Rescue of SP-B Knockout Mice with a Truncated SP-B Proprotein

FUNCTION OF THE C-TERMINAL PROPEPTIDE

(Received for publication, November 13, 1996, and in revised form, January 23, 1997)

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The function of the 102-amino acid C-terminal propeptide of surfactant protein B (SP-B) was analyzed by characterizing the phenotype associated with loss of expression of this peptide domain in transgenic mice. A construct encoding the signal peptide, N-terminal propeptide, and mature peptide of human SP-B (hSP-Bc) was cloned under the control of the 3.7-kilobase human SP-C promoter and injected into fertilized eggs of the FVB/N mouse strain. Founder mice expressing the hSP-Bc transgene were bred with heterozygous SP-B knockout mice (SP-B+/−). Offspring containing the transgene and one allele of mouse SP-B were identified and subsequently crossed to generate a transgenic line that expressed SP-Bhc in a null background (SP-B(−/−)/hSP-Bhc(+/+)). Expression of hSP-Bhc in SP-B(−/−) mice was restricted to type II cells and resulted in a 2-fold increase in mature SP-B relative to wild type littermates. These mice survived without any evidence of respiratory problems and had normal lung function, normal alveolar surfactant phospholipid pool sizes, and typical tubular myelin indicating that the 102-residue C-terminal propeptide of SP-B is not required for normal structure and function of extracellular surfactant. However, proteolytic processing of the SP-C proprotein was perturbed resulting in the accumulation of a processing intermediate, Mw = 11,000, similar to the phenotype detected in SP-B(−/−) mice; furthermore, lamellar bodies in type II cells of SP-B(−/−)/hSP-Bhc(+/+)) mice were much larger than in the wild type animal and saturated phosphatidylcholine content in lung tissue was significantly increased although the incorporation of choline into saturated phosphatidylcholine was normal. Collectively, these results demonstrate a role for the C-terminal propeptide of SP-B in SP-C proprotein processing and the maintenance of lamellar body size. The C-terminal propeptide may be an important determinant of intracellular surfactant pool size.

Pulmonary surfactant is a complex mixture of lipids and proteins that is synthesized exclusively by the alveolar type II epithelial cell. Surfactant is stored in large inclusions (lamellar bodies) that are secreted into the alveolar airspace by exocytosis. Newly secreted lamellar bodies unravel into a tubular network (tubular myelin) that subsequently adsorbs and spreads as a phospholipid-rich film that reduces surface tension at the air-liquid interface. Rapid adsorption and spreading of the phospholipid film are critical and require the presence of specific proteins, in particular surfactant proteins B and C (1–3).

Surfactant protein B (SP-B)2 is a hydrophobic peptide of 79 amino acids that avidly associates with surfactant phospholipids in the alveolar airspace (reviewed in Ref. 4). Homozygous mutations leading to the complete absence of SP-B in newborn human infants result in the rapid onset of respiratory distress syndrome which is refractory to mechanical ventilation and surfactant replacement (5, 6). The latter observation suggests that simple addition of mature peptide to the SP-B-deficient airway is not sufficient to restore lung function. Therefore, despite an obvious requirement for SP-B in normal lung function, the precise role(s) of SP-B in the structure, function, and metabolism of surfactant remains unclear.

Human SP-B is synthesized by the type II epithelial cell as a preproprotein of 381 amino acids. The mature peptide is generated by sequential cleavage of the signal peptide (23 amino acids), the N-terminal propeptide (177 residues), and the C-terminal propeptide (102 residues) (7, 8). Propeptide cleavage occurs within the late endosome/multivesicular body which subsequently directs newly synthesized SP-B to the lamellar body (9); mature SP-B is also recycled from the alveolar airspace, via the endocytic pathway to the multivesicular body (10). Previous in vitro evidence supports the concept that trafficking of the mature peptide through the biosynthetic pathway requires the N-terminal but not the C-terminal domain of the propeptide (11, 12). In the absence of both propeptide domains the mature peptide is degraded within the endoplasmic reticulum (11); however, an SP-B construct encoding both the N-terminal propeptide and the mature peptide (hSP-Bhc) produces a truncated proprotein that is sorted to the multivesicular body where the propeptide is appropriately cleaved to liberate mature SP-B (12). These results clearly demonstrate that the N-terminal propeptide is required for the intracellular trafficking of SP-B; the function of the C-terminal propeptide, however, remains unknown.

