The Collagen-like Region of Surfactant Protein A (SP-A) Is Required for Correction of Surfactant Structural and Functional Defects in the SP-A Null Mouse

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Pulmonary surfactant isolated from gene-targeted surfactant protein A null mice (SP-A-/-) is deficient in the surfactant aggregate tubular myelin and has surface tension-lowering activity that is easily inhibited by serum proteins in vitro. To further elucidate the role of SP-A and its collagen-like region in surfactant function, we used the human SP-C promoter to drive expression of rat SP-A (rSP-A) or SP-A containing a deletion of the collagen-like domain (AG8-P80) in the Clara cells and alveolar type II cells of SP-A-/- mice. The level of the SP-A in the alveolar wash of the SP-A-/- rSP-A and SP-A-/- AG8-P80 mice was 6.1- and 1.3-fold higher, respectively, than in the wild type controls. Tissue levels of saturated phosphatidylcholine were slightly reduced in the SP-A-/- rSP-A mice compared with SP-A-/- littermates. Tubular myelin was present in the large surfactant aggregates isolated from the SP-A-/- rSP-A lines but not in the SP-A-/- AG8-P80 mice or SP-A-/- controls. The equilibrium and minimum surface tensions of surfactant from the SP-A-/- rSP-A mice were similar to SP-A-/- controls, but both were markedly elevated in the SP-A-/- AG8-P80 mice. There was no defect in the surface tension-lowering activity of surfactant from SP-A-/- rSP-A mice, indicating that the inhibitory effect of AG8-P80 on surface activity can be overcome by wild type levels of mouse SP-A. The surface activity of surfactant isolated from the SP-A-/- rSP-A but not the SP-A-/- AG8-P80 mice was more resistant than SP-A-/- littermate control animals to inhibition by serum proteins in vitro. Pressure volume relationships of lungs from the SP-A-/- rSP-A, and SP-A-/- AG8-P80 lines were very similar. These data indicate that expression of SP-A in the pulmonary epithelium of SP-A-/- animals restores tubular myelin formation and resistance of isolated surfactant to protein inhibition by a mechanism that is dependent on the collagen-like region.

Pulmonary surfactant is a heterogeneous mixture of phospholipids and at least four surfactant-associated proteins that is essential for effortless respiration (1). Surfactant secreted into the air space forms a phospholipid-rich film between the epithelial lining fluid and the gas in the lumen of the alveolus, which reduces the natural interfacial surface tension forces. The most abundant protein component of surfactant (by weight) is surfactant protein A (SP-A), a highly conserved glycoprotein that is synthesized and secreted by the alveolar type II cells and Clara cells of the distal lung epithelium (2). SP-A is a hydrophilic, Ca²⁺-dependent phospholipid-binding protein with in vitro activities that suggest contributions to surfactant function and macrophage-dependent microbial clearance (3). The understanding of the role of SP-A in pulmonary homeostasis has evolved significantly over the past decade due in part to the development of improved in vitro models of SP-A function. Although the importance of SP-A in pulmonary host defense has become clearer, there is still no consensus regarding the role of SP-A in surfactant function, where the greatest discrepancies between in vitro and in vivo data exist. Early experimental evidence that SP-A regulates the uptake and secretion of surfactant from isolated type II cells suggested that the protein might play a role in the regulation of intra-alveolar levels of surfactant phospholipids (4, 5). However, neither the absence of SP-A a 7-fold overexpression of SP-A significantly affected surfactant phospholipid pool sizes in recently developed genetically engineered mice (6, 7). Previous in vitro analyses from several laboratories have indicated that SP-A is necessary for the formation and/or stability of tubulin myelin (TM), a lattice-like structure that functions as the phospholipid reservoir for the surfactant monolayer (8). As predicted, TM was absent in the SP-A-/- mice, but normal survival and resting respiration in the presence of TM deficiency was unexpected (6). Early in vitro studies indicated that SP-A preserves the surface activity of surfactant during challenge with serum protein inhibitors in vitro (9, 10). This finding was supported by examination of surfactant isolated from mouse models of SP-A deficiency and excess, which revealed impaired and improved surfactant function in the presence of protein inhibitors, respectively (7, 11). Recent data from SP-A-/- mice suggest that SP-A does not play a major role in surfactant homeostasis or surface activity with exercise, exposure to hypoxia, or chemically induced lung injury (12). However, it

