi intermediate compartment (ERGIC), and the formation of electron-dense inclusion bodies. A missense mutation of the Insulin 2 gene (Cys96Thr), that results in misfolding of proinsulin, was also associated with dilation of the pGI complex in pancreatic β cells in Akita mice (Zuber et al. 2004). The authors of this study suggested that the pGI is the site of retrotranslocation of misfolded proteins. This concept was supported by the co-localization of Sec61, a core protein component of the mammalian translocon, and UDP-glucose:glycoprotein glucosyltransferase, an enzyme important for ER quality control, with the ERGIC (Greenfield et al. 1999; Zuber et al. 2004). Inclusion bodies in SP-C\textit{Δ}exon4 cells were bounded by a limiting membrane, contained the mutant protein and morphologically resembled autophagosomes. Transient expression of SP-C\textit{Δ}exon4 in HEK293 cells was previously reported to induce formation of SP-C-positive aggresomes (Wang et al. 2003). However, whether inclusion bodies/aggresomes actually cause cytotoxicity in cell culture and contribute to the disease process \textit{in vivo} remains unclear.

Based on the findings of this study the following model is proposed. SP-C\textit{wt} protein is correctly folded and exported from the ER whereas SP-C\textit{Δ}exon4 is terminally
misfolded and degraded by a proteasome-dependent pathway. Constitutive expression of terminally misfolded SP-C<sub>Δexon4</sub> results in chronic ER stress and an NF-κB-dependent cytoprotective response. Superimposition of a secondary stress, such as RSV infection, on constitutive expression of SP-C<sub>Δexon4</sub> leads to proteasome dysfunction, accumulation of SP-C<sub>Δexon4</sub> and cytotoxicity. Accumulation of SP-C<sub>Δexon4</sub> and cytotoxicity may contribute to the pathogenesis of ILD associated with mutations in SFTPC.
Materials and Methods

Reagents

Recombinant TNFα protein was from Peprotech (Rocky Hill, NJ). The MTS-reduction assay kit (cat# G3582) and Dual-Luciferase® Reporter Assay System were from Promega (Madison, WI); MTS-reduction assays were performed in 96-well plates per manufacturer’s protocol. MG-132, SN50 and control peptide (SN50mut) were from EMD Biosciences (San Diego, CA).

SP-C cDNA constructs and generation of stably transfected cell lines

Full-length human, wild-type SP-C (SP-Cwt) cDNA and SP-CΔexon4, generated as previously described (Bridges et al. 2003), were subcloned into pTRE2-Hyg (BD Biosciences, Palo Alto, CA) to generate SP-Cwt/pTRE2-Hyg and SP-CΔexon4/pTRE2-Hyg. To generate stably transfected cell lines, HEK293 Tet-Off cells (Clontech) were transiently transfected with SP-Cwt/pTRE2-Hyg or SP-CΔexon4/pTRE2-Hyg using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), in the presence of 250µg/ml hygromycin B as the selection agent. Doxycycline was present in the media during the selection process in order to obtain regulatable lines (i.e. expression of SP-Cwt or SP-CΔexon4 was dependent on doxycycline withdrawal). However, all lines that were initially regulatable eventually reverted to constitutive expression of the transgene. Therefore, SP-Cwt or SP-CΔexon4 cells for this study were cultured in the absence of doxycycline and G418 (selection agent for the tTA cassette). Clonal colonies were isolated, amplified and screened for integration of the transgene by RT-PCR using SP-C-specific primers and Western analyses using an antibody directed against the N-terminal peptide of proSP-C (Bridges et al. 2003).

RNA isolation, RT-PCR and microarray analysis

Total RNA for RT-PCR analysis was isolated using the acidified guanidinium method (Chomczynski et al. 1987), treated with Dnase I (DNA-free; Ambion, Austin, TX) and reverse transcribed into cDNA using SuperScript™ II Reverse Transcriptase
Forward and reverse primer sequences for human XBP-1 are as follows: 5’-GGA CTT AAG ACA GCG CTT GG-3’, 5’-TGA GAG GTG CTT CCT CGA TT-3’. PCR reactions were performed for 35 cycles and products were separated on 4% agarose gels.

RNA samples for microarray analysis were prepared as previously described (DeFelice et al. 2003) and hybridized to the GeneChip® Human Genome U133 Set (HG-U133A and HG-U133B (Affymetrix, Santa Clara, CA) according to the manufacturer’s protocol. Affymetrix Microarray Suite 5.0 was used to scan and quantitate the gene chips under default scan settings. Normalization was performed using the Robust Multichip Average model (Irizarry et al. 2003a; Irizarry et al. 2003b). Data were further analyzed using Significance Analysis Of Microarrays (SAM (Tusher et al. 2001)) and Genespring 7.2 (Silicon Genetics, Redwood City, CA). Detection of differential expression was performed using random permutation and Welch’s approximate t-test for mutant and control groups at the P value ≤ 0.01, FDR ≤ 10%, a minimal of 2-fold changes in absolute ratio and a minimal of 2 Present calls by Affymetrix algorithm in 3 samples with the relative higher expression. Gene Ontology analysis was performed using David (database for annotation, visualization, and integrated discovery) (Dennis et al. 2003). Potential protein/protein interactions were identified using PathwayAssist (Ariadne Genomics). NF-kB binding sites (GGGACTTTCC) were scanned in -2kb promoter region of all differentially expressed genes from microarray analysis using MatInspector (Genomatix) allowing maximal of 1 mismatch.

**Cell culture and Western analysis**

HEK293 stable cell lines were propagated as previously described (Bridges et al. 2003). Protein standardization and Western analysis were performed as previously described (Bridges et al. 2003) using antibodies directed against the N-terminal peptide of SP-C (Vorbroker et al. 1995a), caspase 3 (Cell Signaling, Beverly, MA) or actin (Bridges et al. 2003).
Confocal, fluorescence, electron microscopy and immunogold labeling

Cells for confocal microscopy were prepared as previously described (Conkright et al. 2001). Primary antibodies included a polyclonal anti-proSP-C (Bridges et al. 2003) and a monoclonal anti-LAMP1/CD107A (RDI, Flanders, NJ). Secondary antibodies included anti-rabbit FITC-conjugated and anti-mouse Texas Red(TR)-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA). Fluorescence was visualized with a Zeiss LSM 510 confocal microscope using FITC/TR filters.

Fluorescence and phase microscopy of live cells was performed on an Olympus microscope equipped with a UV lamp and FITC/TR filters and images were captured with an Optronics MagnaFire digital camera. For cell death assays, propidium iodide solution (2 µl of 1mg/ml) was added to live cells for 10min at room temperature prior to visualization.

For electron microscopy, HEK293 cells expressing SP-C∆exon4 or SP-C1-197 were fixed and processed as previously described (Rice et al. 2002). Electron micrographs of HEK 293 cells were digitally acquired using a JEM-1230 TEM (JEOL, Peabody, MA) and an AMT HR-B digital TEM camera (AMT, Danvers, MA).

For immunogold labeling, HEK293 cells were fixed and processed as previously described (Markart et al. 2004). Localization of SP-C∆exon4 was determined using rabbit antisera raised against the N-terminus of proSP-C (Bridges et al. 2003) and 10 nm protein A gold probes (Ueno et al. 2004).

Labeling specificity of antisera to biosynthetic and lysosomal compartments, i.e., endoplasmic reticulum and lysosomes, was assessed using the relative labeling index (RLI) method (Mayhew et al. 2002). Any cellular compartments that had high partial Chi-squared values and RLI greater than 1 were considered to be preferentially labeled by the antisera.

Differential distributions in untreated and MG-132-treated cells were compared using contingency table analysis (Mayhew et al. 2004). Raw scores of gold counts from within endoplasmic reticulum (ER), Golgi (G), vesicles (V), lysosomes (L), and membrane bound inclusions (I) were normalized by a factor of 10 and compared with predicted distributions to determine if MG-132 altered SP-C∆exon4 localization in specific compartments. Any labeled compartments that contributed to greater than 10% of the
total Chi-squared value were considered to have significantly different labeling distributions.

*Flow cytometry*

HEK293 cells were detached from the culture plate 24hrs following RSV infection/transient transfection by treatment with trypsin/EDTA, washed 1X with PBS and resuspended in FACS buffer consisting of PBS with 0.1%FBS and 0.05% sodium azide. Cell associated fluorescence was measured on a FACScalibur flow cytometer using CellQuest software (BD Biosciences). For each sample, 20,000 events were acquired.

*Immunohistochemistry*

Immunohistochemistry was performed on mouse fetal lung samples described in a previous study (Bridges et al. 2003) using an antibody that detects the cleaved form of caspase 3 (cat# AF835, R&D systems, Minneapolis, MN) at a 1:5,000 dilution. Localization of antigen-antibody complexes was performed as previously described (Bridges et al. 2003).

*RSV production and infection protocol*

The A2 strain of RSV was amplified, purified and quantitated in HepG2 as previously described (LeVine et al. 1999). Clonal cell lines were infected with RSV using a previously established protocol for adenoviral infection (Bridges et al. 2003). For studies in which proteasome activity was assessed following RSV infection, the proteasome sensor plasmid, pZsProSensor1 (Clontech, Mountain View, CA), was transfected into the cells (as described above) 24hrs prior to RSV infection.

*Luciferase constructs and assays*

The NF-κB-dependent ELAM-1 promoter-driven firefly luciferase plasmid (pELAM-luc) was from MJ Fenton (University of Maryland, Baltimore, MA). The p5xATF6GL3 plasmid (also known as the UPRE-luciferase reporter) was from Ron Prywes (Columbia University, New York, NY). The IκB “super repressor” expression plasmid (SR) was from R. Hay (University of St. Andrews, St. Andrews, Scotland). The
pRL-TK plasmid was from Promega. Firefly luciferase constructs (400ng) were co-transfected with pRL-TK (50ng) using Lipofectamine 2000. Cells were harvested 48hrs post-transfection and luciferase activity was quantified using the Dual-Luciferase® Assay system in a Berthold multi-tube luminometer. Data are plotted as the ratio of firefly/renilla activity to correct for transfection efficiency among samples.

**Statistics**

Data were analyzed with InStat version 3.0 (GraphPad Software, San Diego, CA). Values are presented as means ± s.d.. Multiple comparisons were made by ANOVA using the Tukey-Kramer multiple-comparisons test and paired samples were analyzed by t-tests. In both cases, statistical significance was defined as p<0.05 or less.