Lung morphogenesis and surfactant phospholipid synthesis in SP-B(−/−) mice proceed normally prior to birth (13). However, lamellar body formation is disrupted, resulting in abnormally inclusions consisting of multivesicular bodies and disorganized lamellae. Neither mature lamellar bodies nor tubular myelin were detected in the SP-B(−/−) mice. In addition to effects on lamellar body biogenesis, SP-B deficiency resulted in

6 This work was supported by National Institutes of Health Grants HL36055 and HL56285 (to T. E. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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1 The abbreviations used are: SP-B, surfactant protein B; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; Sat PC, saturated phosphatidylcholine.
aberrant processing of the SP-C proprotein leading to accumulation of an M, 11,000 processing intermediate and a decrease in mature SP-C peptide levels. The purpose of the present study was to assess the function of the C-terminal propeptide of SP-B by expressing hSP-BΔc in SP-B(-/-) mice and determining which aspects of the SP-B(-/-) phenotype were not corrected by transgene expression.

Materials and Methods

Generation and Identification of SP-B(-/-)/hSP-BΔc(+/-) Mice

FVB/N transgenic mice expressing a transgene construct encoding the signal peptide, 1.3-kilobase proximal promoter, and mature peptide of human SP-B (hSP-BΔc) under the control of the 3.7-kilobase human SP-C promoter were generated as described previously (12). Two of four transgenic mouse lines were used in the present study. Mice from transgenic Lines 6.1 and 1.4 were bred with hemizygous 129J x Swiss Black SP-B mice generated by gene targeting (10). Offspring bearing one wild type mouse SP-B allele and the hSP-BΔc transgene were identified as described below, and siblings crossed to generate mice expressing hSP-BΔc in a null background (SP-B(-/-)/hSP-BΔc(+/-)). hSP-BΔc transgenic mice were identified by PCR amplification of a 600-base pair fragment, spanning the junction of the human SP-C promoter and human SP-B cDNA, as described previously (12). SP-B(+/-) genotypes were identified by amplification of a 1.3-kilobase fragment spanning the neomycin resistance gene intron 4 of the targeted SP-B gene. One μl each of a 5' primer specific for the neomycin resistance gene (5' ATGTCCTCCTGGTTGTTGGTGTTGTG) and a 3' primer specific for the SP-B gene (5' GTTGGGAGGGCTATTTGCCGTATGA) were incubated with 100 ng of genomic DNA in 2.5 μl MgCl₂, 50 μl Tris-HCl (pH 7.6), 50 μl KCl, 100 μM dNTPs, and 0.5 units of AmpliTaq (Perkin-Elmer) for 2 min at 94 °C, followed by a 30-cycle PCR amplification comprised of 94 °C denaturation for 30 s, 63 °C annealing for 30 s and 2 min of elongation at 72 °C. The SP-B(-/-) genotype was identified by the absence of a 260-base pair fragment spanning introns 3 and 4 of the mouse gene SP-B following amplification with 1 μl each of 5' primer (5' CTTCTCTGTCTTCTTGGCAGGA) and 3' primer (5' CGGTGTGCCGACATGTGAGTGTCATACCTG) using the PCR conditions described above. The SP-B(-/-) genotype was confirmed by Southern blotting of BamHI-restricted genomic DNA with a species-specific mouse SP-B cDNA and neomycin resistance probes as described previously (13).

SP-B RNA and Protein Analyses—RNAs encoding mouse and human SP-B were analyzed by S1 nuclease assays using 32P-end-labeled, species-specific probes and 2 μg of total RNA as described previously (14). Total SP-B protein was analyzed by ELISA (15) and Western blotting (11, 12), using equal amounts of protein from lung homogenates of 6–7-week-old mice of each genotype. Mature SP-B, M, ~18,000, and SP-C, M, ~4,000, were separated by SDS-polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulfate and detected by blotting with antiserum 28031 (11), which detects only SP-B, or antisera 5530, which detects both SP-B and SP-C. Proteins that were detected by antiserum 68514 (16) which recognizes antigenic determinants in the 20-amino acid N-terminal propeptide of SP-C.