* Portions of this work were supported by the Medical Research Service of the Department of Veterans Affairs (to F. X. M.), National Institutes of Health Grants HL-61612 (to F. X. M.) and HL-61646 (to J. W., T. K., and M. I.), the National Institutes of Health Grants HL-61612 (to F. X. M.) and HL-61646 (to J. W., T. K., and M. I.), the American and Canadian Lung Associations (to N. P.), and the Medical Research Council of Canada (to N. P.).

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The abbreviations used are: SP-A, surfactant protein A; hSP-A and rSP-A, human and rat SP-A; PCR, polymerase chain reaction; sat PC, saturated phosphatidylcholine; ELISA, enzyme-linked immunosorbent assay; bw, body weight; kb, kilobase; n.s., no significant difference; mN/m, millinewtions per meter; TM, tubular myelin.
remains possible that SP-A contributes to surfactant structure and function under other conditions of lung stress or injury.

Mutagenesis studies have elucidated the interactions between SP-A and phospholipids that are important for surfactant structural and functional integrity (13–15). Rat SP-A is a large oligomer composed of 18 subunits derived from a single gene, which are distinguished by variable glycosylation at one or both N-linked carbohydrate attachment sites (16) and minor heterogeneity in the N-terminal amino acid sequences (17). The predicted primary structure for rat SP-A is composed of several discrete domains including (18): 1) a short N-terminal segment, 2) a collagen-like sequence of Gly-Xaa-Yaa repeats containing a midpoint interruption (Gly44) and abundant hydroxyproline, 3) a hydrophobic neck domain, and 4) a carbohydrate recognition domain. Trimeric association of monomeric subunits occurs by the folding of the collagen-like domains into triple helices (19).

The role of the neck domain in the oligomeric assembly of SP-A has not been reported, but in related collectins, the formation of α-helical coiled-coil bundles in this region is critical for trimerization (20). Fully assembled SP-A is a hexamer of trimers that and functional defects and 2) to determine whether the collagen-like domains of oligomers to the target. Deletion of the collagen-like domain (ΔG8-P80) reduces but does not extinguish lipid binding (14), probably by limiting the extent of oligomerization to simple trimers and hexamers (25). Most notably, the ΔG8-P80 mutation does not affect aggregation of lipid vesicles by the protein or the enhancement of the surface tension-lowering properties of surfactant lipids in the bubble surfactometer (14).

These in vitro data suggested that a partial SP-A molecule containing a deletion of the collagen-like domain retained the structural elements that are required for the surface tension enhancing properties of SP-A. To extend the structural analyses to the whole animal, we developed mice in which endogenous mouse SP-A was replaced with either full-length rat SP-A or a collagen-region deleted rat SP-A. The objectives of this study were 2-fold: 1) to determine whether genetic replacement of SP-A in the SP-A−/− mouse corrects surfactant structural and functional defects and 2) to determine whether the collagen-like region of SP-A is required for tubular myelin formation and resistance of surfactant to protein inhibitors.

**EXPERIMENTAL PROCEDURES**

**Construction of Rat and Mutant SP-A Transgenes**—The construction of a chimeric transgene composed of the 3.7-kb human surfactant protein C promoter (SP-C) (26) and 1.6-kb rat SP-A cDNA has been described previously (17). Using the same approach, a 1.4-kb mutant cDNA for SP-A containing a deletion of the collagen-like domain (ΔG8-P80) (14) was ligated into the unique EcoRI site of the 3.7-kb hSP-C plasmid. Orientation was confirmed with BamHI, and portions of the recombinant plasmid that were unnecessary for expression in mammary cells were removed by digestion with Ndel and NotI. The gel-purified fragment containing the promoter and the ΔG8-P80 transgene was used to produce transgenic mice (7).