**Antibody (Ab) labeling specificity by RLI analysis**

Ab labeling specificity was determined using Chi-squared analysis. Predicted gold counts are determined using the formula P(i) * (Σ(N_o)/Σ(P)), where P(i) is the observed number of point registered on the ith compartment by the counting grid, Σ(N_o) is the summation of observed gold counts, and Σ(P) is the summation of number of points registered on cells. For example, the predicted gold counts on the nucleus is P(Nuc) * (Σ(N_o)/Σ(P)) = 4776 * (4905/100376) = 2258. Relative labeling index (RLI) is calculated using the formula N_o/N_e. RLI for the nucleus is N_o(Nuc)/N_e(Nuc) = 2230/2248 = 0.99. The partial Chi-squared value is obtained using the formula (N_o-N_e)^2/N_e, which gives (2230-2258)^2/2258 = 0.34 for the nucleus. Summaries of labeling specificity by Chi-squared analysis are listed in Supplementary Figure 5. Any cellular compartment that has high partial Chi-squared value and RLI values greater than 1 is considered to be preferentially labeled by the antisera. Chi-squared analysis demonstrated that Ab labeling detected in MG-132 untreated and treated HEK 293 cells was not random (p<0.001). RLI analysis showed that Ab labeled preferentially to biosynthetic and lysosomal compartments, while Ab labeling detected in the nucleus and cytosol were random and nonspecific (RLI<1).
Comparison of gold particle distributions between untreated and MG-132 treated cells expressing SP-C^Δexon4

Contingency table analysis was used to determine whether MG-132 treatment may affect mutant SP-C^Δexon4 distribution in the biosynthetic and lysosomal compartments. The compartmental gold labeling predicted by contingency table analysis is given using the formula $\sum(C_i) \times \sum(C_j) / \sum(C_{ij})$, where $\sum(C_i)$ is the summation of gold counts from treatment group i, $\sum(C_j)$ is the summation of gold counts from compartment j, and $\sum(C_{ij})$ is the summation of gold counts from all cell groups. For example, the predicted gold counts for ER in the MG132 untreated cells and treated cells are $125 \times (29+64)/478=24.32$ and $353 \times (29+64)/478=68.68$ respectively. The Chi-squared values for the ER for untreated and treated cells are $(29-24.32)^2/24.32=0.90$ and $(64-68.68)^2/68.68=0.32$ respectively. Any labeled compartment that contribute to greater than 10% of the total Chi-squared value is considered to have significantly different labeling distribution compared to the same compartment by other treatments. Contingency table analysis demonstrated that the labeling for treated HEK 293 cells was significantly different to untreated cells ($p<0.001$ with degrees of freedom =4). Examination of partial Chi-squared values showed that vesicles in untreated cells had significantly higher than expected number of gold particles. Although vesicles in MG132 treated cells had fewer than expected number of gold particles, it was not significant different to the predicted gold counts because the Chi-squared contribution was less than 10% of the total Chi-squared value. The number of gold particles in the membrane bound inclusions was significantly between untreated and treated cells, whereas there were no significant differences in labeling for ER, Golgi, and lysosomes in untreated and treated cells. The untreated cells had significantly fewer gold particles than expected number of gold particles, while treated cells had significantly greater than expected number of gold particles in inclusions, suggested that treatment with MG-132 altered mutant SP-C^Δexon4 distribution in transfected HEK 293 cells.
Acknowledgements

The authors would like to thank ChenXia Duan, Mei Wang, Senad Divanovic, Al Senft, Mukund Raghavan and Xiaofei Shangguan for technical assistance and/or advice.

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**Figure legends**

**Figure 3.1 – Generation of clonal, stably transfected cell lines**
(a) Phase light micrographs of stably transfected HEK293 cell lines expressing SP-C\textsuperscript{wt} or SP-C\textsuperscript{Δexon4}. Scale bars = 20 µm. (b) Expression of SP-C mRNA in the two cell lines. (c) Cell lysates (30 µg) were subjected to Western analysis using antibody directed against the N-terminus of proSP-C to detect SP-C\textsuperscript{wt} (Mr=21k) and SP-C\textsuperscript{Δexon4} (Mr=17k). (d) Clonal cell lines expressing SP-C\textsuperscript{wt} or SP-C\textsuperscript{Δexon4} were plated on poly-lysine coated coverslips, treated with 5 µM MG-132 for 4 hours, fixed, permeabilized and sequentially stained with antibodies directed against the N-terminus of proSP-C and LAMP1. FITC-conjugated (proSP-C) and Texas Red-conjugated (LAMP1) secondary antibodies were added and images were captured by confocal microscopy. Scale bars = 10 µm.

**Figure 3.2 - Functional associations of differentially expressed genes in SP-C\textsuperscript{Δexon4} cells associated with apoptosis.**
Functional associations analyzed with a separate tool, PathwayAssist (Ariadne Genomics), also identified multiple genes involved in the regulation of apoptosis. Genes up-regulated are highlighted in red; genes down-regulated are highlighted in green. Each line indicates a regulatory relationship based upon literature references (a purple line linking two nodes with a dot in between represents binding, a blue line with a box in between represents expression, a gray dashed line with a box in between represents regulation and a brown line and dot represents protein modification). Regulatory relationships are denoted by arrows and signs within the box (plus sign indicates positive regulation, minus sign indicates negative regulation and no sign when the regulatory relationship is unknown).

**Figure 3.3 - Basal NF-κB activity is increased in cells expressing SP-C\textsuperscript{Δexon4}**
(a) The clonal cell lines expressing empty vector (hatched bar), SP-C\textsuperscript{wt} (open bar) or SP-C\textsuperscript{Δexon4} (closed bar) were transiently co-transfected with a NF-κB reporter construct (pELAM-Luc) and a plasmid encoding renilla luciferase (pRL-TK). Cell lysates were analyzed for luciferase activity forty-eight hours post-transfection and normalized to renilla luciferase activity. Similar results were obtained with two additional NF-κB-
luciferase reporter constructs including a minimal IL-8 promoter and a synthetic NF-κB promoter (data not shown). * = p<0.05 compared to SP-C\textsuperscript{wt}. (b) SP-C\textsuperscript{wt} (open bars) or SP-C\textsuperscript{Δexon4} (closed bars) clonal cell lines were transiently transfected with pELAM-Luc, pRL-TK and increasing amounts (10pg-1ng) of a plasmid encoding a super repressor of NF-κB activity (SR). Co-transfection of 1ng of empty vector (EV) with pELAM-Luc had no effect on luciferase activity. Twenty-four hours post-transfection, one group transfected with the SR (1ng) and one without were treated with TNFα (10ng/ml) for 12 hours. Cell lysates were harvested 36hrs post-transfection and analyzed for luciferase activity. Results are presented as means ± s.d. of the ratio of firefly:renilla, expressed as relative luciferase units (RLU); each determination was performed in triplicate and the experiment was repeated 4 times. * = p<0.05 compared to SP-C\textsuperscript{Δexon4} untreated; # = p<0.05 compared to SP-C\textsuperscript{wt} unRx.

Figure 3.4 - Inhibition of NF-κB nuclear translocation induces death of SP-C\textsuperscript{Δexon4} cells.

The clonal cell line expressing SP-C\textsuperscript{Δexon4} was treated with SN50, a cell-permeable peptide that inhibits nuclear translocation of NF-κB (panel a) or a mutant SN50 peptide (SN50\textsuperscript{mut}, panel b) at the indicated doses. Cells were stained with propidium iodide 24hrs after addition of peptide and phase (top row) and fluorescent (bottom row) images were captured directly from the culture plate. Scale bars = 50μm. (c) Clonal cell lines expressing SP-C\textsuperscript{wt} or SP-C\textsuperscript{Δexon4} were treated with the SN50 peptide at indicated doses for 24hrs and cell death was quantitated by MTS assay. Results are presented as means ± s.d.; each determination was performed in triplicate and the experiment was repeated 3 times. * = p<0.05 compared to SP-C\textsuperscript{Δexon4} unRx; # = p<0.05 compared to SP-C\textsuperscript{wt} unRx.

Figure 3.5 - SP-C\textsuperscript{Δexon4} increases susceptibility to respiratory syncytial virus-induced cell death

(a) Cells expressing SP-C\textsuperscript{wt} (panels a, b, e, f) or SP-C\textsuperscript{Δexon4} (panels c, d, g, h) were infected with RSV at 10MOI (panels b, f, d, h). Cells were stained with propidium iodide (PI) 24hr post-infection and phase (panels a, b, c, d) or fluorescent (panels e, f, g, h) images were captured directly from the culture plate. Scale bars = 50μm. (b) Cells expressing
SP-C\textsuperscript{Δexon4} were infected with 0.1, 1 or 10 MOI RSV for 24hrs. Cells were harvested and equivalent amounts of lysate (30 ug) were separated by SDS-PAGE and subjected to Western blotting with an anti-proSP-C antibody or an anti-actin antibody. (c) Clonal cell lines expressing SP-C\textsuperscript{wt} or SP-C\textsuperscript{Δexon4} were transiently transfected with a reporter construct consisting of 5 UPRE elements driving luciferase (UPRE-Luc) and pRL-TK. Cells were infected with 5 MOI RSV 24 hrs post-transfection and cell lysates were analyzed for luciferase activity 24 hrs post-infection. Results are presented as means ± s.d. of the ratio of firefly:renilla, expressed as relative luciferase units (RLU); each determination was performed in triplicate and the experiment was repeated 4 times. # = p<0.05 compared to SP-C\textsuperscript{wt} unRx; * = p<0.05 compared to SP-C\textsuperscript{Δexon4} unRx; ♦ = p<0.05 compared to RSV-infected SP-C\textsuperscript{wt}.

Figure 3.6 - Proteasome function is decreased in RSV-infected SP-C\textsuperscript{Δexon4} cells

The clonal cell lines expressing SP-C\textsuperscript{Δexon4} (panel a) or SP-C\textsuperscript{wt} (panel b) were transiently transfected with the proteasome sensor pZS-ProSensor-1 construct and infected with RSV at the indicated titers 24 hours post-transfection. Phase (top row) and fluorescent (bottom row) images were captured directly from the culture plate 24 hours post-infection. Following image capture, cells were harvested and subjected to FACS analysis to determine MFI (reported in the top right corner of the fluorescent panels). (c) Clonal cells expressing SP-C\textsuperscript{wt} (panels a and c) or SP-C\textsuperscript{Δexon4} (panels b and d) were transfected with a plasmid encoding EGFP as a control for transfection efficiency (panels a and b) or with the pZs-ProSensor1 vector in the presence of MG-132 (panels c and d) as a positive control. Cells were harvested and subjected to FACS analysis 24 hours post-transfection. Values for GFP+ cells and mean fluorescence intensity (MFI) are shown in the top right corner of each panel. Data is representative of 3 independent experiments. Scale bars = 50 µm.