Surfactant Metabolic Measurements—SP-B(-/-)/hSP-BΔc(+/-) mice and FVB/N mice (wild type) at 7–8 weeks of age were used for measurements of saturated phosphatidylcholine (Sat PC) pool size and choline incorporation in vivo. Wild type and SP-B(-/-)/hSP-BΔc(+/-) mice were given 8 μg/g body weight intraperitoneal injections of 0.2 μCi/μl [3H]choline chloride (DuPont NEN). Groups of 5–7 wild type and SP-B(-/-)/hSP-BΔc(+/-) mice were killed, and an alveolar lavage was recovered from each animal at five preselected times from 3 to 48 h after isotope injection based on our previous experiences with precursor labeling in mice (17). Mice were given intraperitoneal pentobarbital to achieve deep anesthesia, and the distal aorta was cut to exanguinate each animal. The chest of the animal was opened; a 20-gauge blunt needle was tied into the proximal trachea; 0.9% NaCl was instilled into the airways until the lungs were inflated, and the fluid was withdrawn by syringe. The lavage procedure was repeated four times, and samples from each lung were pooled for analysis. Lung tissue after alveolar lavage was weighed and homogenized in 2 ml of 0.9% NaCl. Alveolar lavage fluid and lung homogenates were extracted with chloroform:methanol (2:1) and Sat PC was isolated (18). The Sat PC was divided for measurement of phosphorous (19) and radioactivity. The total radioactivity recovered in Sat PC for the alveolar lavage, lung tissue, and total lung (alveolar and tissue) of each animal was calculated. Percent secretion of labeled Sat PC was calculated as the radioactivity in alveolar Sat PC divided by the total radioactivity in the alveolar lavage plus lung tissue × 100. Pool sizes of Sat PC in alveolar lavage, lung tissue, and total lung were calculated as μmol per kg body weight. The DNA measurements for lung tissue were according to Hill and Whatley (20). For measurements of phospholipid composition, chloroform:methanol extracts of pooled alveolar lavages from 2 SP-B(-/-)/hSP-BΔc(+/-) mice and 3 wild type mice were used for two-dimensional thin layer chromatography (21). The spots were visualized with iodine vapor, scraped, and assayed for phosphorous content (19).

Analysis of Lung Function—Static lung compliance was measured in 12 SP-B(+/-)/hSP-BΔc(+/-), 8 SP-B(-/-)/hSP-BΔc(+/-), and 10 SP-B(-/-)/hSP-BΔc(+/-) mice at 6–7 weeks of age. Mice were injected intraperitoneally with sodium pentobarbital (200 mg/kg) and placed in a chamber containing 100% O₂. In this manner, the lungs were completely deflated at the time of death (22). A catheter (20-gauge angiocatheter) was inserted into the trachea and connected to a silicon pressure sensor (X-ducer, Motorola, Phoenix, AZ). The chest wall and diaphragm were opened carefully to avoid injuring the lungs. The lungs were inflated with air in small increments (0.1 ml every 5–8 s) to a maximal pressure of 40 cm H₂O. Lungs were deflated in stepped fashion. Airway opening pressure and lung volume were recorded at each inflation and deflation increment. All measurements were completed within 15 min after death. Pressure-volume curves were generated for each animal. Lung compliance was determined by calculating the slopes and intercepts of the straight portion of the deflation curve. Pressure ranged from -10 to +10 cm H₂O (23). Specific lung compliance was calculated as lung compliance divided by the lung weight.

In Situ Hybridization—Mature sense and antisense riboprobes were synthesized as described previously (24); human riboprobes were synthesized from full-length SP-B cDNA (25) subcloned from pkc4 as an EcoRI fragment into the EcoRI site of pGEM3Z (26). In situ hybridization was performed under stringent conditions as reported by Damore-Bruno et al. (24) with the addition of an extensive post-hybridization washing schedule (26) to minimize possible cross-reactivity between the mouse and human probes.

Electron Microscopy—Transgenic and control lungs from 6-week-old mice were fixed by tracheal infusion (27) with 1.5% paraformaldehyde, 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Following overnight immersion in the same fixative, lungs were minced into 1-mm cubes and processed for electron microscopy as described by Fehrenbach et al. (28). Briefly, after fixation, blocks were rinsed for 1 h in 4–6 changes of 1.0 mM sodium cacodylate buffer (pH 7.3) (SCB), post-fixed 2–3 h in 1% OsO₄ in SCB, rinsed several times in SCB and several times in distilled H₂O, and incubated overnight in 4% aqueous uranyl acetate. After several rinses in distilled H₂O, blocks were dehydrated through a graded series of ethanols and transferred to Epon 812 (Shell Chemical Co.) via propylene oxide. Ultrathin sections (60–90 nm) were prepared, counterstained with lead citrate, and evaluated under a Jeol-100-CXII transmission electron microscope.