**Replacement of Mouse SP-A by Rat SP-A and ΔG8-P80 in Transgenic Mice**—Swiss Black SP-A−/− mice were developed from embryonic stem cells after disruption of the mouse SP-A gene by homologous recombination as described previously (6). Lung-specific overexpression of rat SP-A in FVB/N mice was accomplished using the hSP-C promoter as described (7). The rat SP-A transgene was bred into the null background by crossing the rat SP-A transgene bearing FVB/N mice (SP-A−/−, x G8-P80) with Swiss Black SP-A−/− mice. Progeny that screened positively for the rat SP-A transgene in the first round were bred again with SP-A−/− mice. Progeny of the second generation that screened positively for the rat SP-A transgene and that were homozygous for the gene-targeted allele by Southern analysis (SP-A−/−, x rat SP−/−) were then bred with Swiss Black SP-A−/− mice through multiple (4–8) generations. All genotypes were confirmed by Southern analysis. SP-A−/−, G8-P80 and SP-A−/−, ΔG8-P80 mice were developed in a similar manner. Controls used for all experiments were transgene-negative littermate controls.

**DNA Analyses**—For identification of the targeted mouse SP-A gene and the rat SP-A transgene in the genomic DNA of mice, both PCR and Southern analysis were used. Tail clips (0.5–1 cm) were digested overnight in buffer containing 50 μg Tris, 100 mM EDTA, 0.5% SDS, and 100 μg/ml proteinase K at 55 °C and purified using the Wizard genomic DNA purification kit (Promega, Madison, WI). PCR was performed using primers that were complementary to the neomycin insert (5′-CTTAACTCACCAAGGTGGTC-3′ and 5′-TTTACAAAGGCGCCATC-5′) for amplification of the targeted mouse SP-A gene and to the 3′-end of the hSP-C promoter and nucleotides 628–652 of the rat SP-A cDNA (5′-CTTAACTCACCAAGGTGGTC-3′ and 5′-TTTACAAAGGCGCCATC-5′) for amplification of the rat and ΔG8-P80 transgenes. For Southern analysis, DNA was phenol/chloroform-extracted, digested with BamHI, and transferred to nitrocellulose by capillary action. The membrane was cross-linked with ultraviolet light and blocked with prehybridization solution at 65 °C. Random primer 32P-labeled probe was synthesized from the PsiI fragment of the rat SP-A cDNA (probe 1) or from nucleotides 3339–3485 of the endogenous mouse gene (probe 2) (Prime-It II, Stratagene).

**Protein Analyses**—Polyacrylamide gel electrophoresis and transfer to nitrocellulose by capillary action. The membrane was cross-linked with ultraviolet light and blocked with prehybridization solution at 65 °C. Random primer 32P-labeled probe was synthesized from the PsiI fragment of the rat SP-A cDNA (probe 1) or from nucleotides 3339–3485 of the endogenous mouse gene (probe 2) (Prime-It II, Stratagene). The membrane was hybridized with the probe overnight at 65 °C, washed three times with 20 mM NaHPO4, 1 mM EDTA, and 1% SDS and placed on film. The rat SP-A transgene was identified by PCR or Southern analysis, using probe 1 as described previously (7).

