Expression of a Human Surfactant Protein C Mutation Associated with Interstitial Lung Disease Disrupts Lung Development in Transgenic Mice*

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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 whether 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 embryonic day 17.5 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 propeptide, a dose-dependent increase in BiP transcription, trapping of the propeptide 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 propeptide with subsequent induction of the unfolded protein response and endoplasmic reticulum-associated degradation pathways ultimately resulting in disrupted lung morphogenesis.

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)1 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 (1–3).

SP-C is exclusively expressed in type II epithelial cells of the lung and is highly conserved among all species studied to date (4–6). Human SP-C is synthesized as a 197-amino acid proprotein (molecular mass = 21 kDa) 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 propeptide traverses the regulated secretory pathway of the type II cell from the endoplasmic reticulum (ER) to the late endosome/multivesicular body where cleavage of the flanking N- and C-terminal domains liberates the extremely hydrophobic, bioactive mature peptide (7, 8). Fusion of the multivesicular body 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 air space.

SP-C is an integral membrane protein that contains a single membrane-spanning domain located within the mature peptide (9). The newly synthesized propeptide 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 (10, 11). Trafficking of the propeptide through the regulated secretory pathway of the type II cell is dependent upon signals encoded in the N-terminal propeptide (11, 12) and may be facilitated by oligomerization because the SP-C propeptide has been shown to form dimers and oligomers in transfected A549 cells (13). The C-terminal peptide is dispensable for trafficking and secretion of the mature peptide (11); 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 nonspecific interstitial pneumonitis and usual interstitial pneumonitis, pulmonary fibrosis is generally regarded as the final common outcome. The index case was a patient diagnosed at 1 year of age with nonspecific interstitial pneumonitis (14). Sequence analysis of genomic

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3 The abbreviations used are: SP, surfactant protein; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ILD, interstitial lung disease; Ex, embryonic day; WT, wild-type; CCSP, Clara cell secretory protein; UPR, unfolded protein response.
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 adenine for a guanosine at the first base pair of intron 4 (genomic DNA base 1728 (g.1728 G → A)), resulting in the insertion of the normal donor splice site at the exon 4/intron 4 boundary (see Fig. 1A). Splicing of the mutant mRNA results in deletion of exon 4, which encodes 37 amino acids in the C-terminal peptide of SP-C, ultimately resulting in the formation of a truncated/splice variant of Cda (SP-C<sub>△exon4</sub>). The mutant splicing variant 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<sub>△exon4</sub> 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 IILD, including adults presenting with usual interstitial pneumonia and children with cellular nonspecific interstitial pneumonitis (15). 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, 11 mutations in the SFTPC locus have been linked to IILD, and all except one map to the C-terminal peptide of SP-C (16). This study was designed to test the hypothesis that a disease-linked mutation in the C-terminal peptide of SP-C causes lung disease.

MATERIALS AND METHODS

DNA Constructs, Generation of Transgenic Mice, and Adenovirus Production—Full-length human SP-C (SP-C<sub>FL</sub>) DNA was cloned into pcDNA3 (Invitrogen) to generate SP-C<sub>FL</sub>/pcDNA3. SP-C<sub>FL</sub> was generated by deleting nucleotides 325–435 (adenosine of start ATG is base pair 1) via overlapping polymerase chain reaction mutagenesis using SP-C<sub>FL</sub>/pcDNA3 as a template and cloned into pcDNA3. A transgene construct was generated by subcloning SP-C<sub>FL</sub>/pcDNA3 into a puromycin-resistant vector containing the 13-kb mouse SP-C promoter (17), rabbit globin intron/exon sequence, and a bovine growth hormone polyadenylation signal (Fig. 1A) as previously described (18). 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 the presence of a reporter gene construct (the SP-C<sub>FL</sub>/pcDNA3 vector into the Adv2 shuttle vector (19). Recombination and adenoavirous production were performed as described previously (19).