Statistical Analysis—For the lung function studies, differences among normal animals were evaluated by comparing lung compliances, specific lung compliances, intercepts, and inflation lung volumes at 30 cm of H₂O by two-way analysis of variance; significant differences between mean values was determined by Bonferroni/Dunn test. Differences between groups of animals in surfactant metabolic studies and ELISA analysis were tested by two-tailed Student’s t test. Values are expressed as mean ± S.E.

Results

Rescue of SP-B(-/-) Mice by hSP-BΔc

Generation of transgenic mouse lines expressing truncated human SP-B (hSP-BΔc, in which the sequence encoding the 102-residue C-terminal propeptide was deleted) in type II epithelial cells has been previously described (12). Since these mice expressed both endogenous SP-B and the human SP-BΔc transgene, there was a 3–4-fold increase of mature SP-B peptide in lung homogenates of animals that were homozygous for the transgene. Surfactant composition and phospholipid pool sizes, static compliance and type II ultrastructure were all normal in adult SP-BΔc transgenic mice, indicating that increased levels of SP-B did not perturb surfactant homeostasis or lung function. hSP-BΔc transgenic mice (line 6.1) were bred with SP-B(-/-) mice and offspring that were homozygous for hSP-BΔc and heterozygous for the mouse SP-B...
allele (SP-B<sup>+/−</sup>/hSP-B<sub>c</sub><sup>+/−</sup>) were identified by PCR. These mice were subsequently interbred to achieve expression of hSP-B<sub>c</sub> in the null background (SP-B<sup>−/−</sup>/hSP-B<sub>c</sub><sup>+/−</sup>) (Fig. 1). Of 105 offspring screened, 21 were identified as both mSP-B<sup>−/−</sup> and hSP-B<sub>c</sub><sup>+/−</sup>, consistent with Mendelian inheritance of a recessive allele and postnatal survival of all animals carrying the hSP-B<sub>c</sub> transgene. In the absence of the transgene no SP-B<sup>−/−</sup> offspring were detected confirming the perinatal lethality associated with ablation of the SP-B locus (13) and the ability of hSP-B<sub>c</sub> to rescue these animals. “Rescued” SP-B<sup>−/−</sup> mice were indistinguishable from wild type littersmates with respect to body weight, lung weight, reproductive capacity, and longevity, indicating that the C-terminal propeptide of SP-B is not essential for survival.

A separate transgenic line (line 1.4) in which the hSP-B<sub>c</sub> transgene was expressed at approximately 1% of wild type SP-B was also bred into the SP-B knockout background. Of 60 offspring from 6 litters no rescued SP-B<sup>−/−</sup> survivors were detected by PCR analyses. To confirm transgene transmission in this line, 2 litters were delivered on day 19 of gestation and analyzed for genotype by PCR. Of 15 offspring, 5 were both transgene-positive and homozygous for the disrupted SP-B allele. In contrast to wild type littersmates, these animals rapidly succumbed to respiratory failure, as described previously for SP-B knockout mice (13), indicating that the level of hSP-B<sub>c</sub> transgene expression in the 1.4 line was not sufficient to rescue SP-B<sup>−/−</sup> offspring.

**Characterization of hSP-B<sub>c</sub> Transgene Expression in Rescued SP-B<sup>−/−</sup> Mice**

S1 nuclease analyses of lung tissues from SP-B<sup>−/−</sup>/hSP-B<sub>c</sub><sup>+/+</sup> mice detected only human SP-B RNA confirming the complete absence of mouse SP-B in these animals (Fig. 2). These results were corroborated by in situ hybridization studies (Fig. 3) in which only human SP-B RNA was detected in SP-B<sup>−/−</sup>/hSP-B<sub>c</sub><sup>+/+</sup> mice. Expression of hSP-B<sub>c</sub> RNA was restricted to alveolar type II epithelial cells in contrast to wild type littersmates in which endogenous SP-B RNA was expressed in both type II cells and nonciliated bronchiolar cells (Clara cells). The expression of hSP-B mRNA in type II cells