**Surface Tension Measurements**—Surface activity was measured with the captive bubble surfactometer (27) using large aggregate surfactant pooled from three mice of the same genotype. The concentration of each sample was adjusted to 3 nmol of saturated phosphatidylcholine (sat PC)/μl, and 3 μl of surfactant was applied to the air-water interface of the bubble by microsyringe. Sensitivity to protein inhibition was measured in the presence of 0.98 mg/ml sheep plasma protein.
Measurement of SP-A and Total Protein Levels—SP-A levels in bronchoalveolar lavage were determined using a rabbit polyclonal IgG against rat SP-A using a sandwich ELISA (22). The lower limit of sensitivity of the assay was 0.20 ng/ml, and the linear range extended from 0.16 to 10.0 ng/ml. Routine protein concentrations were determined with the bicinchoninic protein assay kit (BCA, Pierce) using bovine serum albumin as the standard. Purified SP-A proteins were separated by 8–16% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue (28). For immunoblot analyses, protein species were transferred to nitrocellulose membranes and reacted serially with rabbit anti-rat SP-A IgG and horseradish peroxidase-conjugated, goat anti-rabbit IgG antibody. Blots were developed by horseradish peroxidase/H2O2-dependent oxidation of Luminol and autoradiography using the Enhanced Chemiluminescence kit (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Measurement of Total Sat PC and Incorporation of Radiolabeled Precursors into Sat PC—Sat PC was determined 8 h after a 25-

Pressure-Venue Curves—Pressure-venue relationships of the lungs of SP-A−/−, rSP-A, and SP-A−/−ΔG8-P80 mice were determined as described (12). Briefly, mice were injected with an overdose of sodium pentobarbital (100 mg/kg, intraperitoneal injection) and placed in a chamber containing 100% O2 to collapse the alveoli. After sacrifice by exsanguination, the tracheas were cannulated and connected to a pressure sensor (Mouse Pulmonary Testing System, TSS, Inc., Cincinnati, OH). After the diaphragm was opened, the lungs were inflated in

RESULTS

Development and Physiological Characterization of Mouse Lines—The human SP-C promoter was used to direct the expression of rat and mutant SP-A transgenes in the distal lung epithelium of transgenic mice (Fig. 1). The development of SP-A−/− gene-targeted mice and rat SP-A overexpressing transgenic lines (SP-A−/−, rSP-A) has been described previously (6, 7). Of the two previously reported SP-A−/−, rSP-A lines, only the higher producing rSP-A mice (SP-A2) were used for breeding into the SP-A−/− background. To develop mice that overexpressed the collagen region deleted form of SP-A (ΔG8-P80), a hSP-C/ΔG8-P80 chimeric gene was constructed as outlined under "Experimental Procedures" and injected into fertilized FVB/N oocytes. From a total of three separate rounds of injection and uterine implantation, only one SP-A−/−ΔG8-P80 founder mouse was identified from 120 mice screened. This mouse transmitted the transgene to ~50% of offspring with equal distribution between male and female mice. To produce mice that expressed rat SP-A or ΔG8-P80 in the null background, the SP-A−/−ΔG8-P80 mice were bred with Swiss Black SP-A−/− mice (Fig. 2). Progeny from the first generation were screened for the presence of the transgenes by PCR and Southern analysis and were bred again with the SP-A−/− line. Transgene-positive F2 mice that were homozygous for the gene-targeted mouse SP-A allele were identified by Southern analysis and then bred through 4–8 generations with the SP-A−/− line to provide for a more homogeneous genetic background.

