Ablation of a Critical Surfactant Protein B Intramolecular Disulfide Bond in Transgenic Mice*

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The 79-amino acid, mature SP-B peptide contains three intramolecular disulfide bonds shared by all saposin-like proteins. This study tested the hypothesis that the disulfide bond formed between cysteine residues 35 and 46 (residues 235 and 246 of the SP-B proprotein) is essential for proper function of SP-B. To test the role of this bridge in SP-B function in vivo, a construct was generated in which cysteine residues 235 and 246 of the human SP-B proprotein were mutated to serine and cloned under the control of the 3.7-kilobase hSP-C promoter (hSP-B235S/246S). In two transgenic mouse lines, expression of the mutant peptide in the wild-type murine SP-B background was invariably lethal in the neonatal period. In four additional lines, survival was inversely related to the level of transgene expression. To test the ability of the mutant peptide to functionally replace the wild-type protein, transgenic mice were crossed into the SP-B null background. No animals that expressed hSP-B235S/246S in the murine SP-B background survived the neonatal period. hSP-B235S/246S proprotein accumulated in the endoplasmic reticulum and was not processed to the mature, biologically active peptide. The results of these studies demonstrate that the intramolecular bridge between residues 235 and 246 is critical for intracellular trafficking of SP-B and suggest that overexpression of mutant SP-B in the wild-type background may be lethal.

Pulmonary surfactant is a complex mixture of phospholipids and surfactant proteins (SP-). A, B, and C that is synthesized and secreted by the Type II epithelial cell into the alveolar airspace. Surfactant forms a film along the alveolar epithelium that reduces surface tension to very low levels during lung deflation. Deficiency of pulmonary surfactant results in alveolar collapse, leading to respiratory distress syndrome, a leading cause of morbidity and mortality among neonates worldwide. Substantial benefit is derived from treating these infants with surfactant replacement preparations, particularly those containing SP-B or SP-C. Human infants with mutations in the SP-B gene that result in complete absence of SP-B protein develop severe respiratory distress syndrome at birth and, despite intensive respiratory therapy, ultimately succumb to the disease (1). Similarly, SP-B null mice die of acute respiratory distress syndrome within minutes of birth, have reduced lung volumes, decreased concentrations of mature SP-C peptide, and highly disorganized lamellar bodies, the intracellular storage form of surfactant (2, 3). Collectively, these results indicate that SP-B is critical for lung function.

SP-B is translated as a 381-amino acid preproprotein that is co-translationally cleaved to generate the SP-B proprotein. The SP-B proprotein is sorted to the multivesicular body where the 102-amino acid carboxyl and 177-residue amino-terminal peptides are sequentially cleaved to generate the 79-amino acid mature SP-B peptide (4). The hydrophobic SP-B peptide is stored with surfactant phospholipids in specialized secretory granules (lamellar bodies) in which phospholipids are arranged as concentric membrane lamellae. In the absence of SP-B, lamellar bodies contain numerous vesicles, but few or no lamellae, consistent with a key role for SP-B in the packaging of lamellar body phospholipids. The contents of the lamellar body are secreted into the alveolar space where SP-B and SP-C facilitate the rapid formation of a surfactant film.

The intra-alveolar form of mature SP-B exists in an oxidized state in which six cysteine residues participate in three intramolecular sulfhydryl bridges, while the seventh cysteine forms an intermolecular bridge that results in SP-B homodimers (5, 6). The precise arrangement of the three intramolecular disulfide bridges places SP-B in the saposin-like protein (SAPLIP) family, which also includes saposins A-D, amoebapore, and NK-lysin. SAPLIP proteins serve a variety of functions including glycosphingolipid metabolism, host defense, and in the case of SP-B, surfactant function in the alveoli. The mature SP-B peptide differs from the other family members in that it is more hydrophobic and forms sulfhydryl-mediated dimers.