Figure 3.7 - SP-C\textsuperscript{Δexon4} sensitizes cells to proteasome inhibition

(a) SP-C\textsuperscript{wt} and SP-C\textsuperscript{Δexon4} clonal cell lines were plated at equal cell densities and treated with the proteasome inhibitor MG-132 at the indicated concentrations. Phase
photographs of the cells were captured directly from the culture plate 18 hours post-treatment. Scale bars = 50µm. (b) MTS cytotoxicity assay of cells treated as indicated in panel (a). Results are presented as means ± s.d.; each determination was performed in triplicate and the experiment was repeated 3 times. * = p<0.05 compared to SP-C∆exon4 unRx; # = p<0.05 compared to SP-Cwt. unRx. (c) Immunoblots of whole cell lysates from clonal cell lines expressing SP-Cwt or SP-C∆exon4 following MG-132 treatment. Immunoblots were probed with antibodies against the N-terminus of proSP-C antibody or actin (loading control). (d) Clonal cell lines expressing SP-Cwt or SP-C∆exon4 were cultured in the presence (+) or absence (-) of 500nM MG-132 for 18hrs. Total RNA was isolated from cells and subjected to RT-PCR analysis with primers spanning the intron of unspliced XBP-1 mRNA. XBP-1(u) denotes unspliced, inactive XBP-1; XBP-1(s) denotes spliced, active XBP-1; products were separated on a 4% agarose gel.

Figure 3.8 - Accumulation of SP-C∆exon4 is associated with cell death
(a) SP-Cwt (left panel) and SP-C∆exon4 (right panel) clonal cell lines were plated at equal cell density on poly-lysine coated coverslips and treated with 500nM MG-132. Eighteen hours post-treatment the cells that remained attached to the coverslips were fixed, permeabilized and stained with H33342 dye to visualize the nuclear architecture. Higher magnification of one representative cell is shown in the insets. Scale bars = 50µm for large panels, 10µm for insets. (b) Cells expressing SP-C∆exon4 were treated with MG-132 at the indicated concentrations, harvested 18 hours post-treatment and subjected to Western blotting with a caspase3 antibody that detects both the precursor and cleaved isoforms. Cell lysates of untreated HEK293 cells and HEK293 cells treated with the pro-apoptotic agent staurosporine (1µM) were used as negative and positive controls, respectively. (c) Clonal cell lines expressing SP-Cwt or SP-C∆exon4 were cultured in the presence or absence of MG-132 at the indicated concentrations, harvested 18 hours post-treatment and subjected to Western blotting with a caspase3 antibody that detects both the precursor and cleaved isoforms. (d) Immunohistochemistry for activated caspase3 on lung sections from wild-type (WT) or transgenic mice (TG#2) in which SP-C∆exon4 was
expressed in the distal lung epithelium. Filled arrowheads represent caspase3-positive cells and empty arrowheads represent red blood cells. Scale bars = 20µm.

**Figure 3.9 - Dilation of pre-Golgi intermediate (pGI) compartment and detection of inclusion bodies in MG-132 treated SP-C\(^\Delta\)exon\(^4\) cells.**

Stably transfected cell lines expressing SP-C\(^{wt}\) or SP-C\(^{\Delta\)exon\(^4\) were cultured in presence or absence of 500nM MG-132. Cells were harvested 18 hours following MG-132 treatment and ultrathin sections were prepared and examined by transmission electron microscopy. (a) SP-C\(^{wt}\) without MG-132. (b) SP-C\(^{\Delta\)exon\(^4\) without MG-132. (c) SP-C\(^{\Delta\)exon\(^4\) treated with MG-132. (d) SP-C\(^{\Delta\)exon\(^4\) treated with MG-132. (e) Accumulation of mutant SP-C\(^{\Delta\)exon\(^4\) in membrane bound inclusions after treatment with 500nM MG-132. SP-C\(^{\Delta\)exon\(^4\) was detected by cryoimmunogold labeling using rabbit antisera directed against the N-terminus of proSP-C and 10nM protein A gold (PAG10). SP-C\(^{\Delta\)exon\(^4\) was localized to ER, small vesicles, Golgi complex, lysosomes, and membrane bound inclusions (arrowheads). ER = endoplasmic reticulum, N = nucleus, G = Golgi, M = mitochondria, pGI = pre-Golgi intermediate. Scale bars = 250nm.
Table 3.1 - Transcriptional profiling reveals differential expression of genes associated with apoptosis in SP-C\(^{\Delta\text{exon4}}\) cells.

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The clonal cell lines expressing SP-C\textsuperscript{wt}, SP-C\textsuperscript{Δexon4} or empty vector were subjected to transcriptional profiling using the human U133 gene chip set from Affymetrix. Genes that were specifically increased or decreased in SP-C\textsuperscript{Δexon4} over both SP-C\textsuperscript{wt} and an empty vector control (i.e. mutant-specific changes) are reported. \textsuperscript{a}Anti and pro designations signify anti and pro-apoptosis; evidence of apoptosis is from Gene Ontology database. The far right column lists the number of putative NF-κB binding sites located in the 5’ flanking sequence (1kb) of the gene on the left. Data represent the average of 3 analyses for each genotype.
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<td>Cysteine-rich, angiogenic inducer, 61</td>
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<td>V-akt murine thymoma viral oncogene homolog 2</td>
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<td>Scavenger receptor class B, member 1</td>
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<td>PBEF</td>
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<td>BAX</td>
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<td>Pleiomorphic adenoma gene-like 1</td>
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<td>NM_006504</td>
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<td>EIF4EBP1</td>
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<td>ZNF42</td>
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<td>NM_006055</td>
<td>Zinc finger protein 42 (myeloid-specific retinoic acid-responsive)</td>
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<td>INCENP</td>
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<td>TNFRSF6F</td>
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<td>NM_000043</td>
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<td>CCND3</td>
<td>1.54</td>
<td>NM_001760</td>
<td>Cyclin D3</td>
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<tr>
<td>FGFR3</td>
<td>1.53</td>
<td>NM_001142</td>
<td>Fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)</td>
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<tr>
<td>MAPK14</td>
<td>1.51</td>
<td>NM_001315</td>
<td>Mitogen-activated protein kinase 14</td>
<td>2</td>
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<td>SRF</td>
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<td>NM_003131</td>
<td>Serum response factor (c-fos serum response element-binding transcription factor)</td>
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<td>V-myb myeloblastosis viral oncogene homolog (avian)</td>
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<td>Fibronectin 1</td>
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<td>NM_002632</td>
<td>Placental growth factor, vascular endothelial growth factor-related protein</td>
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<td>FXD5</td>
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<td>NM_014164</td>
<td>FXD domain containing ion transport regulator 5</td>
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<td>Neural precursor cell expressed, developmentally down-regulated 9</td>
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<td>CXCR4</td>
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<td>NM_001346</td>
<td>Chemokine (C-X-C motif) receptor 4</td>
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<td>CD9</td>
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<td>NM_001769</td>
<td>CD9 antigen (p24)</td>
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<tr>
<td>TGFB1</td>
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<td>NM_000660</td>
<td>Transforming growth factor, beta 1 (Cammurai-Engelmann disease)</td>
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<td>SFRP1</td>
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<td>NM_001946</td>
<td>Dual specificity phosphatase 6</td>
<td>1</td>
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<td>CDH1</td>
<td>-4.58</td>
<td>NM_004360</td>
<td>Cadherin 1, type 1, E-cadherin (epithelial)</td>
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</tr>
</tbody>
</table>

Table 3.2 - Descriptive information of apoptosis-associated genes from PathwayAssist model (Fig 3.2)
Table 3.3 - Antibody labeling specificity for untreated HEK-293 SP-C<sup>exon-4</sup> cells

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Observed number of hits from counting grid, ( P(i) )</th>
<th>Observed gold counts, ( N_o )</th>
<th>Predicted gold counts, ( N_e )</th>
<th>RLI</th>
<th>Partial Chi-squared values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus (Nuc)</td>
<td>4776</td>
<td>2230</td>
<td>2258</td>
<td>0.99</td>
<td>0.34</td>
</tr>
<tr>
<td>Cytosol (Cyto)</td>
<td>4529</td>
<td>900</td>
<td>2141</td>
<td>0.42</td>
<td>719.31</td>
</tr>
<tr>
<td>Mitochondria (M)</td>
<td>362</td>
<td>515</td>
<td>171</td>
<td>3.01</td>
<td>691.00</td>
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<tr>
<td>Endoplasmic reticulum (ER)</td>
<td>168</td>
<td>293</td>
<td>79</td>
<td>3.69</td>
<td>574.40</td>
</tr>
<tr>
<td>Golgi Complex (G)</td>
<td>104</td>
<td>124</td>
<td>49</td>
<td>2.52</td>
<td>113.92</td>
</tr>
<tr>
<td>Vesicles (V)</td>
<td>277</td>
<td>468</td>
<td>131</td>
<td>3.57</td>
<td>867.59</td>
</tr>
<tr>
<td>Lysosomes (L)</td>
<td>145</td>
<td>224</td>
<td>69</td>
<td>3.27</td>
<td>352.56</td>
</tr>
<tr>
<td>Inclusions (I)</td>
<td>15</td>
<td>151</td>
<td>7</td>
<td>21.29</td>
<td>2920.63</td>
</tr>
<tr>
<td>Total</td>
<td>100376</td>
<td>4905</td>
<td>4905</td>
<td></td>
<td>6239.74*</td>
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</table>

* p<0.001 for degrees of freedom= 7.
Table 3.4 - Antibody labeling specificity for MG-132-treated HEK-293 SP-C<sup>Δexon-4</sup> cells