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**Fig. 1. Identification of SP-B<sup>−/−</sup>/hSP-B<sub>c</sub><sup>+/−</sup> mice.** Gel electrophoresis analysis of products of PCR amplification using primers to exons 3 and 4 of mSP-B and 50 ng of genomic DNA isolated from offspring of hSP-B<sup>−/−</sup>/hSP-B<sub>c</sub><sup>+/−</sup> transgenic mice (line 6.1) crossed into the SP-B<sup>−/−</sup> background. The 290-base pair fragment represents the amplification product from an intact wild type (WT) mSP-B allele. Animals in lanes 1, 3, 6, and 7 contain two disrupted SP-B alleles; these mice also carry the hSP-B<sub>c</sub> transgene (not shown). Thyroid-stimulating hormone β (TSHβ) was co-amplified as an internal control. Control DNA from an offspring of hSP-B<sup>−/−</sup> mice is indicated by C, bp, base pair; M, DNA size marker.

**Fig. 2. S1 nuclease assay for hSP-B<sub>c</sub> RNA and mSP-B mRNA.** 2 μg of total RNA from offspring of hSP-B<sub>c</sub> transgenic mice (line 6.1) crossed into the SP-B<sup>−/−</sup> background were assayed for human and mouse SP-B using species-specific 32P-labeled probes that protect a 186-nucleotide fragment of hSP-B RNA and a 158-nucleotide fragment of mSP-B RNA. * denotes 20 nt fragment of hSP-B RNA and a 186-nucleotide fragment of mSP-B RNA. A separate transgenic line (line 1.4) in which the hSP-B<sub>c</sub> transgene no SP-B<sub>c</sub> offspring were detected confirming the absence of mouse SP-B in these animals (Fig. 2).

**Fig. 3. In situ analyses of lung tissues from a wild type littermate (A and C) and an SP-B<sup>−/−</sup>/hSP-B<sub>c</sub><sup>+/+</sup> mouse (B and D).** Lungs were harvested from 6-week old mice and in situ hybridization performed using 32S-labeled probes specific for either mouse SP-B (A and B) or human SP-B (C and D) as described. Arrow indicates bronchiolar epithelium; arrowhead indicates alveolar type II epithelial cells. Magnification, 1397 ×.
was consistently observed in 12 SP-B(-/-)hSP-Bc(+/+) animals of different ages from six separate litters. Quantitation of SP-B protein by ELISA indicated that the level of mature peptide in bronchoalveolar lavage fluid was elevated approximately 2-fold in SP-B(-/-)hSP-Bc(+/+) mice relative to wild type littermates (Fig. 4A). Western blot analyses of lung homogenates from SP-B(-/-)hSP-Bc(+/+) demonstrated that all of the hSP-Bc precursor was fully processed to the mature 8-kDa peptide (Fig. 4B). In contrast to normal processing of hSP-Bc, the SP-C proprotein was aberrantly processed resulting in the accumulation of an 11-kDa intermediate as previously reported in SP-B(-/-) mice and human infants (5, 13) (Fig. 5B). However, unlike SP-B(-/-) mice, levels of the mature SP-C peptide, M, = 5000, did not appear to be decreased.

Characterization of Lung Structure in Rescued SP-B(-/-) Mice

Previous ultrastructural analyses of lung tissue from hSP-Bc(+/+) transgenic mice (line 6.1) indicated that type II cell structure was not affected by expression of hSP-Bc or increased levels of SP-B mature peptide (12). In contrast, expression of hSP-Bc in SP-B(-/-) mice resulted in the formation of greatly enlarged lamellar bodies (Fig. 6C) containing normal concentric lamellar membranes in approximately 20% of type II cells. Closely apposed lamellar bodies were detected in all type II cells of transgenic mice. Typical tubular myelin profiles were readily detected in SP-B(-/-)hSP-Bc(+/+) mice indicating that SP-Bc was sufficient to restore this ultrastructural feature of extracellular surfactant in SP-B(-/-) mice (not shown). Other ultrastructural abnormalities were not detected in the lungs of SP-B(-/-)hSP-Bc(+/+) mice, and ultrastructural features of nonciliated bronchiolar cells were indistinguishable from Clara cells in wild type mice.