NK-lysin is the only SAPLIP family member for which the three-dimensional structure has been solved, although sequence homology among family members suggests that all members share the saposin fold of NK-lysin (7). Studies of SP-B secondary structure and comparisons with the NMR structure of NK-lysin predict that SP-B contains five amphipathic α-helices folded into a globular protein domain (7). A structural model of the SP-B mature peptide has been proposed in which a hairpin turn in the mature peptide results in antiparallel alignment of the helices, bringing cysteine residues 235 and 246 into close apposition (8). It is likely that the cysteine bridge formed between residues 235 and 246 stabilizes this turn and contributes to the structural integrity of mature SP-B. The amino acid sequence located between cysteines 235 and 246 in the SP-B mature peptide is highly hydrophobic, suggesting that this region of the peptide interacts with surfactant lipids.
and might be critical for SP-B function. The current study was undertaken to test the hypothesis that the intramolecular cysteine bridge formed between residues 235 and 246 of SP-B is critical for SP-B structure and function in vivo.

MATERIALS AND METHODS

Generation of DNA Constructs—To generate the hSP-B<sub>235S/C246S</sub> construct, site-directed mutagenesis was employed using a sequential PCR protocol (Current Protocols in Molecular Biology 8.5) to substitute serine for the cysteine residues at positions 235 and 246 in the SP-B preproprotein. Primers were chosen which would amplify a 1.6-kb fragment of human SP-B cDNA that included the endogenous Kozak sequence and sequence encoding the 381-amino acid SP-B preproprotein (5′-GCCGAAATTGAGGCTCCATCGTCTAC and 5′-CAGCTTCGCGGAAGCCTGGGCGTGTGATACACT). Internal primers used in each of the PCR result in the substitution of serine for cysteine 235 (upstream primer 5′-GGTGTCGGCGTGTACCTCTCT and downstream primer 5′-GGCCGACACCTGGCCACGCTGAC). This construct, hSP-B<sub>235S/C246S</sub>, was cloned into the EcoRI and SstII sites of pEGFP-N1 (CLONTECH, Palo Alto, CA) which resulted in a fusion of SP-B cDNA containing the preproprotein but lacking the carboxy terminus. To generate the transgene construct, primers (5′-GCCGAAATTGAGGCTCCATCGTCTAC and 5′-CAGCTTCGCGGAAGCCTGGGCGTGTGATACACT) were used to amplify hSP-B<sub>235S/C246S</sub> using the SP-B/GFP fusion construct as template. This sequence was cloned into the EcoRI site of plasmid pCC10kbpA which contained the mouse CCSP promoter, rabbit β-globin intron 2 and bovine growth hormone polyadenylation signal (kind gift of Francesco DeMayo, Baylor University). To generate transgenic mice in which hSP-B<sub>235S/C246S</sub> expression was restricted to the distal respiratory epithelium, the 3.7-kb human SP-C promoter fragment (9) was substituted for the CCSP promoter. The final vector is referred to as the BGI-hSP-C expression vector.

Intracellular Trafficking of hSP-B<sub>235S/C246S</sub> in PC12 Cells—PC12 cells (American Type Culture Collection, Rockville, MD) were cultured in 10-cm<sup>2</sup> tissue culture dishes that were incubated with 70% confluent PC12 cells grown in 10-cm<sup>2</sup> tissue culture dishes that were incubated with a medium containing 10% fetal bovine serum (number 28031) exactly as described (11).

Immunoprecipitations were performed using anti-mature SP-B antibody for 1 h followed by secondary antibody for 30 min at room temperature. Cells were fixed, permeabilized, and incubated with primary antibody for 1 h followed by secondary antibody for 30 min at room temperature. Cells were viewed with a Leica DMRB/E inverted microscope and scanned with a Leica 3-laser scanning confocal microscope system. Antibody directed to Chromogranin A was purchased from Incstar (Stillwater, MN) and the Texas Red-conjugated goat anti-rabbit secondary antibody was purchased from Vector Labs (Burlington, CA). Metabolic labeling was performed essentially as described (11) using 70% confluent PC12 cells grown in 10-cm<sup>2</sup> tissue culture dishes that were transfected with 20 μg of DNA in 246 μl of LipofectAMINE. Immunoprecipitations were performed using anti-mature SP-B anti-serum (number 28031) exactly as described (11).