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Observed point hits from counting grid, P(i)</th>
<th>Observed gold counts, N&lt;sub&gt;o&lt;/sub&gt;</th>
<th>Predicted gold counts, N&lt;sub&gt;e&lt;/sub&gt;</th>
<th>RLI</th>
<th>Partial Chi-squared values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus (Nuc)</td>
<td>2404</td>
<td>2455</td>
<td>3190</td>
<td>0.77</td>
<td>169.34</td>
</tr>
<tr>
<td>Cytosol (Cyto)</td>
<td>2403</td>
<td>1762</td>
<td>3188</td>
<td>0.55</td>
<td>637.58</td>
</tr>
<tr>
<td>Mitochondria (M)</td>
<td>308</td>
<td>481</td>
<td>409</td>
<td>1.18</td>
<td>12.82</td>
</tr>
<tr>
<td>Endoplasmic reticulum (ER)</td>
<td>180</td>
<td>640</td>
<td>239</td>
<td>2.68</td>
<td>640.01</td>
</tr>
<tr>
<td>Golgi Complex (G)</td>
<td>99</td>
<td>293</td>
<td>131</td>
<td>2.23</td>
<td>198.97</td>
</tr>
<tr>
<td>Vesicles (V)</td>
<td>138</td>
<td>807</td>
<td>183</td>
<td>4.41</td>
<td>2126.18</td>
</tr>
<tr>
<td>Lysosomes (L)</td>
<td>279</td>
<td>650</td>
<td>370</td>
<td>1.76</td>
<td>211.58</td>
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<tr>
<td>Inclusions (I)</td>
<td>390</td>
<td>1139</td>
<td>517</td>
<td>2.20</td>
<td>746.74</td>
</tr>
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<td>Total</td>
<td>6201</td>
<td>8227</td>
<td>8227</td>
<td></td>
<td>4777.74*</td>
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</tbody>
</table>

*: p< 0.001 for degrees of freedom= 7.
Table 3.5 - Mutant SP-C<sup>Exon4</sup> protein distribution by immunogold quantitation using normalized gold counts‡

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Untreated cells observed (predicted)</th>
<th>MG132 treated observed (predicted)</th>
<th>Row Totals</th>
<th>Chi-squared values (untreated, treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoplasmic reticulum (ER)</td>
<td>29(24.32)</td>
<td>64(68.68)</td>
<td>93</td>
<td>0.90, 0.32</td>
</tr>
<tr>
<td>Golgi Complex (G)</td>
<td>12(10.72)</td>
<td>29(30.28)</td>
<td>41</td>
<td>0.15, 0.05</td>
</tr>
<tr>
<td>Vesicles (V)</td>
<td>47(33.47)</td>
<td>81(94.53)</td>
<td>128</td>
<td>5.47*, 1.94</td>
</tr>
<tr>
<td>Lysosomes (L)</td>
<td>22(22.75)</td>
<td>65(64.25)</td>
<td>87</td>
<td>0.02, 0.01</td>
</tr>
<tr>
<td>Membrane bound Inclusions (I)</td>
<td>15(33.73)</td>
<td>114(95.27)</td>
<td>129</td>
<td>10.40**, 3.68*</td>
</tr>
<tr>
<td>Total</td>
<td>125(125)</td>
<td>353(353)</td>
<td>478</td>
<td>22.95†</td>
</tr>
</tbody>
</table>

‡: Raw score of gold counts (Table 1 and 2) were normalized by a factor of 10 and rounded to the first decimal point for contingency table analysis.

*: Compartment had significantly higher than expected number of gold particles.

**: Compartment had significantly lower than expected number of gold particles.

†: p< 0.001 for degrees of freedom =4.
Figure 3.1 – Generation of clonal, stably transfected cell lines
Figure 3.2 – Functional associations of differentially expressed genes in SP-C\textsuperscript{∆exon4} cells associated with apoptosis
Figure 3.3 – Basal NF-κB activity is increased in cells expressing SP-C\textsuperscript{Δexon4}
Figure 3.4 – Inhibition of NF-κB nuclear translocation induces death of SP-C\textsuperscript{Δexon4}
cells
Figure 3.5 - SP-C<sup>Δexon4</sup> increases susceptibility to respiratory syncytial virus-induced cell death
Figure 3.6 – Proteasome function is decreased in RSV-infected SP-C<sup>Δexon4</sup> cells
Figure 3.7 - SP-C^{Δexon4} sensitizes cells to proteasome inhibition
Figure 3.8 – Accumulation of SP-C^{Δexon4} is associated with cell death
Figure 3.9 – Dilation of pre-Golgi intermediate (pGI) compartment and detection of inclusion bodies in MG-132-treated SP-C$^{\Delta\text{exon4}}$ cells
CHAPTER IV

Discussion/Future Directions
Summary

Mutations in SFTPC are associated with both sporadic and familial ILD in children and adults; however, it is unclear if this relationship is causal in nature. The overall goal of this dissertation study was to determine whether a disease-linked mutation in the C-terminal peptide of SP-C causes lung disease. Expression of the index mutation, SP-CΔexon4, in type II epithelial cells of transgenic mice resulted in a dose-dependent perturbation of lung development associated with epithelial cell cytotoxicity (Chapter 2). Transient expression of SP-CΔexon4 in isolated type II cells or HEK293 cells resulted in: 1) incomplete processing of the mutant proprotein, 2) a dose-dependent induction of the unfolded protein response, 3) trapping of the mutant proprotein in the ER and 4) rapid degradation via ERAD (Chapters 2 and 3). Further, constitutive expression of SP-CΔexon4 resulted in an adaptive, cytoprotective response involving NF-κB and increased susceptibility to RSV-induced cell death associated with accumulation of the mutant proprotein (Chapter 3).

Based on the collective findings of these studies the following model is proposed (Figure 4.1). SFTPC encodes a protein that is subjected to quality control in the ER. While SP-Cwt protein is correctly folded and exported from the ER for secretion, mutations in the C-terminal peptide of SP-C, including SP-CΔexon4, result in terminal misfolding and degradation of the mutant proprotein by a proteasome-dependent pathway. In transiently transfected cells SP-CΔexon4 has been shown to redirect SP-Cwt for degradation, thereby acting as a dominant negative (Wang et al. 2003). Therefore, it is possible that the loss of SP-C in the airway due to a dominant negative effect of a SP-C mutant contributes to disease in patients with mutations in SFTPC. Consistent with this hypothesis, SP-CΔ mice on the 129J background developed a severe progressive pulmonary disorder with histologic features consistent with interstitial pneumonitis (Glasser et al. 2003) as did three members of a family that lacked detectable SP-C in their airways in the absence of a SFTPC mutation (Amin et al. 2001). In addition, cell culture studies suggested that NF-κB promoted an adaptive response to chronic ER stress imposed by the expression of SP-CΔexon4, allowing for cell survival under basal conditions. However, when a secondary insult is imposed on SP-CΔexon4-expressing cells, such as a viral infection, the degradative capacity of the proteasome is overwhelmed, resulting in
accumulation of the misfolded proprotein and apoptosis of type II epithelial cells. It is thought that apoptosis of type II epithelial cells is a pivotal event in the generation of ILD, resulting in a hyperactive wound healing response consisting of inflammation, myofibroblast proliferation and excessive deposition of extracellular matrix components, including collagen, in the lung interstitium (Selman et al. 2001; Noble et al. 2005). This unremitting cycle of epithelial injury and wound healing, initiated by SP-C-induced cytotoxicity, may contribute to the pathogenesis of ILD associated with mutations in *SFTPC*. 

Figure 4.1 – Model outlining proposed mechanism underlying misfolded SP-C and pathogenesis of interstitial lung disease
**Mechanism underlying NF-κB activation in response to SP-CΔexon4**

The NF-κB family consists of five genes that give rise to seven proteins: p105, p100, p50, p52, p65/RELA, c-REL and RELB. Each NF-κB family member participates in the formation of homo- or heterodimers that are thought to confer target-gene specificity. Regulation of NF-κB activity occurs primarily through post-transcriptional events (for review see (Chen et al. 2004)). Under basal conditions, NF-κB dimers are sequestered in the cytoplasm in an inactive state through binding to IκB repressor proteins, including IκBα. Phosphorylation of IκB proteins by upstream kinases induces their ubiquitination and subsequent degradation via the proteasome, allowing translocation of NF-κB into the nucleus where it binds to κB enhancers of target genes to induce transcription. Post-translational modifications of p65 and p50, including phosphorylation and acetylation, have been shown to optimize the NF-κB response by increasing binding to κB enhancers or by modifying the architecture of neighboring chromatin (Chen et al. 2004). Results of studies in stably transfected cells suggested that the SP-CΔexon4 cell line adapted to constitutive expression of misfolded SP-C and that NF-κB played a pivotal role in the adaptive response. Defining the pathway that leads to NF-κB activation in response to the expression of SP-CΔexon4 as well as the constitution of the active NF-κB complex in SP-CΔexon4 cells are the first important steps toward understanding the basis of NF-κB activity in the cytoprotective response against SP-CΔexon4.

ER stress, induced by the xenotoxic agents such as tunicamycin and thapsigargin, resulted in translational repression via the PERK/eIF2α pathway and caused NF-κB activation (Jiang et al. 2003). A separate report indicated that phosphorylation of eIF2α, independent of ER stress, was sufficient to activate NF-κB (Deng et al. 2004). While both studies demonstrate the requirement of eIF2α phosphorylation for this event, these reports were at odds with respect to the mechanism underlying NF-κB activation; Jiang et al concluded that the mechanism involved dissociation of IκBα from NF-κB but not degradation, while Deng et al demonstrated that eIF2α phosphorylation-dependent repression of IκBα synthesis was responsible for the activation of NF-κB. To determine
if decreased levels of IkBα account for the constitutive levels of active NF-κB in SP-CΔexon4 cells, IkBα levels will be compared in cells stably expressing SP-CΔexon4 or SP-Cwt by immunoblot analysis (IkBα antibody from Santa Cruz Biotechnologies, Santa Cruz, CA). To determine the role of PERK activation in regulation of IkBα, levels of phosphorylated PERK and eIF2α will also be assessed by immunoblot analysis (phospho-PERK antibody available from Cell Signaling, Beverly, MA; phospho-eIF2α antibody available from Research Genetics, Huntsville, AL). While IkBα levels may be decreased in SP-CΔexon4 cells, it is not anticipated that PERK and eIF2 will be phosphorylated since activation of this pathway leads to translational repression and the cell lines expressing SP-CΔexon4 or SP-Cwt show similar doubling rates, inconsistent with a unremitting decrease in global protein synthesis (data not shown). An alternative mechanism for decreased levels of IkBα could be due to heightened activity of the kinases involved in the phosphorylation and subsequent degradation of IkB, namely IkB kinases (IKK) 1 and 2. The activity of these kinases will be assessed by in vitro kinase assays using cell lysates from SP-CΔexon4 or SP-Cwt cells.