Characterization of Lung Function in Rescued SP-B(-/-) Mice

Surfactant Pool Sizes and Phospholipid Composition—Intra- and extracellular pool sizes of Sat PC were estimated in 32 SP-B(-/-)/hSP-Bc(+/+) mice and 36 wild type mice from...
preincorporation studies (Fig. 7). The alveolar Sat PC pool size, estimated from alveolar lavage samples, was similar in the two groups. The lung tissue pool of Sat PC was increased 35% in SP-B(-/-)hSP-B\(_{A1}(+)/+\) mice compared with wild type mice, and the amount of Sat PC in total lung was increased 23% (calculated as the sum of the alveolar and tissue Sat PC). The percent of the total lung Sat PC recovered by alveolar lavage was 20.9 ± 0.4% in SP-B(-/-)hSP-B\(_{A1}(+)/+\) mice and 27.7 ± 0.5% in wild type mice (p < 0.0001) consistent with increased size and number of lamellar bodies noted in the ultrastructural studies. There were no differences between the two groups of mice in body weight (SP-B(-/-)hSP-B\(_{A1}(+)/+\)) mice: 23.8 ± 0.5 g, wild type: 22.4 ± 0.2 g) and total lung DNA (SP-B(-/-)hSP-B\(_{A1}(+)/+\): 997 ± 37 μg, wild type: 923 ± 49 μg). The compositions of the phospholipids recovered by alveolar lavage were similar in the two groups of mice (Table I).

Precursor Incorporation and Secretion—The incorporation of radiolabeled choline (Fig. 8), into lung phospholipid, was measured as total incorporation into Sat PC. \(^{3}H\)Choline incorporation into Sat PC was similar in SP-B(-/-)hSP-B\(_{A1}(+)/+\) and control mice. The loss of labeled Sat PC after precursor injection, measured at 24 and 48 h, indicated that \(^{3}H\)choline-labeled Sat PC was significantly higher in lung tissue of SP-B(-/-)hSP-B\(_{A1}(+)/+\) mice. The percent choline-labeled Sat PC secreted to the alveolar space was decreased by about 20% in SP-B(-/-)hSP-B\(_{A1}(+)/+\) mice relative to control mice, and the difference was significant at 3 and 15 h (p < 0.01).

Pulmonary Function—Static lung compliance was determined by analyzing pressure-volume curves. There were no differences in static lung compliance corrected for body weight when SP-B(-/-)hSP-B\(_{A1}(+)/+\), SP-B(+/minus)/hSP-B\(_{A1}(+)/+\), and SP-B(+/+)hSP-B\(_{A1}(+)/+\) mice were compared (data not shown). There were also no differences in y intercepts, inflation lung volumes at 30 cm of H\(_2\)O, or lung compliances normalized to lung wet weight or volume. The values for static lung compliance/g body weight were not significantly different from values obtained in wild type FVB/N mice (3.54 ± 0.34 μl/cm H\(_2\)O per g body weight), indicating that the Swiss Black genetic background had no influence on these values. Additionally, lung compliance in hSP-B\(_{A1}(+)/+\) transgenic mice (line 6.1) was not significantly different from that in wild type control mice, indicating that hSP-B overexpression also did not influence lung compliance (data not shown).

DISCUSSION

Recent studies from this laboratory demonstrated that the 102-residue C-terminal propeptide of SP-B was not required for intracellular trafficking of the hydrophobic, mature peptide (11), for targeting of the proprotein to lamellar bodies (12), or for proteolytic processing of the proprotein (12). The present study was therefore undertaken to identify the function of the C-terminal propeptide of SP-B by analyzing the phenotype...
associated with loss of expression of this peptide domain in transgenic mice. Expression of an hSP-BΔc transgene in type II cells of SP-B(−/−) mice resulted in postnatal survival, normal lung function, and normal surfactant composition indicating that the C-terminal propeptide was not required for reversal of neonatal lethality associated with SP-B deficiency (13); however, these “rescued” mice (SP-B(−/−)/hSP-BΔc(+/+)) contained abnormally large lamellar bodies with increased tissue pools of saturated phosphatidylcholine and accumulated an SP-C processing intermediate consistent with a role for the C-terminal propeptide in SP-C processing and lamellar body biogenesis.