Histological and Western Analysis of Lung Tissue—Potential founder mice (F<sub>0</sub>) 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 (20). For Western analysis, lung tissues were homogenized in phosphate-buffered saline containing 1% protease inhibitor mixture (Sigma). Total protein concentration of the lung homogenate was determined by BCA assay, and equal amounts of protein were separated by SDS-PAGE. Concentration of the lung homogenate was determined by BCA assay 1% per volume protease inhibitor mixture (Sigma). Total protein content was assessed by BCA assay, and equal amounts of protein were separated by SDS-PAGE, and equal amounts of protein were separated by SDS-PAGE.

Separate proteins were electrophoretically transferred to nitrocellulose membranes and probed with a polyclonal antibody directed against the N-terminal peptide of SP-C (21) or actin (a kind gift from Dr. James Lebowitz, Children’s Hospital, Cincinnati, OH). Total protein concentration of the lung homogenate was determined by BCA assay (Pierce), and equal amounts of protein were separated by SDS-PAGE. The same proteins were incubated with a rabbit polyclonal antibody directed against the N-terminal peptide of SP-C and Clara cell secretory protein (CCSP) (the latter antibody was kindly provided by Dr. Barry Stripp, University of Pittsburgh) at the indicated dilutions. Biotinylated secondary antibodies and a streptavi- din-biotin-peroxidase detection system (Vector Laboratories, Inc.) were used to localize the antibody-antigen complexes in the tissues, as previously described (20). In situ hybridization was performed as previously described (5) using a 35S-UTP-labeled, transgene-specific riboprobe directed against the bovine growth hormone polyadenylation signal (283-bp fragment).

Type II Cell Isolation, Adenoviral Infection, Metabolic Labeling, Immunoprecipitation, and In Vitro Transcription/Translation—Type II epithelial cells were isolated from SP-C<sub>197</sub>/mice (22, 23) using the method described by Rice et al. (24). 1 × 10<sup>6</sup> cells/well were plated on 100% Matrigel (BD Pharmingen, San Diego, CA) in growth medium containing all anti-myoepithelial additives except hydrocortisone (Chemicon, Temecula, CA). The medium also included 10% charcoal-stripped fetal bovine serum (Sigma) and 10 ng/ml KGF (Promega, Rocky Hill, NJ). The cells were cultured at 37 °C in a humidified incubator containing 5% CO$_2$. Seventy-two hours post-isolation, the cells were infected with multiplicity of infection of purified adenoviral particles encoding SP-C<sub>197</sub> or SP-C<sub>△exon4</sub> in infection medium containing 2% fetal bovine serum; infection medium was replaced with complete medium 90 min following infection. Forty-eight hours post-infection, the cells were metabolically labeled with 0.5 mCi/ml of [35S]methionine/cysteine (ICN, Aurora, OH) for 4 h. The cell lysates were immunoprecipitated exactly as described previously (25) with 5 μl of an antibody directed against the mature SP-C (26). SDS-PAGE and autoradiography was performed as previously described (25). SP-C<sub>197</sub>/pcDNA3 and SP-C<sub>△exon4</sub>/pcDNA3 were transcribed and translated in vitro in the presence of [35S]methionine/cysteine (ICN) using the TNT® coupled reticulocyte lysate system (Promega, Madison, WI). The completed reactions were analyzed by SDS-PAGE/autoradiography.

HEK293 Cell Culture and Transfection—HEK293 cells were purchased from ATCC (Manassas, VA). Growth medium consisted of Rich-ter’s medium (Biowhittaker, Walkersville, MD) containing 10% fetal bovine serum (Sigma), 2 mM L-glutamine and 1 unit/ml penicillin and streptomycin (Sigma). The cells were cultured at 37 °C in a humidified incubator containing 5% CO$_2$. For proteasome inhibitor experiments, 2 × 10<sup>5</sup> cells were plated into a 12-well plate 24 h prior to transfection. The cells were transiently transfected with 1 μg/well SP-C<sub>197</sub>/pcDNA3 or SP-C<sub>△exon4</sub>/pcDNA3 using LT-1 reagent (Mirus, Madison, WI). Four-hour hours prior to harvest, the cells were treated with 5 μM MG-132 (Cal-biochem, La Jolla, CA) or Me$_6$SO as a vehicle control. The cells were harvested in phosphate-buffered saline, sonicated immediately, the total protein content was assessed by BCA assay, and equal amounts of protein were analyzed by SDS-PAGE/Western blotting with SP-C or actin antisera as described above.