Generation and Characterization of Transgenic Mice Expressing hSP-B<sub>235S/C246S</sub>—The transgene was excised from the BGI-hSP-C expression vector by NotI/Ndel digestion, isolated by gel electrophoresis, and purified using Qiaex resin (Qiagen, Germany). The transgene was dialyzed for 48 h against 5 mM Tris (pH 7.5, 1 mM EDTA) and microinjected into fertilized FVB/N oocytes by the Children’s Hospital Transgenic Core Facility. Founder mice were identified by a transgenic specific PCR with primers that amplified a 336-base pair fragment of the hSP-B cDNA (upstream primer 5′-AGCAGCAATTCCCCATTCCTCCT and downstream primer 5′-ATGGCCTGCTGGCTGTTGTC). Primers that amplified a 270-base pair fragment of the endogenous murine SP-B allele were included in the transgene reaction as a positive control (12). PCR conditions were 30 cycles, 58 °C annealing temperature, 0.5 μM primer concentration, 0.25 μM hSP-B primer, 0.25 μM murine SP-B primer, 0.2 μM dNTPs, 1 unit of AmpliTaq Gold, and 1× Amplitaq Gold buffer (Perkin Elmer, Foster City, CA). The PCR included primers specific for the endogenous murine SP-B allele and reaction conditions were 30 cycles, 60 °C annealing temperature, 0.5 μM neomycin primers, 0.25 μM mSP-B primers, 0.25 μM dNTPs, 2.5 μM MgCl<sub>2</sub>, 1 unit of display Taq (PGC Scientific) and 1× PCR Buffer II (Perkin Elmer, Foster City, CA). Individual lanes were excised from the gel and incubated at room temperature for 2 h in 1× glyoxine gel running buffer with 20 μl diithiothreitol (Sigma) in order to reduce hyaluridyl bridges. The excised lane was then placed in a large well of a 10% glyoxine gel and subjected to SDS-PAGE in the second dimension under reducing electrophoretic conditions. Western blotting was performed using antibodies directed against murine SP-B (number 28031).

Expression of hSP-B<sub>235S/C246S</sub> in the SP-B<sup>−/−</sup> Background—To generate mice that expressed hSP-B<sub>235S/C246S</sub> in the absence of endogenous SP-B, hSP-B<sub>235S/C246S</sub> mSP-B<sup>−/−</sup> mice were crossed with SP-B hemizygous mice (mSP-B<sup>+/−</sup>). Transgenic mice with a single copy of the endogenous SP-B allele (hSP-B<sub>235S/C246S</sub> mSP-B<sup>+/−</sup>) were identified by a PCR that is specific for the neomycin interrupted, murine SP-B allele (upstream primer 5′-CCACACGAGCAATCGCT and downstream primer 5′-CCAGTCGCTGGCGCCGGA). This PCR included primers specific for the endogenous murine SP-B allele and reaction conditions were 30 cycles, 60 °C annealing temperature, 0.5 μM neomycin primers, 0.25 μM mSP-B primers, 0.25 μM dNTPs, 2.5 μM MgCl<sub>2</sub>, 1 unit of display Taq (PGC Scientific) and 1× PCR Buffer II (Perkin Elmer, Foster City, CA). These mice (hSP-B<sub>235S/C246S</sub> mSP-B<sup>+/−</sup>) were subsequently crossed with SP-B hemizygous mice to generate offspring that expressed the transgene in the SP-B<sup>−/−</sup> null background (hSP-B<sub>235S/C246S</sub> mSP-B<sup>−/−</sup>).

Lung Morphology in Transgenic Animals—Immunostaining for surfactant proteins was performed exactly as described with antisera directed against the carboxy terminus of SP-B preproprotein, mature SP-B, and the amino terminus of SP-C proprotein (15). Type II cell ultrastructure was examined by electron microscopy as described (16).