Effect of SP-A Expression on Saturated PC Pool Sizes and Synthesis—The level of SP-A in the alveolar wash of all mouse lines was compared with wild type mice. Three to six age-matched (6–8 week old) mice/genotype were lavaged with 5 ml of normal saline, and SP-A was measured in the recovered lavage fluid by ELISA (4.6–4.9 ml in all cases). The SP-A−/−, rSP-A mice had 849 ± 121 ng SP-A/ml lavage, 6-fold higher than SP-A−/− mice (138 ± 6 ng SP-A/ml). The level of SP-A in the SP-A−/−,ΔG8-P80 mice (179 ± 24 ng/ml) was 1.3-fold higher than SP-A−/− control mice. No SP-A was detected in the lavage of SP-A−/− littermate control mice from the SP-A−/−, rSP-A or the SP-A−/−ΔG8-P80 lines. To examine the role of SP-A in surfactant homeostasis, the pool sizes of SP-A and sat PC in lavage and homogenized lung tissue of the SP-A−/−, rSP-A mice (n = 8), and their transgene negative littermates (n = 13) were quantified and normalized to body weight (bw) (Fig. 3). The pool sizes of rSP-A in lavage and lung tissue of the SP-A−/−, rSP-A mice were 94 ± 16 μg of SP-A/kg bw and 182 ± 70 μg of SP-A/kg bw, respectively, compared with undetectable SP-A in the SP-A−/− mice. The mutant SP-A levels in the alveolar lavage and homogenized lung tissue of SP-A−/−ΔG8-P80 mice (n = 3) were 8 ± 3 μg of ΔG8-P80/kg bw and 22 ± 6 μg of ΔG8-P80/kg bw, respectively, similar to the pool sizes of SP-A reported in SP-A−/− mice (11 ± 5 μg of SP-A/kg bw and 16 ± 6 μg of SP-A/kg bw,
respectively (7). Comparison of surfactant phospholipid pool sizes from the SP-A–/–,rSP-A mice and their SP-A–/– littermates revealed that the elevated rSP-A levels had minor effects on surfactant levels in lavage (9 ± 1 μmol of surfactant/kg versus 10 ± 1 μmol of surfactant/kg, p = 0.02) or lung homogenates (23 ± 4 μmol of surfactant/kg versus 28 ± 2 μmol of surfactant/kg, p < 0.01) of the SP-A–/–,rSP-A mice compared with their SP-A–/– littermates. The effect of SP-A overexpression on the synthesis of surfactant phospholipids was examined by measuring incorporation of [14C]palmitate and [3H]choline chloride into surfactant 8 h after a body weight-adjusted precursor injection (not shown). In the SP-A–/–,rSP-A mice (n = 6), as compared with the SP-A–/– littermates (n = 8), there was a slight increase in [14C]palmitate incorporation into surfactant wash sat PC (3985 ± 670 versus 3177 ± 254 dpm, respectively, p < 0.01) and total lung sat PC (19678 ± 3471 versus 15956 ± 2520 dpm, p < 0.05) but no significant difference in tissue incorporation (15693 ± 3019 versus 12788 ± 2408 dpm, p = 0.07). The incorporation of [3H]choline chloride into SP-A–/–,rSP-A lung fractions was not different (p = n.s.) compared with the SP-A–/– littermates, including surfactant wash (3573 ± 437 versus 3133 ± 495 dpm), lung homogenate (131492 ± 1965 versus 13073 ± 2403 dpm), and total lung sat PC (16772 ± 2256 versus 16205 ± 2777 dpm). The percent secreted phospholipid (surfactant wash counts/lung homogenate counts) for SP-A–/–,rSP-A mice compared with SP-A–/– mice was 20.4 ± 2.7 versus 20.2 ± 2.6% for [14C]palmitate (p = n.s.) and 21.4 ± 2.3% versus 19.5 ± 2.0% for [3H]choline chloride (p = n.s.).

Role of the Collagen-like Region in the Association of SP-A with Large Aggregate Surfactant—An immunoblot analysis of SP-A in whole lavage fractions (normalized to total protein) is shown in Fig. 4A and in the large aggregate surfactant (normalized to sat PC content) in Fig. 4B. There was good general agreement between the concentration of SP-A estimated by ELISA and the relative levels of SP-A that were apparent by immunoblot analysis. Mouse SP-A from the SP-A+/+ line migrated as two bands at 32 and 36 kDa (Fig. 4A, lane 1), whereas the rSP-A in the SP-A–/–,rSP-A line migrated as three species at 26, 32, and 38 kDa (Fig. 4A, lane 3). The ΔGS-P80 SP-A mice migrated as two bands at 25 and 32 kDa (Fig. 4A, lane 5), most likely representing the glycosylated and nonglycosylated forms of the protein. For the SP-A+/+ ΔΔG-P60 mice, immunoreactive bands appeared at 25, 32, and 38 kDa, consistent with expression of mouse SP-A and the ΔGS-P80 (Fig. 4A, lane 6). There were no immunoreactive species in the 20–40 kDa region detected in the SP-A–/– littermates of the SP-A–/–,rSP-A or SP-A–/–ΔΔG-P60 mice (Fig. 4A, lanes 2 and 4). The immunoblot analysis of SP-A species associated with large aggregate surfactant (Fig. 4B) revealed migration patterns that were similar to whole surfactant for all proteins, except that the ΔGS-P80 was under-represented in the large aggregate fraction when the lanes were loaded for equal sat PC content.