Immunofluorescence—HEK293 cells transfected with SP-C<sub>△exon4</sub> or SP-C<sub>197</sub> were plated on poly-lysine coated coverslips. 4 h prior to fixation, the cells were treated with 5 μM MG-132 or Me$_6$SO vehicle control (Calbiochem, La Jolla, CA) as indicated. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.1% Triton X-100, and stained with a polyclonal antibody directed against the N-terminal propeptide of SP-C for 2 h at 37 °C. The cells were washed and incubated with anti-rabbit, fluorescein isothio- cyanate-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) for 2 h at 37 °C. The cells were washed three times with phosphate-buffered saline, washed once with distilled H$_2$O, and mounted on slides with Vectashield mounting medium (Vector Laboratories, Inc.). Fluorescence was imaged with a Nikon microscope using a UV lamp and a fluorescein isothiocyanate filter. The images were captured using an Onpramons 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) (27). The β-galactosidase plasmid, pSV-β-galacto-sidase, was purchased from Promega. 2 × 10<sup>5</sup> HEK293 cells were plated into a 12-well plate 24 h prior to transfection. The cells were co-transfected with three plasmids including: 1) 75 or 250 ng of one of the test plasmids (SP-C<sub>197</sub> or SP-C<sub>△exon4</sub>, or pcDNA3 as an empty vector control), 2) 250 ng of BiP/luciferase reporter plasmid, and 3) 75 ng of β-galactosidase plasmid. The cells were harvested 48 h post-transfection in Glo Lysis Buffer (Promega). The luciferase activity was measured with a Bright LB96V luminometer (Berthold, Bad Wildbad, Germany). In situ hybridization as previously described (5) using a 35S-UTP-labeled, transgene-specific riboprobe directed against the bovine growth hormone polyadenylation signal (283-bp fragment)
Higher magnification revealed the presence of vacuolated (Fig. 2) eosin staining of fetal lung tissue (Fig. 2), whereas the remaining three F0 mice were morphologically indistinguishable from the wild-type control (WT). The SP-C exon4 transgene into the genome. Despite two rounds of pronuclear injections into FVB/N eggs, no transgene-positive progeny were recovered, suggesting that disruption was also disrupted, albeit to a lesser extent than that observed in TG#1 and TG#2. 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 distal airways. The airway debris was more prominent in TG#2 and consisted of sloughed epithelial cells and distal airways. The airway debris was more prominent in TG#2 as compared with the wild-type control (WT). TG#3 was morphologically indistinguishable from the wild-type control. Scale bar, 500 μm.

Results

Generation of SP-CΔexon4 Transgenic Mice—To test the hypothesis that mutations in the C-terminal peptide of SP-C cause lung disease, SP-CΔexon4 was expressed in type II epithelial cells of transgenic mice. The SP-CΔexon4 construct was synthesized by deleting the nucleotides encoding the amino acids of exon4 (residues 109–145) via overlapping PCR mutagenesis using human SP-C cDNA as a template. Expression of SP-CΔexon4 was specifically targeted to type II epithelial cells of the lung using the 13-kb mouse SP-C promoter (Fig. 1B). Despite two rounds of pronuclear injections into FVB/N eggs, no transgene-positive progeny were recovered, suggesting that expression of SP-CΔexon4 was associated with neonatal lethality. The construct was therefore injected again, and the lungs were harvested from potential founder mice (F0) 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-CΔexon4 Disrupts Lung Organogenesis in Transgenic Mice—Two of five transgene-positive F0 fetuses displayed disrupted lung organogenesis, as assessed by hematoxylin and eosin staining of fetal lung tissue (Fig. 2), whereas the remaining three F0 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 sacculles, little branching morphogenesis, and loss of typical distal acinar structures (Fig. 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 (Fig. 2B). The lung structure of the other three F0 mice (one of which is depicted in Fig. 2C as TG#3) was completely normal.