Surfactant Protein mRNA Expression—SI nuclease mapping was performed similarly to that described (17). Briefly, the left lobe of four 4- to 6-week-old lungs (two lungs were used to generate homogenates in 4 ml guanidine isothiocyanate with 0.3 M 2-mercaptoethanol and total lung RNA isolated. SI probes specific for murine cytoplasmic β-actin, murine SP-C, murine SP-B, and human SP-B were radiolabeled with [γ-32P]ATP (17, 18) and hybridized with 3 μg of RNA at 55 °C overnight, followed by SI nuclease (Life Technologies, Inc.) digestion at room temperature for 1 h. Protected fragments were separated in a 6% polyacrylamide, 8% urea gel that was dried and subject to PhosphorImaging (Molecular Dynamics, Sunnyvale, CA). All PhosphorImaging data were analyzed in ImageQuant (Molecular Dynamics, Sunnyvale, CA) and hSP-B levels were normalized to cytoplasmic β-actin.

Surfactant Protein Synthesis in Lung Explant Culture—To determine if expression of the hSP-B<sub>235S/C246S</sub> transgene affected expression of endogenous surfactant proteins, synthesis of SP-A and SP-C was assessed in lung explant cultures from day 18 mouse fetuses. Offspring from timed pregnancies were harvested by cesarean section on day 18 after timed pregnancies were harvested by cesarean section on day 18 after. Lungs were quickly isolated and minced into 1-mm<sup>3</sup> pieces with a McIlwain lung tissue Chopper (Brinkman, Westbury, NY). Lung pieces were incubated (37 °C, room air, rotating at 8 rpm) in saline and methionine-deficient Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) for 40 min and then supplemented with 0.5 mM of Promix <sup>7</sup>LS-labeled cysteine and methionine (Amersham Pharmacia Biotech). Four hours later, tissues were harvested by gentle centrifugation (<750 × g), boiled for 4 min, and subjected to extensive sonication until all tissue had been dispersed. Tricarboxylic acid precipitable counts were estimated for tissue and media and equal disintegrations/min were immunoprecipitated sequentially with normal rabbit serum followed by antibodies directed against SP-C proprotein, mature SP-B and SP-A (SP-A antibody was a gift from Frank McCormack, University of Cincinnati). Immunoprecipitations were subject to SDS-PAGE and dried gels were analyzed by PhosphorImaging.
Ablation of SP-B Intramolecular Disulfide Bond

The results show that the intramolecular disulfide bond formed between SP-B residues 235 and 246 is essential for SP-B folding and/or sorting to the regulated secretory pathway, PC12 cells were transfected with the hSP-BC235S/C246S/GFP fusion construct in which both cysteine residues were mutated to serine and compared with cells transfected with wild type hSP-B/GFP. Transfected cells were radiolabeled with [35S]cysteine and serine and compared with cells transfected with wild type hSP-B/GFP. Transfected cells were immuno precipitated from the cell lysates (lanes 1–4) and media (lanes 5–8). Both mutant and wild-type proteins present in the media were Endo H resistant indicating that they had trafficked through the endoplasmic reticulum. GFP fluorescence in cells transfected with hSP-B C235S/C246S/GFP (a) colocalized with the dense core granule marker, chromogranin A (b). Panel c shows only those pixels in which GFP and chromogranin A are colocalized. A DIC image of the same cell is shown in d.