Role of post-translational modifications of NF-κB in cytoprotective response

Preliminary EMSA results indicated that NF-κB binding to a κB enhancer was similar between SP-CΔexon4 and SP-Cwt cells (Figure 4.2), despite the ~8.6-fold increase in NF-κB activity in SP-CΔexon4 cells (Figure 3.2), suggesting that post-translational modifications may be responsible for constitutive NF-κB activation in SP-CΔexon4 cells. Prior to testing this hypothesis, the constitution of the NF-κB subunits in SP-CΔexon4 cells must first be determined. To determine the constitution of NF-κB subunits in SP-CΔexon4 cells, supershift EMSAs with antibodies against NF-κB family members will be performed in both SP-CΔexon4 and SP-Cwt cells. Initial experiments will focus on the p65 and p50 subunits as both have been implicated in the anti-apoptotic function of NF-κB and p65 null mice show increased apoptosis of hepatic cells and consequential developmental abnormalities (Beg et al. 1995; Beg et al. 1996).

Assuming that the active NF-κB complex in SP-CΔexon4 cells consists of p50 and/or p65 subunits, additional experiments will be performed to determine if these subunits
Figure 4.2 – Similar NF-κB binding in SP-CΔexon4 and SP-Cwt cells. Nuclear extracts were prepared from SP-CΔexon4 and SP-Cwt cells. 10µg of nuclear protein from each sample was incubated with one of two 32P-labeled oligonucleotides consisting of a κB enhancer (sequence for probe#1 – AGT TGA GGG GAC TTT CCC AGG C; sequence for probe#2 – GCC ATT GGG GAT TTC CTC) for 30min. The protein/oligonucleotide mix was separated by SDS-PAGE followed by gel drying and autoradiography for 24 hours at room temperature. Untransfected HEK293 cells were treated with 20ng/ml TNFα for 1hour as a positive control; however, these cells require at least a 4 hour exposure to TNFα to induce NF-κB binding (data not shown).

contain post-translational modifications, specifically phosphorylation of p65 and acetylation of p65 and p50, as these modifications have been shown to modulate and
optimize the NF-κB response (Chen et al. 2004). The phosphorylation status of p65 will be determined by Western blot analysis of nuclear extracts from SP-CΔexon4 and SP-Cwt cells using a phospho-specific antibody for p65 (Rockland, Gibertsville, PA). The acetylation status of endogenous p65 and p50 will be assessed as described previously (Chen et al. 2001b). Briefly, the SP-CΔexon4 and SP-Cwt cell lines will be radiolabeled for 1 hour with Na-[3H]-acetate (1 mCi/ml) in the presence of cycloheximide (25 µg/ml). conjugated agarose beads (Santa Cruz Biotechnologies, Santa Cruz, CA), separated by SDS-PAGE followed by audioradiography for 4-6 weeks to evaluate the potential acetylation of p50 and/or p65.

The histone deacetyltransferase (HDAC)-3 has been shown to de-acetylate the p65 subunit, causing retrotranslocation of p65 out of the nucleus into the cytosol (Chen et al. 2001b). To determine if HDAC-3 can de-acetylate p65 in SP-CΔexon4 cells, an expression plasmid encoding HDAC3 will be transiently transfected in the stable cell lines and acetylation assays will be performed. In addition, confocal analysis will be performed using a p65 antibody to determine the effects of HDAC3 expression on the cellular localization of p65. Blocking nuclear export of NF-κB in stably expressing SP-CΔexon4 cells resulted in cell death (Figure 3.3). To determine if the cessation of NF-κB activity by dephosphorylation and/or deacetylation of p65 in SP-CΔexon4 cells results in a similar outcome, cell death assays will be performed using PI staining and MTS assays following dephosphorylation of p50/p65 with pharmacologic inhibitors against known p50/p65 kinases (e.g. PKCζ and PKAc) and/or de-acetylation with transient expression of HDAC3. Collectively, these studies are designed to identify the subunits of constitutively active NF-κB in SP-CΔexon4 cells and to determine if post-translation modifications of these subunits are critical in modulating the NF-κB-mediated cytoprotective response in the SP-CΔexon4 cell line.

**Mechanism underlying NF-κB function in cytoprotective response**

Microarray analysis revealed that several anti-apoptotic genes were up-regulated in cells stably expressing SP-CΔexon4 (Table 3.1). To test the hypothesis that NF-κB promotes survival of SP-CΔexon4 cells by directly up-regulating anti-apoptosis genes, chromatin immunoprecipitation (ChIP) analysis will be performed on SP-CΔexon4 cells.
This analysis will confirm which anti-apoptotic genes are truly NF-κB-dependent in a cellular context. Briefly, SP-C\(^{∆}\)exon4 cells will be fixed, sonicated and immunoprecipitated with anti-NF-κB antibodies; use of specific antibodies will be determined from the supershift experiments described above. Following immunoprecipitation, DNA fragments will be analyzed by PCR analysis with primers that span the κB enhancer located in the proximal promoters of the anti-apoptotic genes. These experiments will identify target genes that contain a functional κB enhancer and will demonstrate active binding of NF-κB to the enhancer in SP-C\(^{∆}\)exon4 cells.

Following identification of bona fide NF-κB target genes, each one will be tested individually for its ability to promote survival of SP-C\(^{∆}\)exon4 cells independent of NF-κB blockade. Anti-apoptotic genes that were upregulated in the array will be inhibited by RNAi and the effects on cell survival will be assessed. NF-κB consisting solely of p50 homodimers has been shown to repress transcription of genes containing κB enhancers due to the lack of a transactivation domain in p50 (Ghosh et al. 1998). The down regulation of pro-apoptotic genes in SP-C\(^{∆}\)exon4 cells suggests that p50 homodimers may constitute active NF-κB in this cell line which may repress the expression of these genes (Table 3.1). In the event that p50 homodimers are detected in the supershift assays and are found to occupy κB enhancers of target genes in the ChIP assays, NF-κB translocation will be inhibited by SN50 peptide, as outlined in Chapter 3, and levels of these genes will be determined by real-time PCR. This experiment will determine if NF-κB blockade relieves the repression of pro-apoptotic genes. The aforementioned studies are designed to test the hypothesis that NF-κB promotes survival of SP-C\(^{∆}\)exon4 cells by directly regulating apoptosis-associated genes and to determine which genes are directly involved in the cytoprotective response.

In addition to serving as the major protein-folding compartment, the ER also serves as the primary site for intracellular calcium storage. Calcium is an intracellular second messenger that is rapidly mobilized in response to various stimuli to mediate a wide variety of cellular responses including cell proliferation, motility, secretion and neurotransmission. In addition, calcium also serves as a trigger for apoptosis in physiological and pathophysiological conditions. Disruption of ER homeostasis, induced by misfolded proteins, heat shock or inhibition of calcium re-uptake into the ER, causes
an efflux of calcium from the ER to the cytosol. The resulting increases in intracellular calcium levels trigger apoptosis in different cell types including fibroblasts, neurons and tumor cells (Mattson et al. 2000; Kim et al. 2002; Rudner et al. 2002). Recent reports indicated that activation of inositol 1,4,5-triphosphate (IP$_3$) receptors resulted in calcium release from the ER and NF-κB activation and that suppression of calcium release from IP$_3$-sensitive stores, through direct downregulation of IP$_3$R, mediated the anti-apoptotic function of NF-κB (Glazner et al. 2001; Camandola et al. 2005). In light of this data, it is possible that accumulation of SP-C$^{\Delta$exon4} in HEK293 cells perturbed ER homeostasis leading to calcium release from the ER and the subsequent activation of NF-κB. Activation of NF-κB may have then promoted the restoration of calcium homeostasis in the ER and the suppression of apoptosis by downregulating IP$_3$ receptors. To determine if intracellular calcium pools are altered in SP-C$^{\Delta$exon4} cells, basal cytosolic and ER calcium levels will be measured in SP-C$^{\Delta$exon4} and SP-C$^{wt}$ cells using ratiometric imaging of Fura-2 fluorescence in live cells as described previously (Leissring et al. 2000). In addition, IP$_3$R levels will be assessed by Western blotting and RT-PCR analysis to determine if decreases in these proteins directly correlate with differences in calcium storage pools. These experiments will also be performed in transiently transfected cells to determine if acute SP-C$^{\Delta$exon4} expression alters intracellular calcium pools. Collectively, these experiments may provide an alternative mechanism of the cytoprotective response of NF-κB in response to SP-C$^{\Delta$exon4} expression.

Animal model of misfolded SP-C

Expression of the SP-C$^{\Delta$exon4} proprotein in type II cells of transgenic mice led to disrupted lung morphogenesis associated with apoptosis and cytotoxicity (Figures 2.2-2.4, and 3.7). Human patients with the corresponding mutation in SFTPC did not present with gross developmental abnormalities of the lung at birth; instead, they developed disease in the perinatal period or in adulthood. Probable explanations for the differences in phenotype likely stem from the fact that a 13kb fragment of the endogenous mouse SP-C promoter was used to drive expression of the SP-C$^{\Delta$exon4} proprotein in transgenic animals. This promoter fragment has been used previously to generate transgenic mice with type II epithelial cell-specific expression of the transgene (Conkright et al. 2002;
Glasser et al. 2005), the precise reason it was chosen for this study. However, the “strength” of the promoter appeared to be quite high leading to a large production of transgenic protein, as evidenced in a study in which it was used to drive expression of mature SP-C^{24-58} (Conkright et al. 2002) and this study (Figure 2.4 and 2.5). The ability of the 13kb fragment to drive expression higher than that of the endogenous promoter is likely due to the absence of cis-regulatory units in the 13kb fragment. Another possibility for the disrupted lung phenotype may be due to the inexact timing of expression between the 13kb promoter fragment and the endogenous promoter; however, exact comparisons addressing this issue are difficult to assess. While other SP-C promoter fragments exist, such as the 4.8kb mouse fragment and the 3.7kb human fragment, and have been used successfully to generate transgenic mice, expression of a transgene from these fragments is often not type II epithelial cell-specific and is observed in other lung epithelial cells such as Clara cells (Lin et al. 1999; Glasser et al. 2000).