Throughout the study, a gradient of SP-CΔexon4 expression, as assessed via reverse transcription polymerase chain reaction (RT-PCR) and in situ hybridization, was observed in TG#1 as compared with a wild-type control (WT). The epithelial cells are highlighted in WT (arrowheads) for comparison with those shown in TG#1. Scale bar, 100 μm.

Analysis of the hematoxylin and eosin-stained sections at higher magnification revealed the presence of vacuolated (Fig. 2). The SP-CΔexon4 transgene construct was therefore injected again, and the lungs were harvested from potential founder mice (F0) 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.

Fig. 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 intron 4. This base pair substitution ablates a normal donor splice site, resulting in deletion of exon 4 upon splicing of the mRNA. Translation of the mutated mRNA results in a truncated protein, designated SP-CΔexon4. The open boxes represent exons, whereas the lines represent introns. The black box in exon2 represents a point mutation at position 1728/H11001 g.1728→H11001.

3A, arrowheads) and hypertrophic (Fig. 3A, arrowheads) epithelial cells in TG#1. The epithelium of TG#2 appeared largely intact and contained few vacuolated cells (Fig. 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 air spaces. In contrast, the epithelium in TG#3 and WT were intact, devoid of airway debris, and supported by condensed mesenchymal tissue indicative of the canalicular stage of murine lung development (Fig. 3, C and D). Collectively these data demonstrate that expression of SP-CΔexon4 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 whether variability in phenotype was associated with the level of SP-CΔexon4 expression, in situ hybridization was performed with a radiolabeled riboprobe specific for transgenic mRNA. A gradient of SP-CΔexon4 mRNA expression was detected among F0 mice with the highest expression observed in TG#1 and the lowest expression observed in TG#3.
Fig. 4. Level of SP-C^exon4 expression correlates with the severity of phenotype. In situ hybridization (ISH) and immunohistochemistry (IHC) were performed on serial lung sections from E17.5 F0 mice. In situ hybridization (A–D) was performed using a transgene-specific radiolabeled riboprobe directed against the poly(A) tail of the transgenic construct (see Fig. 1B). Immunohistochemistry was performed with a polyclonal antibody directed against the N-terminal propeptide of SP-C (E–H) or against the proximal epithelial marker CCSP (I–L). Primary antibody dilutions for proSP-C are indicated at the top right of E–H. Primary antibody dilution for CCSP was 1:5,000 for I–L. Scale bar, 500 μm for A–L and 60 μm for insets.

(Fig. 4, A–C). The antisense signal in WT (Fig. 4D) was indistinguishable from sense controls (data not shown). These results demonstrate that the expression level of SP-C^exon4 was correlated with the severity of the lung phenotype. The expression pattern of SP-C^exon4 was assessed by immunohistochemistry using an antibody specific for the N-terminal propeptide of SP-C (proSP-C) (Fig. 4, E–H). Because this antibody detects both endogenous SP-C and SP-C^exon4, the primary antibody was titered to a concentration at which only SP-C^exon4 was detected (1:44,000); endogenous SP-C staining was detected at a primary antibody dilution of 1:1,000 but not 1:4,000. Intense SP-C immunoreactivity was observed in the epithelial cells lining the cystic saccules of TG#1 (Fig. 4E), confirming successful targeting of SP-C^exon4 to the distal epithelium. In addition, high expression of SP-C^exon4 in TG#1 was associated with sloughing of the epithelium, leading to an accumulation of SP-C-positive cellular debris in the air spaces (Fig. 4E, inset). Interestingly, SP-C immunoreactivity was only detected in the air spaces of TG#2 and not in the epithelium at the 1:44,000 dilution, suggesting that intracellular SP-C^exon4 protein was turned over rapidly in this animal (Fig. 4F and inset). Lung tissue from TG#3 was completely devoid of SP-C staining at the 1:44,000 dilution (Fig. 4G), even though SP-C^exon4 mRNA was detected in this mouse (Fig. 4C). For comparison, the endogenous SP-C staining pattern of distal epithelial cells is shown on wild-type tissue at a 1:1,000 dilution of primary antibody (Fig. 4H). The staining pattern for a proximal epithelial cell marker, CCSP, appeared normal, suggesting that cell specification was not perturbed even in the most severely affected lungs (Fig. 4, I–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.