Several observations argue for a specific role for the C-terminal propeptide in lamellar body biogenesis and SP-C processing in type II epithelial cells. First, the phenotype observed in SP-B(−/−)/hSP-BΔc(+/+) mice is not related to the site of transgene integration; both the 1.6 and 1.4 transgenic lines express the hSP-BΔc transgene in the wild type background without any detectable effect on lung structure or function (12). Second, although the genetic background of the knockout mouse can clearly influence the phenotype (29, 30), this does not appear to be the case for the SP-B(−/−)/hSP-BΔc(+/+) mice; both F1 and F6 SP-B(−/−) offspring of SP-B knockout mice crossed into the FVB/N background (the strain used to generate the 1.6 and 1.4 transgenic lines) exhibit the same phenotype as that originally reported for the SP-B knockout in mice crossed into the FVB/N background (the strain used to generate the 1.6 and 1.4 transgenic lines). Third, it is unlikely that the loss of SP-B expression in Clara cells of type II cells of these animals. Although lamellar body morphology was dramatically altered in type II cells, Clara cell ultrastructure was not perturbed; furthermore, the C-terminal propeptide is not secreted into the airway in healthy animals (7, 9) making it unlikely that the Clara cell is a source of C-terminal propeptide for lamellar body biogenesis or SP-C processing in type II cells. Finally, it is unlikely that elevated levels of mature SP-B peptide in SP-B(−/−)/hSP-BΔc(+/+) mice contribute to increased lamellar body size and accumulation of the SP-C processing intermediate since overexpression of SP-B in the hSP-BΔc(+/) transgenic line produced no alterations in type II cell ultrastructure, tubular myelin formation, or SP-C processing. Therefore, the C-terminal propeptide is either directly or indirectly involved in processing of the SP-C protein in type II cells. Effects on lamellar body size may be secondary to SP-C misprocessing or may represent a separate function of this peptide domain.

As evident from the study of SP-B(−/−) mice (13), the formation of lamellar bodies is clearly aberrant in the absence of SP-B, resulting in large inclusions containing multivesicular bodies and occasionally loosely organized membrane lamellae. Expression of hSP-BΔc in SP-B(−/−) mice leads to formation of inclusion bodies with tightly organized concentric lamellae, similar to lamellar bodies detected in wild type littermates. In SP-B(−/−)/hSP-BΔc(+/+) mice, lamellar body formation proceeds in the absence of the C-terminal propeptide and in the presence of misprocessed SP-C demonstrating that the mature SP-B peptide and/or the N-terminal propeptide are sufficient for lamellar body formation. While the formation of lamellar bodies in SP-B(−/−)/hSP-BΔc(+/+) mice appears normal, the regulation of lamellar body size is clearly compromised. Accumulation of tissue PC and dramatically enlarged lamellar bodies are similar to findings in the lipid storage disease seen in beige mice, an animal model of Chediak-Higashi syndrome (31). Although the biochemical basis of Chediak-Higashi syndrome is not known, other lipid storage diseases result from an accumulation of substrate in lysosomes. It is now clear that several of these storage diseases are related to a deficiency of one or more saposins, small 80-amino acid peptides that act as co-enzymes for lysosomal glycolipid hydrolysis (reviewed in

**Table I**

| Phospholipid composition of alveolar lavage | Wild type | SP-B(−/−)/hSP-BΔc(+/+) |
|---------------------------------------------|-----------|------------------------|
| Number of samples*                          | 5         | 5                      |
| Phosphatidylcholine                         | 80.7 ± 0.6| 84.2 ± 0.7             |
| Phosphatidylglycerol                        | 13.6 ± 0.5| 9.8 ± 0.4              |
| Phosphatidylinositol                        | 1.3 ± 0.1 | 1.7 ± 0.1              |
| Phosphatidylethanolamine                    | 2.8 ± 0.3 | 2.2 ± 0.2              |
| Phosphatidylserine                          | 0.9 ± 0.1 | 0.9 ± 0.1              |
| Lysophosphatidylcholine                     | 0.1 ± 0.1 | 0.4 ± 0.1              |
| Sphingomyelin                               | 0.5 ± 0.1 | 0.7 ± 0.1              |

* Samples were pooled to include two mice/sample.

![Figure 7](http://www.jbc.org/)  
**FIG. 7.** Pool sizes of Sat PC. The amount of Sat PC in alveolar lavages from 36 wild type and 32 SP-B(−/−)/hSP-BΔc(+/+) mice was not different. Sat PC was increased in the lung tissue after lavage and in the total lung (alveolar plus tissue) for the SP-B(−/−)/hSP-BΔc(+/+) mice (*p < 0.0001).
Ref. 32). Interestingly, the C-terminal propeptide of SP-B bears strong homology to the saposins, including conservation of six cysteine residues, a site for Asn-linked oligosaccharide, and various hydrophobic amino acids; furthermore, the lamellar body is a lysosomal-like compartment that contains hydrolases and membrane glycoproteins common to lysosomes (9, 33). Collectively, these observations are consistent with the hypothesis that the C-terminal propeptide plays an important role in regulating lamellar body size and intracellular surfactant pool size. The mechanism leading to increased lamellar body contents and size is unclear but may include increased fusion of individual lamellar bodies and/or reduced catabolism of surfactant phospholipids.