**RESULTS**

**Intracellular Trafficking of hSP-B C235S/C246S PC12 Cells**—Intramolecular sulfur bridges stabilize protein structure and in some instances provide information for intracellular sorting (19–21). To determine if the sulfhydryl bridge formed between SP-B residues 235 and 246 is essential for SP-B folding and/or sorting to the regulated secretory pathway, PC12 cells were transfected with the hSP-B C235S/C246S/GFP fusion construct in which both cysteine residues were mutated to serine and compared with cells transfected with wild type hSP-B/GFP. Transfected cells were radiolabeled with [35S]cysteine and [35S]methionine followed by immunoprecipitation of cell lysates and media with SP-B antiserum and treatment with endoglycosidase H (Endo H). Both wild-type and mutant SP-B/GFP fusion proteins were detected in the media as Endo H-resistant proteins were localized by confocal microscopy 24 h after transfection of PC12 cells. GFP fluorescence partially colocalized with chromogranin A in cells transfected with wild-type (not shown) or mutant (Fig. 1B) SP-B/GFP constructs, consistent with sorting of hSP-B C235S/C246S/GFP to dense core secretory granules of the regulated secretory pathway. For both wild-type and mutant constructs, there were populations of SP-B/GFP and chromogranin A proteins which did not colocalize. In addition, the fluorescence intensity in dense core granules was consistently higher in cells transfected with the wild-type SP-B construct than with the mutant construct, while there appeared to be increased fluorescence in the Golgi region of cells transfected with hSP-B C235S/C246S/GFP. While the kinetics of SP-B/GFP trafficking may have been altered in cells transfected with the mutant peptide, detection of hSP-B C235S/C246S/GFP in dense core secretory granules and media strongly suggested that the Cys235/246 sulfhydryl bridge was not critical for sorting and secretion of SP-B/GFP and that ablation of this cysteine bridge would not alter the intracellular transport of SP-B in vivo.

**Generation and Characterization of hSP-B C235S/C246S Transgenic Mice**—To identify the function of the Cys235/246 sulfhydryl bridge in vivo, the hSP-B C235S/C246S construct was expressed in transgenic mice. hSP-B C235S/C246S expression was directed to the distal respiratory epithelium using the 3.7-kb human SP-C promoter to recapitulate the endogenous expression pattern for SP-B (Fig. 2A). Seven of 20 (35%) offspring from fertilized oocyte injections were transgene positive, as identified by both PCR and Southern blot analyses of tail DNA (not shown). These seven animals were referred to as transgenic founders H through N and were bred with wild-type FVB/N mice to establish eight separate transgenic lines. Transgenic line I carried two insertion sites that segregated independently during four consecutive crosses with wild-type littersmates to produce transgenic lines 1a and 1b. Western analyses of total lung homogenates were performed on offspring from transgenic animals using antibodies that detected antigenic epitopes in the mature peptide (M, ~42,000) or NH2-terminal propeptide of SP-B (M, ~42,000). SP-B propeptide was readily detected in transgene positive animals but was not detected in wild-type mice, consistent with expression of human SP-B (Fig. 2B). Transgenic RNA and protein was detected in seven of eight transgenic lines (lines H, 1a, Ib, K, L, M, and N).

To determine if the mutant protein reversed the neonatal lethal phenotype in SP-B−/− mice, mice expressing the hSP-B C235S/C246S transgene were bred with SP-B hemizygous (mSP-B+/−) mice to generate transgenic animals carrying one functional endogenous SP-B allele (hSP-B C235S/C246S, mSP-B+/−). These animals were subsequently crossed with mSP-B+/− animals in order to achieve expression of the hSP-B C235S/C246S transgene in the null background (hSP-B C235S/C246S, mSP-B−/−). Despite the fact that one of every seven surviving offspring...
Ablation of SP-B Intramolecular Disulfide Bond

Five separate hSP-B<sub>C235S/C246S</sub> transgenic lines were bred into the mSP-B<sup>−/−</sup> background to determine if hSP-B<sub>C235S/C246S</sub> could reverse the neonatal lethal SP-B<sup>−/−</sup> phenotype. One of every seven offspring was predicted to be hSP-B<sub>C235S/C246S</sub>, mSP-B<sup>−/−</sup> (33 of 231 mice). No surviving hSP-B<sub>C235S/C246S</sub>, mSP-B<sup>−/−</sup> offspring were identified, which indicated that hSP-B<sub>C235S/C246S</sub> was unable to functionally replace endogenous mSP-B.