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 because of rapid processing to the mature peptide in the biosynthetic pathway of the type II cell (Fig. 5A, WT). Immunoreactive SP-C was readily detected in TG#1 (Fig. 5A), and the size of the proSP-C positive band (M_r = 17,000) 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 (Fig. 5B). These data demonstrate that the expression of SP-C^exon4 in TG#1 detected by Western analysis correlates 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 C-terminal peptide domain resides in the lumen of the ER (10,
metabolically labeled with [35S]cysteine/methionine, and the cell lysates were immunoprecipitated with a polyclonal antibody directed against the mature SP-C peptide. The immunoprecipitates were separated by SDS-PAGE and subjected to autoradiography. The top portion of the gel containing preproteins was exposed to film for 24 h, and the bottom portion was exposed for 72 h. The molecular mass markers are indicated on the left.

Although the function of the C-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 whether 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 (duplicates, lanes 1 and 2) or SP-CΔexon4 (duplicates, lanes 3 and 4). Forty-eight hours post-infection, the cells were metabolically labeled with [35S]cysteine/methionine, and the cell lysates were immunoprecipitated with a polyclonal antibody directed against the mature SP-C peptide. The immunoprecipitates 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-actin antibody for a loading control. The presented data represent three experiments. The molecular mass markers are indicated on the left.

To determine whether SP-CΔexon4 was degraded early in the biosynthetic pathway, SP-C1–197 or SP-CΔexon4 was transiently transfected into HEK293 cells expressing either SP-C1–197 or SP-CΔexon4. SP-C1–197 exhibited a punctuate staining pattern that was unaltered by proteasome inhibition (Fig. 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 (28). In contrast, low levels of protein were detected in cells that expressed SP-CΔexon4 in the absence of a proteasome inhibitor, consistent with results obtained by Western analysis (Fig. 8, bottom panels). Treatment with MG-132 prevented degradation of the SP-CΔexon4, revealing a diffuse staining pattern indicative of ER localization (Fig. 8, bottom panels). These results support the hypothesis that SP-CΔexon4 is recognized as a misfolded peptide, retained in the ER, and rapidly degraded via the ERAD pathway in a non-type II epithelial cell line.

SP-CΔexon4 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 (29). To determine whether SP-CΔexon4 induced ER stress, mammalian expression vectors encoding SP-CΔexon4, SP-C1–197, 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) (Fig. 8). The cell lysates were harvested 48 h post-transfection and analyzed for luciferase activity and SP-C levels via Western analysis. The cells transfected with BiP/Luc and subjected to a 6-h exposure of 10 μM 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 (Fig. 9A). 75 ng of SP-CΔexon4 co-transfected with the BiP/Luc reporter resulted in a 2-fold increase in luciferase activity compared with cells transfected with the empty vector control. Co-transfection of an equivalent amount of SP-C1–197 and BiP/Luc caused a modest increase in luciferase activity compared with pcDNA3 but failed to reach significance (Fig. 9A). When the amount of input cDNA was increased to 250 ng, SP-CΔexon4 augmented luciferase activity.

Fig. 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 48 h. The 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, the cells were metabolically labeled with [35S]cysteine/methionine, and the cell lysates were immunoprecipitated with a polyclonal antibody directed against the mature SP-C peptide. The immunoprecipitates were separated by SDS-PAGE and subjected to autoradiography. The top portion of the gel containing preproteins was exposed to film for 24 h, and the bottom portion was exposed for 72 h. The molecular mass markers are indicated on the left.
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 whether the g.1728 G → A (SP-CΔexon4) point mutation in the SFTP C locus was directly linked to the pathogenesis of lung disease. This hypothesis was tested by generating transgenic mice that expressed SP-CΔexon4 in type II cells of the respiratory epithelium. SP-CΔexon4 caused a dose-dependent perturbation of lung development 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 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 unfolded protein response (UPR) and ERAD pathways.