In order to avoid the undesirable side effects of transgenesis and to generate a mouse model that genetically recapitulates the human situation, mice were generated in which the point mutation corresponding to c.460+1 G>A (also known as g.1728 G>A) in SFTPC was homologously recombined (i.e. knocked-in) into the endogenous mouse SP-C locus (sftpc). The sequence of sftpc was obtained from UCSC genome informatics database (http://genome.ucsc.edu/) and sequence alignment of SFTPC and sftpc demonstrated conservation of the first nucleotide in intron 4, corresponding to c.460+1 G>A (SP-C^{Δexon4}) (data not shown). To generate the knock-in construct, 6.7kb of sftpc was cloned out of ES cell genomic DNA by PCR-based methods using primers designed by the MIT primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sftpc locus was amplified in two fragments: a 5.3kb 5’ homology arm and a 1.4kb 3’ homology arm; following amplification, the homology arms were cloned into the pNEB193 vector (New England Biolabs, Ipswich, MA). An adenosine was substituted for a guanosine at a position in the 3’ homology arm that corresponded to the first nucleotide of the forth intron using the QuikChange site-directed mutagenesis kit (Invitrogen, Carlsbad, CA). Both homology arms were then sequenced bidirectionally to verify integrity and to confirm the presence of the desired mutation in the 3’ arm; the homology arms were subsequently subcloned into the OSdupdel2 vector (gift from Oliver...
Smithies, University of North Carolina at Chapel Hill, NC). OSdupdel2 was chosen as
the backbone of the targeting vector due to the presence of a flanked loxp (floxed)
neomycin-resistance cassette (NEO), allowing for excision of NEO following positive
selection of the recombined ES cells (Figure 4.3). The 5’ and 3’ arms were cloned into
OSdupdel2 such that the NEO cassette was placed in the third intron of sftpc, a region
that was found to be relatively non-conserved between SFTPC and sftpc by sequence
alignment analysis (data not shown). This strategy also placed NEO ~240bp upstream
from the desired point mutation, thereby decreasing the probability of homologous
recombination between the desired point mutation and the selection cassette.

The resulting targeting vector was electroporated into ES cells derived from the
129J/SvEvTac mouse strain, the same strain from which the homology arms were cloned,
and cultured in the presence of G418 and ganciclovir for positive and negative selection,
respectively. A total of 278 individual, G418-resistant clones were isolated, amplified
and screened for homologous recombination of the targeting vector into the genome.
Screening of ES clones was initially performed by PCR analysis with two separate primer
sets to detect the 5’ and 3’ homology arms (Figure 4.4). Positive clones were sequenced
across the desired mutation to ensure that recombination did not occur between the NEO
cassette and the mutation. Site-specific recombination of the targeted allele in PCR-
positive clones was confirmed by Southern blot analysis (data not shown). A total of
two, independent clones were identified from these analyses, designated D49 and D128.
The targeted ES cells were independently injected into blastocysts, harvested from Black
Swiss mice, and implanted into Black Swiss mice to generate chimeric offspring that
harbor the targeted sftpc allele (Figure 4.5). A total of 18 chimeric mice were obtained
from this injection, 4 from the D49 clone and 14 from the D128 clone, 15 of which were
>85% chimeric as determined by coat color. The chimeric mice, which contained genetic
contributions from both the 129J, contributed from injected ES cells, and Black Swiss
backgrounds, were backcrossed to Black Swiss mice to obtain pure agouti offspring,
designated as such due to the characteristic coat color of the 129J mice. Approximately
50% of the agouti mice should contain the targeted allele; mice harboring
**Figure 4.3 – Diagram of c.460+1 G>A knock-in targeting allele (SP-C<sup>Δexon4-KI</sup>).**

*sftpc* was cloned out of ES cell DNA in two fragments: a 5.3kb 5’ homology arm and a 1.4kb 3’ homology arm. Adenonine was substituted for guanosine at the first nucleotide of intron 4 (corresponding to position c.460+1 of the cDNA and position g.1728 of genomic DNA) in the 3’ arm by PCR mutagenesis. A floxed NEO cassette was inserted into intron 3 approximately 240bp from the desired mutation and a thymidine kinase cassette driven by the phosphoglycerate kinase promoter was inserted at the 5’ end of the construct. Open boxes represent exons, lines represent introns. The black box in exon 2 corresponds to the SP-C mature peptide. Numbers below the exons represent position with respect to the translational start site of SP-C proprotein.

The targeted allele will then be bred to identify SP-C<sup>Δexon4-KI</sup> founders that transmit the targeted allele to their progeny (i.e. germline transmitters). SP-C<sup>Δexon4-KI</sup> mice obtained from this mating will be heterozygous for the targeted allele (SP-C<sup>Δexon4-KI+/+</sup>) and will also contain the floxed NEO cassette. The NEO cassette will be excised *in vivo* by breeding the SP-C<sup>Δexon4-KI+/+</sup> mice to EIIa-Cre transgenic mice in order to eliminate possible undesired side effects of NEO expression in type II epithelial cells. The EIIa-Cre line carry the Cre transgene under the control of the adenovirus EIIa promoter that targets expression of the Cre recombinase enzyme to the early mouse embryo, specifically to the one-cell zygote stage of embryonic development. Cre-mediated recombination occurs in all tissues of the developing animal, including the germ cells that transmit the genetic
Figure 4.4 – Homologous recombination of SP-\(\text{C}^{\Delta\text{exon4-KI}}\) into the \(sftpc\) locus. Genomic DNA was harvested from ES clones D49 and D128 for PCR analysis. Primer sets included one primer against the NEO cassette (downstream primer for the 5’ arm and upstream primer for the 3’ arm) and one primer against regions of the endogenous \(sftpc\) allele outside of the targeting vector (upstream primer for 5’ arm and downstream primer for 3’ arm). Presence of 5.8kb band in 5’ arm and 1.5kb band in 3’ arm indicate homologous recombination of mutant allele in \(sftpc\) locus. Correct size of predicted band in both arms indicates that no additions or deletions occurred during the recombination event. \(\text{H}_2\text{O}\) indicates water control for 5’ arm; vector DNA containing distal portion of \(sftpc\) was used as positive control for 3’ arm (+ control).

alteration to progeny. This line has been used effectively for the \textit{in vivo} excision of floxed cassettes in numerous mouse tissues (Williams-Simons et al. 1999). Confirmation of NEO excision will be performed by PCR and Southern analyses. SP-\(\text{C}^{\Delta\text{exon4-KI+}}\) mice
Figure 4.5 – Diagram outlining generation of $\text{SP-C}^{\Delta\text{exon4-KI}}$ mice. ES cells were harvested from 129J males. Targeted ES cells, containing mutant $\text{sftpc}$ allele, were injected into blastocysts harvested from Black Swiss females. The injected blastocysts were implanted into pseudo-pregnant Black Swiss females for embryonic development. Chimeric mice, containing genetic contributions from Swiss Black (black coat) and 129J (agouti coat) were obtained and scored for degree of chimericism. Chimeras were then crossed with Black Swiss females to generate progeny with pure 129J background. These mice, designated $\text{SP-C}^{\Delta\text{exon4-KI}}$, are heterozygous for the mutant $\text{sftpc}$ allele.

will then be back bred to the 129J strain for eight generations to eliminate the FVB/N genetic component conferred by EIIa-Cre transgenic mice.

Experimental plan for $\text{SP-C}^{\Delta\text{exon4-KI}}$ mice

Studies with $\text{SP-C}^{\Delta\text{exon4-KI}}$ mice will be performed in the 129J strain since $\text{SP-C}^{-/-}$ mice are also on this background and may be used in future experiments to test the effects
of the mutant allele in the absence of endogenous SP-C. Initial experiments will be performed with heterozygous SP-C$^{\Delta\text{exon4-KI}+/}$ mice to mirror the genetics of human patients with this SFTPC mutation. It is anticipated that lung development in SP-C$^{\Delta\text{exon4-KI}+}$ mice will proceed normally as lung structure was not affected in transgenic mice expressing low levels of SP-C$^{\Delta\text{exon4}}$ protein (see TG#3 in Figure 2.3 and 2.4). To determine if mRNA transcripts from the mutant allele are aberrantly processed, lungs will be harvested from newborn SP-C$^{\Delta\text{exon4-KI}+/}$ mice and subjected to RT-PCR analysis with SP-C-specific primers. It is anticipated that the c.460+1 G>A point mutation will behave similarly in SP-C$^{\Delta\text{exon4-KI}+}$ mice and in human patients, leading to the production of mutant SP-C$^{\Delta\text{exon4}}$ proprotein. Western blot analysis of primary type II cells isolated from SP-C$^{\Delta\text{exon4-KI}+/}$ mice will be performed to determine the presence and turnover of SP-C$^{\Delta\text{exon4}}$ proprotein in vivo. Levels of mature SP-C in the airspaces will be assessed by Western blot analysis of bronchoalveolar lavage fluid (BALF) to determine if SP-C$^{\Delta\text{exon4}}$ proprotein has a dominant negative effect on endogenous SP-C protein. If the expression of SP-C$^{\Delta\text{exon4}}$ proprotein results in an absence of SP-C in the airway, the SP-C$^{\Delta\text{exon4-KI}+}$ mice may develop a progressive lung disorder similar to that seen in SP-C$^{-/}$ mice (Glasser et al. 2003).

Data presented in this dissertation demonstrated that accumulated SP-C$^{\Delta\text{exon4}}$ protein was associated with cellular cytotoxicity, achieved either by high expression levels in transgenic mice (Chapter 2) or by prevention of proteasome-dependent degradation in cell culture (Chapter 3). In addition, constitutive expression of SP-C$^{\Delta\text{exon4}}$ in HEK293 cells lead to 1) an adaptive, cytoprotective response regulated, in part, by NF-κB and 2) increased susceptibility to viral-induced cell death (Chapter 3). Further, patients with SFTPC mutations displayed a large variability in the age of onset of lung disease, suggesting the involvement of environmental and/or genetic factors. The finding that a high percentage of children with SFTPC mutations became symptomatic following viral infection is consistent with this concept. Therefore, based on the collective results of studies in transgenic mice, transiently transfected cells and human patients, it is anticipated that SP-C$^{\Delta\text{exon4-KI}+}$ mice will not exhibit lung disease in the absence of a secondary insult to the pulmonary epithelium. It is possible that type II epithelial cells expressing SP-C$^{\Delta\text{exon4}}$ may efficiently degrade the misfolded proprotein and/or induce an
NF-κB-dependent adaptive response. These cellular responses would thus serve to maintain homeostasis, preventing cytotoxic effects and the subsequent formation of lung disease.

To test the hypothesis that the expression of SP-C\textsuperscript{Δexon4} leads to an NF-κB-dependent adaptive response in vivo, NF-κB activity will also be assessed in primary type II cells using assays described above (see Mechanism underlying NF-κB function in cytoprotective response section). In the event NF-κB is constitutively activated in SP-C\textsuperscript{Δexon4-KI/+} mice, NF-κB activity will be blocked in vivo to determine if this is sufficient to lead to toxicity of type II cells and disease pathogenesis. NF-κB activity will be blocked by the in vivo administration of NF-κB decoy oligonucleotides, shown to be effective in blocking and preventing the neuro-protective actions of NF-κB against the excitotoxic agent kainate (Yu et al. 1999). Endpoints of these experiments include confirmation of successful NF-κB blockade, lung histology to assess pathological changes and immunohistochemistry with anti-caspase 3 antibodies to ascertain activation of apoptosis pathways.