The metabolism measurements complement the cellular abnormalities and provide some insight into the effects of the hSP-B transgene. The increased lung tissue pools of Sat PC are consistent with the large and numerous lamellar bodies because a large percentage of Sat PC is associated with lamellar bodies in the normal lung. Radiolabeled choline incorporation into Sat PC is the best indicator of lamellar body formation. The synthetic machinery for Sat PC seems to be insensitive to alterations in surfactant packaging and secretary pathways. The slower rate of secretion of de novo synthesized Sat PC and the lower percent total secretion of Sat PC indicate a processing defect. Of interest, Sat PC metabolism also was abnormal in the beige mouse model of Chediak-Higashi syndrome (34). However, in that mouse (3H)glycerol incorporation into Sat PC was decreased to about 30% that in wild type mice, and the alveolar pool of Sat PC was decreased by about 30%. SP-B(−/−)/hSP-B(+/+) mice have remarkable cellular and metabolic abnormalities in the surfactant processing pathways, but they achieve a normal alveolar pool size for surfactant. The net effect of the hSP-B transgene correction in SP-B(−/−) mice likely reflects regulation of the alveolar pool to maintain normal surfactant homeostasis. Many mechanisms that promote surfactant secretion have been described (35); however, very little is known about pool size regulation. Other transgenic models indicate that cytokines such as granulocyte-macrophage colony-stimulating factor are produced by type II cells and can modulate surfactant component clearance kinetics (17, 36). The hSP-B transgene correction results in a healthy animal because the net effect is the production of normal amounts of surfactant with a normal composition and adequate content of mature SP-B.

Although surfactant function was restored in the SP-B(−/−)/hSP-B(+/+) mice, the accumulation of an SP-C processing intermediate seen in SP-B(−/−) mice was not corrected by the hSP-B transgene. Restoration of normal lung function in SP-B(−/−)/hSP-B(+/+) mice in the continued presence of the M2 = 11,000 SP-C processing intermediate indicates that the primary reason for neonatal lethality in SP-B(−/−) mice is deficiency of mature SP-B and/or mature SP-C, not accumulation of the M2 = 11,000 protein. Although the functional consequences of SP-C deficiency are not yet known, it is clear that reduced levels of SP-B protein are associated with altered lung function. A human infant with partial SP-B deficiency was recently reported to have severe respiratory distress syndrome and chronic lung disease (37). Mice that are hemizygous for the SP-B allele and express SP-B at 50% of the wild type level with normal levels of mature SP-C exhibit air trapping at low lung volumes (38). Further decreases in SP-B levels to approximately 1% of wild type result in neonatal lethality as evidenced by the hSP-B2.1 transgenic line; however, these animals also had decreased levels of mature SP-C peptide similar to SP-B(−/−) mice. Taken together, these observations suggest that decreased SP-B concentration, secondary to premature birth, infection, or other causes, may contribute to the pathogenesis of lung disease.

SP-B is expressed in noniliated bronchiolar Clara cells as well as alveolar type II epithelial cells. Although the function of SP-B in type II cells is likely related to packaging of the surfactant complex, the role of SP-B in the Clara cell is not known. In SP-B(−/−)/hSP-B(+/+) mice, SP-B expression was restricted to type II cells. The observation that tubular myelin formation, dynamic compliance, and airway pool size were normal in SP-B(−/−)/hSP-B(+/+) mice indicates that Clara cell SP-B is not required for surfactant function and metabolism. During hyperoxic lung injury, SP-B expression increases in Clara cells and decreases in type II cells (39, 40); however, it is not known if SP-B progenitor in Clara cells is processed to the mature peptide and secreted or if the Clara cell contributes SP-B to the alveolar surfactant pool following lung injury. It is therefore unclear if SP-B expression in Clara cells is related to surfactant function or some novel function.

In summary, the C-terminal propeptide of SP-B likely plays an important role in regulating SP-C proprotein processing and lamellar body size. Increased lamellar body size in SP-B(−/−)/hSP-B(+/+) mice was accompanied by increased levels of tissue Sat PC suggesting that the C-terminal propeptide may be an important determinant of intracellular surfactant pool size.

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