Lung development was profoundly disrupted despite the fact that expression of the SP-CΔexon4 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. First, epithelial cells expressing high levels of the transgene exhibited cell swelling consistent with necrosis. Second, the sloughed respiratory epithelium and the cellular debris detected in the airways of two independent F0 animals stained intensely for proSP-C at an antibody dilution that detected only the transgene-derived protein. Third, macrophage infiltrates were present in the lungs of both affected animals. Because 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 whether SP-CΔexon4-induced dysmorphogenesis was linked to altered epithelial cell specification, markers of the proximal and distal respiratory epithelium were analyzed in lung tissues from three F0 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 in-
teractions are absolutely required for proper branching morphogenesis in numerous organs including the lung (30). 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 because mice expressing SP-B^C or lysozyme transgenes were viable with no lung abnormalities (31, 32). A phenotype similar to that seen in the SP-C^Δexon4 mice was observed in mice expressing high levels of the SP-C mature peptide, SP-C^Δ458, or diapher in type II cells (33, 34). Collectively, these data suggest that the demise of fetal type II epithelial cells, irrespective of the causal insult, leads to altered lung morphogenesis.

Deletion of exon 4 from SP-C resulted in incomplete processing of the mutant proprotein in isolated type II epithelial cells. Processing of the N-terminal propeptide and the C-terminal peptide occurs in the multivesicular body of the type II cell leading to the generation of the 4-kDa active peptide (7, 8). Processing of the N-terminal propeptide and the C-terminal peptide via proteasome-dependent mechanisms. Proteasome-dependent increase in BiP transcription and rapid degradation of the proprotein via proteasome-dependent mechanisms. Proteasome-dependent degradation of SP-C^Δexon4 in HEK293 cells together with the inability to detect SP-C^Δexon4 protein in TG82 and incomplete processing of the mutant proprotein in type II cells support the hypothesis that SP-C^Δexon4 is recognized as a misfolded protein within the ER and rapidly degraded via ERAD. Wild-type SP-C^C197 also caused an increase in BiP transcription at the higher input dose of cDNA; however, unlike SP-C^Δexon4, SP-C^C197 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^Δexon4 induces ERAD in HEK293 cells resulting in selective degradation of mutant but not wild-type SP-C.

The high level of SP-C^Δexon4 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 air spaces 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^−/− mice (22, 36). However, SP-C deficiency cannot be the sole cause of disease in transgenic mice because lung structure and function is normal in newborn SP-C^−/− mice (23).

A previous study in transiently transfected A549 cells demonstrated that deletion of exon 4 in the context of SP-C/GFP fusion proteins resulted in ubiquitination and aggresome formation (37). 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^Δexon4 in HEK293 cells is consistent with ubiquitination of the SP-C cysteine mutants in A549 cells. However, rapid turnover of SP-C^Δexon4 both in vitro and in vivo is inconsistent with aggresome formation. Although ultrastructural analysis was not performed on SP-C^Δexon4 F0 mice because of the limited amount of lung tissue, aggresomes were not detected in transgenic mice expressing SP-C^C458, which displayed a similar phenotype to SP-C^Δexon4 transgenic mice (33). 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. (37); however, in HEK293 cells transiently expressing high levels of the folding mutant CFTR^F508, only 5–15% of cells contained aggresomes (38). Taken together, we postulate that the constitutive expression of misfolded SP-C^Δexon4 overwhelmed the degradative capacity of ERAD machinery, resulting in chronic induction of ER stress pathways, type II cell injury, and disrupted lung morphogenesis.

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SP-C\textsuperscript{\textasciitilde exon4} Disrupts Lung Morphogenesis

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