In Vivo Structure/Function Analysis of SP-A

Role of the collagen-like Region of SP-A in Tubular Myelin Formation—SP-A–/– mice do not make the surfactant aggregate, TM (6). To assess the role of the collagen-like domain of SP-A in the formation of TM, we examined the ultrastructure of large aggregate surfactant from SP-A–/– mice that overexpress rat SP-A and ΔGS-P80 (Fig. 5). Square lipid lattices that were typical for TM were abundant in the SP-A–/–,ΔΔG-P60 (Fig. 5B) and SP-A–/–,ΔG-P60 mice (Fig. 5D), but the SP-A–/– littermates (Fig. 5A) had only large loopy surfactant aggregates without TM formation, as described previously (6). TM lattices in SP-A–/–,ΔΔG-P60 mice appeared as regularly organized squares with nearly equal side dimensions of 52.8 ± 9.1 × 52.2 ± 6.4 nm (side 1 versus side 2, p < 0.001) (Fig. 5D). The sides of TM from SP-A–/– mice were unequal and less uniform, with mean dimensions of 53.4 ± 16.0 ± 73.6 ± 22.6 nm (side 1 versus side 2, p < 0.01) (Fig. 5B). The dimensions of TM lattices of SP-A–/–,ΔSP-A mice were different from those of SP-A–/– mice (p < 0.01). Overexpression of the ΔGS-P80 in the null background did not correct TM formation (Fig. 5C). These data indicate that SP-A-mediated TM formation and/or stabilization requires SP-A and is collagen-like region-dependent.
Surface Tension-lowering Properties of Surfactant Isolated from Transgenic Mice—To measure surfactant function, large surfactant aggregates were applied directly to the air-liquid interface in the captive bubble apparatus (Figs. 6 and 7). The pressure measured in the lumen of the bubble after a 300-s incubation was defined as the equilibrium surface tension (Fig. 6), whereas the minimum surface tension was determined after cycling the bubble through 5 oscillations of maximum and minimum (65% volume reduction) radii (Fig. 7). The equilibrium and minimum surface tension of surfactant from the SP-A$^{−/−}$ mice (21.7 ± 0.5 and 2.5 ± 0.1 mN/m, respectively) were comparable with those of SP-A$^{+/+}$ mice (21.6 ± 0.6 and 5.6 ± 1.2 mN/m, respectively) and of SP-A$^{−/−}$ control littermates (22.8 ± 0.5 and 7.9 ± 2.3 mN/m, respectively) (panels A, Figs. 6 and 7) (6). However, both equilibrium and minimum surface tensions were elevated in the SP-A$^{−/−}$G8-P80 mice (34.9 ± 5.0 and 15.4 ± 1.8 mN/m, respectively, p < 0.05 compared with SP-A$^{−/−}$ mice) (panels B, Fig. 6 and 7). In contrast, surfactant isolated from the SP-A$^{+/+}$G8-P80 mice, which expressed ΔG8-P80 in the presence of normal levels of endogenous mouse SP-A, reached minimum surface tensions that were as low as their SP-A$^{+/+}$ littermates (2.5 ± 0.5 and 3.2 ± 1.3 mN/m, respectively). The surface activity of surfactant isolated from the SP-A$^{+/−}$,rSP-A (Fig. 7A) but not the SP-A$^{+/−}$ΔG8-P80 (Fig. 7B) mice was much more resistant than SP-A$^{−/−}$ littermate control animals to inhibition by serum proteins. Lung pressure volume curves for the SP-A$^{−/−}$ (n = 4), SP-A$^{+/−}$,rSP-A (n = 5), and SP-A$^{+/−}$ΔG8-P80 (n = 7) mice revealed no significant differences in total lung volumes, hysteresis, or compliance (data not shown). These data indicate that: 1) the defect in surfactant resistance to protein inhibition in the SP-A$^{−/−}$ mouse is corrected by genetic replacement of SP-A, 2) the collagen-like region of SP-A is required to correct the defect, 3) deletion of the collagen-like domain from SP-A converts the protein from a promoter to an inhibitor of surfactant surface activity, 4) surfactant inhibition by ΔG8-P80 can be overcome by normal levels of mouse SP-A, and 5) lung compliance in the SP-A$^{+/−}$ΔG8-P80 mouse is normal despite in vitro evidence that the ΔG8-P80 is an inhibitor of surfactant function.