While increased NF-κB activity may promote an adaptive response to SP-C\textsuperscript{Δexon4} in type II epithelial cells of SP-C\textsuperscript{Δexon4-KI/+} mice, it may also upregulate the production of pro-inflammatory cytokines and chemokines such as IL-2, IL-12, IL-8 and MIP-1α in this model. This, in turn, may promote a robust inflammatory response leading to the infiltration of inflammatory cells, epithelial damage and subsequent pathogenesis of lung disease. In the event that SP-C\textsuperscript{Δexon4-KI/+} mice spontaneously develop lung disease (i.e. independent of a secondary insult) and NF-κB activity is increased in type II cells, cytokine levels and total and differential cell counts of inflammatory cells in BALF will be assessed to determine if an association between increased NF-κB activity and inflammation exists. It is important to note that the transcription of NF-κB-dependent, pro-inflammatory genes were not increased in stably transfected SP-C\textsuperscript{Δexon4} cells (data not shown) despite a significant increase in NF-κB activity. However, the constitution of the subunits in the active NF-κB complex may govern this paradoxical effect. In addition, these results are from one cell type in culture and activation of NF-κB in the context of the intact animal may yield alternative results.
To test the hypothesis that SP-C$^{\Delta\text{exon4}}$-KI/+ mice are more susceptible to viral-induced cell death, RSV will be administered into the respiratory tract of SP-C$^{\Delta\text{exon4}}$-KI/+ mice by intratracheal inoculation using previously established methods (LeVine et al. 1999). Infected SP-C$^{\Delta\text{exon4}}$-KI/+ mice will be compared to wild-type littermates and efficacy of infection will be confirmed by immunohistochemical staining of the RSV F-protein from lung sections 2 days following infection. Total cell counts and the inflammatory cytokines TNF$\alpha$ and MIP-2 will be measured in the BALF 7 days after infection to determine if the administered dose was sufficient to elicit an inflammatory response. Lungs from infected animals will be harvested for histology 2, 4 and 8 weeks following infection to determine if long-term morphological changes are occurring in SP-C$^{\Delta\text{exon4}}$-KI/+ mice compared to wild-type controls. 129J mice clear RSV efficiently from the lungs with no detectable virons 7 days following infection (LeVine et al. 1999). Therefore, it is predicted that any observed phenotype will be due to an increased susceptibility of SP-C$^{\Delta\text{exon4}}$-expressing type II cells to viral-induced death as opposed to a chronic inflammatory reaction. The cell count and cytokine measurements performed 7 days following infection should provide insight into this prediction.

To determine the effects of SP-C$^{\Delta\text{exon4}}$ in the absence of endogenous SP-C, SP-C$^{\Delta\text{exon4}}$-KI/- mice will be generated by mating SP-C$^{\Delta\text{exon4}}$-KI/+ and SP-C +/- mice. These mice will be generated in the 129J background to eliminate the possible side effects of a mixed genetic background. It is anticipated that SP-C$^{\Delta\text{exon4}}$-KI/- mice will also show an abnormal lung phenotype although the effects are likely to be less severe than in SP-C$^{\Delta\text{exon4}}$-KI/+ mice. These experiments will determine if SP-C$^{\Delta\text{exon4}}$ induces cytotoxic effects in the absence of endogenous SP-C.

**Identification of molecular pathways induced by the expression of misfolded SP-C**

In contrast to ER quality control for glycosylated proteins, virtually nothing is known about the mechanisms underlying ER quality control for non-glycosylated, transmembrane proteins such as SP-C. Expression of SP-C$^{\Delta\text{exon4}}$ in transiently transfected HEK293 cells resulted in induction of ER stress and UPR pathways, including the XBP-1/IRE1 pathway; this pathway is known to induce genes involved in protein refolding and degradation. Based on this data, it was postulated that components of the molecular
machinery responsible for the degradation of misfolded SP-C will be transcriptionally induced in response to its expression. To identify components of the quality control machinery that are induced in response to misfolded SP-C, microarray analysis was performed on transiently transfected HEK293 expressing one of two disease-linked, SFTPC mutants, SP-C\textsuperscript{Δexon4} and SP-C\textsuperscript{L188Q}, and compared to cells expressing SP-C\textsuperscript{wt}. These particular SP-C mutants were chosen due to the fact that both have a familial association with lung disease, are located in the C-terminal peptide of the proprotein, a region to which all but one disease-linked SFTPC mutations map, and have been shown to misfold when expressed in cultured cells (Nogee et al. 2001; Nogee et al. 2002; Thomas et al. 2002; Bridges et al. 2003; Chibbar et al. 2004).

Human cDNA encoding SP-C\textsuperscript{Δexon4}, SP-C\textsuperscript{L188Q} or SP-C\textsuperscript{wt} was cloned into the pIRES2-EGFP vector (Clontech, Palo Alto, CA). This bicistronic vector contains an internal ribosomal entry site (IRES), allowing translation of a gene of interest (e.g. an SP-C variant) and EGFP from a single mRNA transcript. Fluorescence-activated cell sorting following transient transfection allows for the isolation of EGFP+ cells from untransfected cells, thereby enriching the cell population of interest and decreasing the background of the microarray analysis. Cells were transfected with either SP-C\textsuperscript{Δexon4}, SP-C\textsuperscript{L188Q} or SP-C\textsuperscript{wt} for 24 hours, harvested from culture dishes and sorted to obtain EGFP+ cells. Transfections and sorting of each SP-C variant was performed in triplicate (Figure 4.6). Total RNA was isolated from the enriched cells using an acidified guanidinium method (Chomczynski et al. 1995). Total RNA was reverse transcribed into cDNA using a T7 promoter-dT primer, amplified and biotinylated through an \textit{in vitro} transcription reaction using T7 RNA polymerase prior to hybridization to the to the GeneChip\textsuperscript{®} Human Genome U133 Plus 2.0 Set (contains 54,681 probe sets representing approximately 47,000 transcripts and variants, including 38,500 well-characterized genes) (Affymetrix, Santa Clara, CA) according to the manufacturer’s protocol. Normalization and data analysis were performed exactly as described in the Materials and Methods section of Chapter 3.
Figure 4.6 – Experimental design to identify quality control receptor for misfolded SP-C. cDNA encoding SP-CΔexon4, SP-C^L188Q or SP-C^wt (SP-C^X) were cloned into the pIRES2-EGFP vector (top). Brief description of experimental design is listed below diagram of construct (see text for details).

K-means cluster analysis of transcripts from transiently transfected cells demonstrated that the SP-CΔexon4 and SP-C^L188Q mutants invoked similar transcriptional responses compared to SP-C^wt (Figure 4.7). In fact, the largest gene cluster obtained from this analysis was one that contained differentially expressed transcripts from both SP-CΔexon4 and SP-C^L188Q over SP-C^wt; a total of 5,554 probe sets were increased ≥ 2-fold in this cluster, herein referred to as cluster1, representing approximately 10% of the total number of probe sets on the chip. Functional categorization of this cluster1, as defined by biological process from the Gene Ontology database, demonstrated that components throughout the secretory pathway were increased in response to misfolded SP-C including those involved in ER-to-Golgi transport, post-Golgi transport and intracellular
**Figure 4.7** - SP-C^{Δexon4} and SP-C^{L188Q} invoke similar transcriptional responses in HEK293 cells. K-means cluster analysis of mRNA transcripts from cells transiently transfected with SP-C^{Δexon4} (M1) and SP-C^{L188Q} (M2) compared to SP-C^{wt} (WT). Each horizontal line indicates the relative expression level of an individual gene with blue representing low expression, yellow representing moderate expression and red representing high expression. Each column represents all transcripts from cluster 1 within an individual sample. Results demonstrate similar transcriptional profiles in SP-C^{Δexon4} and SP-C^{L188Q}-expressing cells compared to cells expressing SP-C^{wt}.

Furthermore, genes involved in chaperone/protein folding, the ubiquitin/proteasome pathway and apoptosis were differentially expressed in this gene cluster, indicative of ER stress and ERAD induction (**Figure 4.8**). Further analysis of these categorical classifications revealed that several bona fide components of ER stress and ERAD pathways were increased in both SP-C^{Δexon4} and SP-C^{L188Q} cells including XBP-1, HERPUD1, HRD1, GRP78 and GRP94 (**Figure 4.9**). This finding is
confirmatory of data that demonstrated a dose-dependent increase in GRP78 transcription in HEK293 cells transiently transfected with SP-C$^{\Delta \text{exon4}}$ (Figure 2.9) and contradictory of microarray data obtained in stably transfected SP-C$^{\Delta \text{exon4}}$ cells (Chapter 3), lending further support to the concept that stably transfected SP-C$^{\Delta \text{exon4}}$ cells have adapted to chronic ER stress imposed by the expression of misfolded SP-C. Taken together, these microarray results demonstrate that two distinct, C-terminal SFTPC mutants invoke similar acute transcriptional responses throughout the secretory pathway that serve to promote refolding and degradation of the resultant misfolded proproteins.

NF-κB promoted a cytoprotective, adaptive response in stably expressing SP-C$^{\Delta \text{exon4}}$ cells. Interleukin 8 (IL-8) is a pro-inflammatory cytokine that is induced by
Figure 4.9 – Known components of ER stress and ERAD pathways are increased in response to misfolded SP-C. Blue bars represent gene ratio of SP-C^Δexon4 to SP-C^wt, red bars indicate ratio of SP-C^L188Q to SP-C^wt.

NF-κB. IL-8, a NF-κB-dependent gene, is increased 3.9-fold and 5.2-fold in cells transiently expressing SP-C^Δexon4 and SP-C^L188Q, respectively, suggesting that the activation of NF-κB occurs early in response to misfolded SP-C; however, this hypothesis remains to be tested. Experiments will be performed to determine if transient expression of misfolded SP-C induces NF-κB activation and the subsequent production of IL-8. In addition to IL-8, several other genes involved in inflammation and its associated signaling pathways were significantly increased in the cells expressing misfolded SP-C including interleukin-6 signal transducer (IL6ST), signal transducer and activator of transcription 1 (STAT1) and interferon gamma receptor 1 (IFNGR1). Collectively, these data suggest that while NF-κB promotes a cytoprotective response to chronic ER stress imposed by misfolded SP-C, this response may come at a cost of
increased inflammation that could initiate the pathogenesis of lung disease in patients with SFTPC mutations.