| Total offspring | H | Ia | Ib | L | M | Total |
|-----------------|---|----|----|---|---|-------|
| Expected, hSP-B<sub>C235S/C246S</sub>, mSP-B<sup>−/−</sup> | 35 | 46 | 72 | 44 | 34 | 231 |
| Observed, hSP-B<sub>C235S/C246S</sub>, mSP-B<sup>−/−</sup> | 0 | 0 | 0 | 0 | 0 | 0 |

From these crosses should have expressed human SP-B in the absence of murine SP-B, no animals of this genotype (hSP-B<sub>C235S/C246S</sub>, mSP-B<sup>−/−</sup>) were Endo H sensitive, consistent with trapping of the mutant protein in the endoplasmic reticulum. One possible explanation for the failure to process the hSP-B<sub>C235S/C246S</sub> protein is that ablation of the Cys<sup>235</sup>/Cys<sup>246</sup> sulfhydryl bridge resulted in retention of the proprotein in the endoplasmic reticulum. To test whether hSP-B<sub>C235S/C246S</sub> proprotein was trapped in the endoplasmic reticulum, SP-B trafficking was characterized in explant tissue from fetal lungs from wild-type and transgenic littermates (Fig. 4). Newly synthesized SP-B proprotein was detected in wild-type lung explants, but was rapidly processed, resulting in low levels of SP-B, M<sub>b</sub> ~ 42,000 (Fig. 4, lanes 1–4); in contrast, newly synthesized proprotein in hSP-B<sub>C235S/C246S</sub> lung explants was not processed and accumulated (Fig. 4, lanes 7–10). SP-B proprotein in hSP-B<sub>C235S/C246S</sub> mice was Endo H resistant, whereas virtually all SP-B proprotein in wild-type mice was Endo H resistant. These findings are consistent with retention of hSP-B<sub>C235S/C246S</sub> protein in the endoplasmic reticulum and suggested that, unlike the hSP-B<sub>C235S/C246S</sub>/GFP fusion protein, hSP-B<sub>C235S/C246S</sub> failed to exit the endoplasmic reticulum.

Transgenic-dependent Mortality in Mice Expressing hSP-B<sub>C235S/C246S</sub>—While characterizing the hSP-B<sub>C235S/C246S</sub> mice, we observed that transgenic lines with higher expression levels of mutant hSP-B protein had lower numbers of surviving transgenic offspring. To determine if transgene RNA levels were inversely correlated with survival, total lung RNA was prepared for S1 nuclease analysis of hSP-B<sub>C235S/C246S</sub> mRNA expression (Fig. 5). Transgenic lines expressing undetectable (line J) or low levels of hSP-B<sub>C235S/C246S</sub> mRNA (lines H and Ia) transmitted the transgene at rates approaching the expected Mendelian frequency of 50%. A transgenic line that expressed intermediate levels of mutant hSP-B (line L) had lower transmission rates, while a line with higher levels of hSP-B<sub>C235S/C246S</sub> mRNA (line M) had very few surviving offspring. Furthermore, in two other lines (N and K) no surviving transgenic offspring were detected. Dead newborn pups recovered from these lines were transgenic and expressed higher levels of hSP-B<sub>C235S/C246S</sub> protein (by Western analysis) relative to the

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immunoblotting. Mice that expressed both hSP-BC235S/C246S peptide, tissue homogenates from fetal lungs were analyzed by gene altered levels of endogenous SP-B proprotein or mature M-derived samples migrated with nonreducing conditions (Fig. 7, lane 1). SP-B proprotein oligomers were never detected. In mice expressing the hSP-B C235S/C246S transgene (lanes 2–4), oligomers of SP-B were consistently identified.