The data presented here indicate that replacement of SP-A in the SP-A$^{−/−}$ mouse restores tubular myelin formation and corrects the defect in the resistance of isolated surfactant to protein inhibition. The association of SP-A with the large aggregate fraction, the formation of tubular myelin, and the protection of surfactant surface activity from inhibitors all require the collagen-like region of SP-A. The collagen-like region and/or full oligomeric assembly also plays an important role in the accommodation of SP-A in the alveolar lining fluid, because the ΔG8-P80 mutation converts SP-A into an inhibitor of surfactant function.

Previously reported mouse models of SP-A deficiency and excess did not support a role for SP-A in the regulation of phospholipid levels in the alveolar space (6, 7). The 6-fold elevation of SP-A provided by overexpression of rSP-A in the SP-A$^{−/−}$ model provide an opportunity to examine the effect of extremes of SP-A production on surfactant homeostasis in a relatively homogeneous genetic background. Comparison of the surfactant pool sizes in the SP-A$^{+/−}$,rSP-A mice and the SP-A$^{−/−}$...
controls revealed a small decrease in the total pool of sat PC due to a decrease in sat PC in the tissue compartment. This decrease in sat PC pool size was accompanied by no change to a small increase in the synthesis of sat PC based on incorporation of sat PC precursors. These data suggest that SP-A may have minor effects on pool sizes of sat PC, perhaps by enhancing the catabolism of sat PC, but it does not appear to be a critical regulator of surfactant homeostasis.

Several investigators have reported that the reconstitution of TM in vitro requires SP-A, SP-B, and surfactant lipids (8). Ultrastructural analyses of TM reveal that SP-A is located in the corners of the lattice (29). In these images, it appears that the globular heads of SP-A are buried at the apex of the intersecting lipid membranes, and the N-terminal regions of the molecule extend outward toward the center. Based on in vitro data that the N-terminal segment but not the collagen-like region is required for aggregation of phospholipid vesicles, we theorized that overexpression of AG8-P80 would result in the formation of TM with altered dimensions (30). We found that overexpression of rat SP-A in the SP-A-/- mouse restored TM formation but overexpression of the ΔG8-P80 at levels that were comparable with mouse SP-A in SP-A+/- lines did not. Tubular myelin isolated from the SP-A+/-,rSP-A had equal side dimensions, consistent with true squares, compared with the more irregular and rectangular dimensions of TM from SP-A-/- mice, similar to the published morphologies of rat (31) and mouse (6) TM, respectively. We conclude that the collagen-like region of SP-A is required for TM formation in vivo.