**Identification of the quality control machinery for misfolded SP-C**

It is anticipated that the quality control receptor(s) for misfolded SP-C is induced by the IRE1/XBP-1 pathway. Upon activation by IRE1, XBP-1 translocates to the nucleus, binds to an enhancer sequence known as the unfolded protein response element (UPRE) and induces transcription of downstream targets. To identify candidate XBP-1 target genes, the 1kb proximal promoter of the genes in cluster1 were scanned for UPRE sequences. From this analysis, 1,181 of the 5,554 probe sets were found to contain 1 or more UPRE sequences in their promoter suggesting that they are putative XBP-1 target genes (Figure 4.10). Because misfolded SP-C is retained in the ER prior to its degradation, it is anticipated that the quality control receptor for SP-C will be localized to the ER. To identify ER resident genes, predicted protein sequences from cluster1 genes were analyzed by the PSORT algorithm (http://psort.nibb.ac.jp/). PSORT predicts the localization sites of a protein within the cell based on analysis of the primary amino acid sequence of the input protein. PSORT identified 83 putative ER resident genes in cluster1, 26 of which contained UPRE sequences in their proximal promoter. Included in these 26 genes were two genes that are strong candidates for the quality control receptor for misfolded SP-C, ERdj4 and ERdj5, based on their established role in protein folding (Figure 4.10).

ERdj4 and ERdj5 belong to a family of proteins known as ER-localized DnaJ homologs, which currently consists of five identified members. DnaJ, a member of the HSP40 family, was first identified as a cofactor of DnaK, the bacterial homolog of HSP70, and was shown to stimulate the ATPase activity of DnaK (Yochem et al. 1978). Since the initial identification of DnaJ, DnaJ homologs have been identified in all species and organelles, including the ER, where they serve to assist in protein folding. ERdj proteins are divided into 3 subgroups based on the degree of homology with bacterial DnaJ. Type I DnaJ proteins possess all 3 domains found in DnaJ, including the N-terminal, highly conserved ~70 amino acid J domain, the glycine/phenylalanine-rich domain, and a cysteine-rich, Zn²⁺ binding domain. Type II DnaJ homologs possess the J
Figure 4.10 – Venn diagram outlining filtering strategy to identify candidate quality control receptors for misfolded SP-C. Box designated 2-fold increase indicates number of probe sets that were increased ≥ 2-fold in both SP-C\textsuperscript{\textDelta exon4} and SP-C\textsuperscript{L188Q} over SP-C\textsuperscript{wt}. Box designated UPRE indicates number of probe sets that contain at least one UPRE in the 1kb proximal promoter. Box designated ER indicates number of genes that are ER-localized as predicated by PSORT algorithm. Box designated UPRE/ER indicates number of probe sets that correspond to a putative UPRE-containing gene whose product is predicted to be ER-localized. \textDelta exon4/197 and L188Q/197 indicate ratio of gene expression of SP-C mutants with respect to wild-type protein. UPRE listed after fold change indicates number of putative UPRE in proximal promoter.

and glycine/phenylalanine-rich domains but lack the zinc finger domain. Type III proteins only contain a J domain. The J domain is essential for interaction with HSP70 members, mediated through a highly conserved HPD motif, and serves to stabilize HSP70 binding to unfolded protein substrates (Tsai et al. 1996; Chevalier et al. 2000). The zinc finger domain has been shown to interact directly with unfolded substrates. The
function of the glycine/phenylalanine motif is unknown although it is also thought to interact directly with unfolded protein substrates.

ERdj4 is a type II DnaJ homolog that was initially identified by searching the GenBank™ database for proteins that contained a J domain and an ER localization sequence (Shen et al. 2002b). ERdj4 is an ER-resident, membrane protein and the majority of the protein, including the J domain, resides in the lumen of the ER (Figure 4.11). Expression of ERdj4 protein was found in all human tissues, including the lung, and was shown to stimulate ATPase activity of BiP in a dose-dependent manner. Interaction of ERdj4 with BiP was dependent of the HPD motif of the J domain. Expression of ERdj4 was highly induced at the transcriptional level in response to ER stress triggered by tunicamycin, suggesting it may play a role in protein folding in the ER or ERAD (Shen et al. 2002b).

ERdj5 is a type III DnaJ homolog that contains a hydrophobic leader sequence and four conserved thioredoxin-like domains (TLDs) in addition to the conserved J domain (Figure 4.11). Unlike ERdj4, ERdj5 is completely localized to the lumen of the ER; similar to ERdj4, it is induced in response to ER stress and stimulates the ATPase activity of BiP in vitro (Cunnea et al. 2003). The TLDs are thought to promote the reduction and/or oxidation of disulfide bonds between cysteine residues of nascent polypeptides, thereby assisting in protein folding and quality control in the ER.

Based on the large transcriptional induction of ERdj4 and ERdj5 in response to misfolded SP-C, their localization to the ER and their proposed function in protein folding and ER quality control, it is predicted that ERdj4 and/or ERdj5 participate in the recognition and degradation of misfolded SP-C. To test the hypothesis that ERdj4 and ERdj5 specifically bind to misfolded SP-C, SP-C protein will be immunoprecipitated from SP-C∆exon4 or SP-Cwt expressing cells followed by Western blot analysis with anti-Erjd4/5 antibodies to determine if these ERdj proteins form a complex with misfolded SP-C. It is anticipated that ERdj4/5 will specifically bind misfolded SP-C and the BiP will also co-precipitate with this complex. If ERdj4/5 are found to associate with misfolded SP-C, experiments will be performed to determine if the overexpression of ERdj4/5 accelerates the degradation of misfolded SP-C. ERdj4/5 will be cloned into a mammalian expression vector and transiently transfected in stably expressing SP-C∆exon4.
or SP-CΔexon4 cells. Levels of misfolded SP-C will be assessed by Western blot analysis. Finally, a loss-of-function experiment will be performed to determine if misfolded SP-C is stabilized in cells with decreased levels of ERdj4/5. Translation of ERdj4/5 in stably expressing SP-CΔexon4 or SP-CΔL188Q cells will be inhibited by RNAi and levels of misfolded protein will be assessed by Western blot analysis. In addition, pulse-chase experiments will be performed to determine the kinetics of SP-C turnover. Cells expressing SP-Cwt will be used as a control for in all of the experiments outlined above.

It is anticipated that ERdj4/5 will specifically recognize and facilitate the proteasome-dependent degradation of misfolded SP-C. In the event that the predicted outcome is observed, the in vivo significance of this finding will confirmed using SP-CΔexon4-KI/+ mice. Knock-out mouse models of ERdj4 and ERdj5 have not been described and based on their ubiquitous expression pattern, direct ablation of either locus may result in a non-viable animal. Therefore, a lung specific, conditional knockout model of ERdj4/5 may have to be generated in order to test the in vivo significance of these
proteins. Alternatively, transgenic mice which express RNAi against ERdj4/5 under the control of a SP-C promoter could also be generated for this experiment.

As an alternative approach to the microarray experiment, a co-immunoprecipitation experiment was performed to identify proteins that specifically bind misfolded SP-C. Stably transfected SP-C^{Δexon4} or SP-C^{wt} cells were labeled with ^{35}\text{S}-cysteine/methionine and immunoprecipitated with an antibody directed against the N-terminal peptide of the SP-C proprotein. The proteasome inhibitor MG-132 was added to the culture media during the labeling period to prevent degradation of misfolded SP-C as was the covalent protein cross-linker dithio-bis(succinimidyl propionate) (DSP). Results revealed that several proteins specifically co-precipitate with SP-C^{Δexon4} (Figure 4.12). Of particular interest was one band corresponding to a molecular weight of approximately 25kDa, close to the predicted molecular weight of ERdj4. Optimization experiments are currently underway to allow for the identification of the unique proteins by mass spectrometry. These experiments, coupled with the experiments stemming from the microarray, should provide significant insight into the molecular machinery involved in identification and degradation of misfolded SP-C.

**Conclusions**

The overall goal of this dissertation was to determine if mutations in SFTPC cause lung disease. The hypothesis underlying this work was that mutations in SP-C lead to misfolding of the proprotein resulting in chronic ER stress, cytotoxicity and the subsequent formation of lung disease. The work outlined in Chapter 1 demonstrated that the index mutation, SP-C^{Δexon4}, was misfolded, leading to ER retention, activation of ER stress pathways and degradation via a proteasome-dependent manner. Further, expression of SP-C^{Δexon4} in type II epithelial cells of transgenic mice led to a profound disruption of lung morphogenesis, associated with epithelial cytotoxicity. The experiments in Chapter 3 were designed to test the hypotheses that the chronic ER stress imposed by the expression of SP-C^{Δexon4} leads to a cytoprotective response and SP-C^{Δexon4}-expressing cells are predisposed to a secondary environmental insult, such as viral infection. The results suggested that the SP-C^{Δexon4}-expressing cell line adapted to chronic ER stress by an NF-κB-dependent mechanism and that the adaptive response increased
Figure 4.12 – Identification of proteins that specifically bind misfolded SP-C. Cell lines expressing SP-C\textsuperscript{Δexon4}, SP-C\textsuperscript{197} or empty vector (last lane) were labeled with \textsuperscript{35}S-cysteine/methionine for 4 hours, immunoprecipitated with proSP-C antibodies followed by autoradiography. Prominent bands on bottom of gel represent SP-C\textsuperscript{197}, also referred to as SP-C\textsuperscript{wt}, and SP-C\textsuperscript{Δexon4}. Blue band highlights protein that specifically binds to SP-C\textsuperscript{Δexon4} (molecular weight \textasciitilde 25kDa).

susceptibility to viral-induced cell death. These findings may explain the wide variability in the age of onset and incomplete penetrance of disease in patients with \textit{SFTPC} mutations. The SP-C\textsuperscript{Δexon4-KI/+} model will be used to determine the relative contribution of these pathways to the pathogenesis of lung disease. The microarray results demonstrated a commonality among two C-terminal mutants in SP-C, lending further support to the involvement of ER stress signaling in disease pathogenesis. Misfolded proteins are associated with numerous diseases including cardiomyopathy, Alzheimers and diabetes.
Identification of the quality control machinery for misfolded SP-C will hopefully lead to a better global understanding of how misfolded, non-glycosylated proteins are handled in a cell. These advancements may allow for therapeutic strategies to prevent the untoward effects of accumulated, misfolded proteins in a variety of diseases.
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