Other transgenic lines (Fig. 2B and data not shown). Two transgenic offspring from the N line observed at birth suffered lethal neonatal respiratory distress within the first 30 min, while nine wild-type littermates survived the neonatal period, strongly suggesting that expression of the transgene in this line was incompatible with life. The N and K founders were likely mosaic animals whose lungs were comprised largely of non-transgenic cells. This conclusion was supported by Western and immunohistochemical analyses on lungs from these founders, which demonstrated low levels of hSP-B C235S/C246S protein and very few positively staining cells in the distal respiratory epithelium, respectively (not shown). In contrast, fetal lungs from line N transgenic offspring stained intensely throughout the distal respiratory epithelium for human SP-B (not shown).

The inverse correlation between transgene expression and survival suggested that hSP-B C235S/C246S proprotein might interfere with the normal processing of wild-type SP-B proprotein. To determine if expression of the hSP-B C235S/C246S transgene altered levels of endogenous SP-B proprotein or mature peptide, tissue homogenates from fetal lungs were analyzed by immunoblotting. Mice that expressed both hSP-B C235S/C246S and wild-type protein (hSP-B C235S/C246S, mSP-B+/+ or hSP-B C235S/C246S, mSP-B+/−) had much lower levels of mature mSP-B peptide than their non-transgenic littermates (Fig. 6). These results, coupled with the accumulation of Endo H-sensitive hSP-B C235S/C246S in the endoplasmic reticulum (Fig. 4), suggested that the mutant protein might impede trafficking of wild-type proprotein to the multivesicular body where proteolytic processing to the mature peptide occurs. Consistent with this hypothesis, higher molecular weight species of SP-B proprotein were consistently detected in transgenic lungs but were never detected in wild-type lungs (Fig. 7). Two-dimensional gel electrophoresis, in which the first dimension was run under nonreducing conditions (Fig. 7, lanes 2–4) and the second under reducing conditions, revealed that the higher molecular weight forms of pro-SP-B (Mₚ = 80,000 and 120,000) in nonreduced samples migrated with Mₑ = 42,000 under reducing conditions (not shown). It is conceivable that oligomers may form between wild-type and mutant SP-B proproteins, thereby preventing the wild-type protein from exiting the endoplasmic reticulum.

Alternatively, the dominant negative phenotype may have resulted from a generalized decrease in surfactant protein synthesis in transgenic animals or from changes in lung morphogenesis. To test the first possibility, SP-A and SP-C protein synthesis levels were assessed by metabolic labeling of fetal lungs. No differences were detected in SP-A and SP-C protein synthesis rates between transgenic and non-transgenic lungs (not shown). It is therefore unlikely that expression of the transgene resulted in a global decrease in surfactant protein synthesis. To determine if lung structure was altered in transgenic mice, lungs of transgenic and wild-type fetal day 18.5 littermates were analyzed. Histological analyses and immunohistochemistry with anti-SP-C antibody revealed no overt changes in lung structure (not shown). Taken together, these data suggest that hSP-B C235S/C246S protein interferes with processing of wild-type SP-B protein but does not affect the synthesis of other surfactant proteins or overall lung structure.

DISCUSSION

The SP-B mature peptide contains three intramolecular sulfhydryl bridges which are conserved in all members of the SAPLIP family. To test the function of the bridge between cysteine residues 235 and 246 of SP-B, hSP-B C235S/C246S was first expressed as a fusion protein with GFP in PC12 cells. The fusion protein was secreted in an Endo H-resistant form and was detected in the dense core granules of PC12 cells by confocal microscopy, consistent with targeting of hSP-B C235S/C246S to the regulated secretory pathway. To test the function of Cys²³⁵/²⁴⁶ bridge in vitro, hSP-B C235S/C246S was expressed in the distal respiratory epithelium of SP-B−/− mice. Replacement of endogenous mSP-B with hSP-B C235S/C246S failed to reverse the neonatal lethality of SP-B null mice. This result indicates that the cysteine 235/246 bridge is essential for normal SP-B function. Closer analyses revealed that mutation of the Cys²³⁵/²⁴⁶ bridge resulted in an SP-B molecule that was unable to exit the endoplasmic reticulum in vivo. The proposed function of this bridge is to stabilize a hairpin turn motif in the SP-B mature peptide. Thus, it is likely that stabilization of this bridge is necessary for the correct folding of SP-B in vivo.