Our previously reported in vitro functional analyses had indicated that the collagen-like region of SP-A is not required for the surface tension enhancing properties of the protein (14). We were surprised to find that surfactant isolated from the SP-A-/-,AG8-P80 mice had impaired surface tension lowering activity at base line. The SP-A-/-,ΔG8-P80 surfactant failed to efficiently form a monolayer and did not reach low surface tensions with cycling. Although the mechanism of this effect is unclear, two potential explanations are that the ΔG8-P80 protein may be competing with surfactant lipids for the interface or that ΔG8-P80 may be interfering with the recruitment of lipids from the hypophase to the interface. Immunoblot and ELISA analysis of whole lavage versus isolated large aggregate surfactant indicates that co-sedimentation of the ΔG8-P80 with the surfactant phospholipids was reduced compared with that from the SP-A-/-,rSP-A mice. These data suggest that ΔG8-P80 exerts inhibitory effects in the aqueous compartment. However, direct visualization by transmission EM has previously confirmed that ΔG8-P80 can interact with lipid (25) in vitro. Whether ΔG8-P80 perturbs surfactant function from the aqueous or lipid compartment, it is clear that SP-A can reverse the inhibitory activity of ΔG8-P80, because surface activity was restored by natural levels of endogenous mouse SP-A in the SP-A-/-,AG8-P80 mouse. Potential explanations for this finding are that mouse SP-A may displace ΔG8-P80 from binding sites on surfactant lipids or that mouse SP-A fortifies the surfactant monolayer such that the ΔG8-P80 is excluded from the interface.

Surfactant protein A has been reported to enhance the surface activity of surfactants that are challenged with serum protein inhibitors in vitro and in vivo (9, 32). Surfactant isolated from the SP-A-/- mouse is susceptible to inhibition by serum proteins, and surfactant isolated from mice that overexpress SP-A by 7-fold is more resistant than that of wild type mice to protein inhibition (6, 7). In this study, we found that expression of rat SP-A in SP-A-/- mice restored the resistance of surfactant to serum protein inhibition. In contrast, surfactant isolated from the SP-A+/-,ΔG8-P80 mice performed no better than the surfactant from SP-A-/- mice in the presence of protein inhibitors. These data indicate that the collagen-like region of SP-A is required to restore the resistance of surfactant to serum protein inhibition.

Despite considerable impairment in the surface activity of surfactant isolated from the SP-A-/-,ΔG8-P80 mice, there were no significant differences in the pulmonary mechanics of lungs from the SP-A-/-,ΔG8-P80 mice compared with the SP-A-/- or SP-A-/-,rSP-A animals. This finding may suggest that the ΔG8-P80 protein, which readily impairs surfactant surface activity at the diluted surfactant concentrations that are used for in vitro analysis, does not affect surfactant function at the very high phospholipid concentrations present in the alveolar film. Alternatively, the alveolar lining layer may contain other molecules that protect the surface activity of surfactant but are lost during the surfactant purification process.

The structural basis of SP-A function has been examined in several laboratories using mutagenesis and in vitro analysis. The SP-A-/- mouse provides an ideal background to test the physiological roles of individual structural domains of SP-A in the whole organism. The objectives of this study were to determine whether SP-A replacement in the SP-A-deficient mouse corrected surfactant structural and functional defects and whether a structurally simplified SP-A composed primarily of the C-terminal domains of the protein contained the elements required for SP-A-related surfactant functions in vivo. Our findings indicate that the collagen-like region of SP-A is required for avid surfactant lipid association, tubular myelin formation, and the protection of surfactant from protein inhibition. Furthermore, deletion of the collagen-like domain from SP-A converts the protein into an inhibitor of surfactant function. Given recent compelling evidence that SP-A is critical for host defense in the lung, we speculate that the evolution has conferred nondisruptive phospholipid binding properties on SP-A as a mechanism for positioning and concentrating the protein at a most hydrophobic and inaccessible interface with the environment.

Acknowledgments—We thank Mamatha Damodarasamy and Maura Unger for expert technical assistance.
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