The discrepancy between the in vitro and in vivo trafficking of the hSP-B mutant could be attributed to fusion with the GFP reporter protein. GFP has been widely used to study the intracellular localization of proteins, including several studies of proteins targeted to the regulated secretory pathway (22–24). Although GFP did not prevent correct intracellular sorting in these studies, a recent report indicated that the fusion of yeast secretory proteins to GFP resulted in aberrant targeting of constitutively secreted molecules to the yeast vacuole (25). In the present study mutant SP-B/GFP may have circumvented the protein quality control mechanisms in the endoplasmic
reticulum due to a chaperone effect of the GFP molecule. Given these results, caution should be exercised in utilizing GFP as a marker for mutant secreted proteins.

The observation that the hSP-B<sup>C235S/C246S</sup> mutant protein failed to reverse the neonatal lethal SP-B null phenotype is consistent with the recent clinical finding that a mutation in this bridge in a compound heterozygote human infant was incompatible with life. A single base pair change that resulted in substitution of arginine for cysteine at position 235 was described in an infant who was a compound heterozygote at the SP-B locus. The second SP-B allele carried the most common mutation in hereditary SP-B deficiency, which involves an insertion of 2 base pairs in codon 121 (121ins2), resulting in a frameshift and no SP-B proprotein in the lungs (26). This infant suffered from neonatal respiratory distress that was not responsive to surfactant replacement therapy and succumbed in the perinatal period. SP-B proprotein was detected in the lungs by immunohistochemistry using an antibody to the COOH-terminal domain of the SP-B propeptide, indicating that full-length SP-B proprotein was synthesized. The results of the current study suggest that respiratory distress syndrome in this infant was the consequence of an inability to generate mature SP-B peptide related to trapping of the propeptide in the endoplasmic reticulum.

Production of mutant hSP-B<sup>C235S/C246S</sup> protein resulted in dose-dependent lethality. Transgenic lines that produced low levels of hSP-B<sup>C235S/C246S</sup> had near-normal survival rates, whereas lines that expressed intermediate levels of hSP-B<sup>C235S/C246S</sup> had significantly decreased survival rates, and lines that expressed high levels of hSP-B<sup>C235S/C246S</sup> had low levels or no surviving offspring. This is the first example in which overproduction of mutant SP-B protein in wild-type mice resulted in phenotypic abnormalities. In transgenic mice expressing high levels of hSP-B<sup>C235S/C246S</sup> protein, there was a marked reduction in the amount of wild-type SP-B mature peptide. Sulphydryl-dependent oligomers of SP-B proprotein were also detected in these animals, indicating that the mutant protein contained exposed cysteine residues not normally available for intermolecular disulfide bonding of SP-B protein. It is possible that the dominant negative phenotype in moderate levels or no surviving offspring. This is the first example in which overproduction of mutant SP-B protein in wild-type mice resulted in decreased survival in the neonatal period. These data suggest that mutations in the cysteine residues that make up the intramolecular sulphydryl bridges of SP-B may predispose individuals to clinical disease even in the presence of a second wild-type allele.

In summary, hSP-B<sup>C235S/C246S</sup> protein did not reverse the neonatal lethal respiratory failure in SP-B gene targeted mice. hSP-B<sup>C235S/C246S</sup> protein failed to exit the endoplasmic reticulum and was likely misfolded. Overproduction of hSP-B<sup>C235S/C246S</sup> protein in wild-type mice decreased the concentration of mature SP-B protein and resulted in diminished survival in the neonatal period. These data suggest that mutations in the cysteine residues that make up the intramolecular sulphydryl bridges of SP-B may predispose individuals to clinical disease even in the presence of a second wild-type allele.

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2 L. Nogee, unpublished data.
Ablation of a Critical Surfactant Protein B Intramolecular Disulfide Bond in Transgenic Mice

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