Activity of Pulmonary Surfactant Protein-D (SP-D) in Vivo Is Dependent on Oligomeric Structure

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Pulmonary surfactant protein-D (SP-D) is a member of the collectin family of C-type lectins that is synthesized in many tissues including respiratory epithelial cells in the lung. SP-D is assembled predominantly as dodecamers consisting of four homotrimeric subunits each. Association of these subunits is stabilized by interchain disulfide bonds involving two conserved amino-terminal cysteine residues (Cys-15 and Cys-20). Mutant recombinant rat SP-D lacking these residues (RrSP-Dser15/20) is secreted in cell culture as trimeric oligomers rather than as dodecamers. In this study, transgenic mice that express this mutant were generated to elucidate the functional importance of SP-D oligomerization in vivo. Expression of RrSP-Dser15/20 failed to correct the pulmonary phospholipid accumulation and emphysema characteristic of SP-D null (mSP-D/−) mice. Expression of high concentrations of the SP-D null (mSP-D/−) mouse. RrSP-Dser15/20 binds to the hemagglutinin of the influenza virus in a CRD-dependent manner. However, the mutant SP-D does not aggregate particulate ligands such as viral particles and competitively inhibits aggregation mediated by wild-type SP-D (15, 16).

High affinity binding of SP-D to various ligands in vitro requires a trimeric CRD (17–19). However, previous studies using trimeric CRDs, RrSP-Dser15/20, and variably multimerized fractions of recombinant human SP-D suggest that trimers are functionally univalent and have a restricted range of biological activities. For example, trimeric CRDs elicit the chemotaxis of neutrophils and mononuclear phagocytes in vitro and neutralize the respiratory syncytial virus in vivo (20). In addition, RrSP-Dser15/20, similar to wild-type SP-D, inhibits stimulated proliferation of T-lymphocytes (22). However, other activities of SP-D involve ligand aggregation and require higher orders of covalently stabilized oligomerization. For example, trimeric CRDs inhibit the hemagglutinin of the influenza virus, but unlike SP-D dodecamers, they cannot mediate viral aggregation, enhance viral binding to neutrophils, or enhance the oxidative response to a bound virus (16, 19, 23).

Analyses of the SP-D gene-targeted mice indicated an important role of SP-D in the regulation of surfactant lipid homeostasis and macrophage function (24). Lungs of SP-D null mice have increased surfactant phospholipid pools associated with abnormally enlarged and foamy alveolar macrophages and hyperplastic type II cells with an increased size of lamellar bodies (25, 26). SP-D/− mice also develop progressive pulmonary emphysema and subpleural fibrosis, associated with chronic inflammation and increased matrix metalloproteinase and increased oxidant production by alveolar macrophages (27). Expression of rat SP-D (rSP-D) in distal respiratory epithelium of the lung completely corrects the pulmonary lipid accumula-

Surfactant protein-D (SP-D) is a member of a family of collagenous host defense lectins termed collectins (1, 2). SP-D is secreted into the distal airways and alveoli of the lung (3–5) but is expressed also in other tissues (6–8). Each 43-kDa SP-D monomer consists of an NH2-terminal domain containing two conserved cysteine residues (Cys-15 and Cys-20), a collagenous domain, a short neck sequence, and a COOH-terminal carbohydrate recognition domain (CRD) (9–12). Electron microscopy and proteinase digestion studies demonstrated that SP-D monomers are assembled into tetrameric subunits (dodecamers) (13, 14). The two NH2-terminal cysteine residues of SP-D are critical in the formation of interchain disulfide bonds that stabilize the dodecameric structure (13).

Substitution of serine for cysteine at positions 15 and 20 of the mature protein results in the efficient secretion of trimeric subunits corresponding to a single arm of the SP-D dodecamer (15). Mutant recombinant rat protein migrates as a monomer on SDS-polyacrylamide gel electrophoresis in the absence of sulphydryl reduction and elutes as a trimer rather than as a dodecamer from gel filtration columns under non-denaturing conditions (16). The collagen domain forms a triple helix as assessed by protease digestion (16). The trimeric protein is also a functional lectin with the same saccharide selectivity as the wild-type protein. RrSP-Dser15/20 binds to the hemagglutinin of the influenza virus in a CRD-dependent manner (15, 16). However, the mutant SP-D does not aggregate particulate ligands such as viral particles and competitively inhibits aggregation mediated by wild-type SP-D (15, 16).

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tation and abnormalities in alveolar macrophages seen in SP-D null mice, whereas the targeted increased expression of wild-type rat SP-D in normal mice had no effect on endogenous SP-D, lung morphology, or surfactant phospholipid content (28).

SP-D dodecamers bind to specific surfactant lipids and influence their state of aggregation (29–32). Based in part on these observations, we hypothesized that the covalent oligomerization of trimeric subunits is required to mediate the effects of SP-D on surfactant homeostasis and macrophage function in vivo. For the present studies, transgenic mice were generated that expressed single trimeric subunits by targeting the expression of RrSP-Dser15/20 to peripheral respiratory epithelial cells of the lung using the human surfactant protein-C promoter (33). The RrSP-Dser15/20 mutant protein did not correct lung phospholipids, alveolar macrophage abnormalities, or emphysema in SP-D−/− null mice. Expression of the mutant protein in wild-type mice interfered with the normal covalent oligomerization of the endogenous mSP-D and caused emphysema and the accumulation of foamy alveolar macrophages similar to that observed in SP-D null animals.

**EXPERIMENTAL PROCEDURES**

**Generation of Transgenic Mice**—The 1.3-kb mutant cDNA of rat SP-D (RrSP-Dser15/20) was generated previously (16) and inserted into the EcoRI site of the BGI-hSPC expression vector. The entire cDNA into the EcoRI site of the BGI-hSPC expression vector. The entire

**Western Blot Analysis**—Animals were weighed, anesthetized by intraperitoneal injection of pentobarbital, and exsanguinated by severing the distal aorta. Bronchoalveolar lavage was performed five times with saline for each lung, and the volume was measured (35). Bronchoalveolar lavage fluid (BALF) (25 μl) from each mouse was diluted and reconstituted in 15 μl of nonreduced Laemmli sample buffer (Bio-Rad). After resolution with a 10–20% SDS-Tris/glycine-polyacrylamide gel (NOVEX, San Diego, CA) and transfer to a nitrocellulose membrane (Bio-Rad), the blots were blocked with 5% nonfat milk and then incubated at room temperature overnight with rabbit anti-mouse SP-D antiserum diluted 1:5,000 in Tris-buffered saline with 0.1% Tween. Blots were washed with Tris-buffered saline/Tween and incubated at room temperature for 4 h with a 1:10,000 dilution of peroxidase-conjugated goat anti-rabbit IgG antibody (Calbiochem). After washing, blots were developed with a chemiluminescence detection system (Amer sham Pharmacia Biotech). Generally, 4–8 mice from each genotype were analyzed for each mouse line and representative results shown. Protein bands on a Western gel were quantified using the computer program ImageQuant 1.2. The density volume of each band is the sum of pixel density above background in the band area.

**Binding to Maltoyl-Sepharose—**A 6-week-old mouse was incubated with maltose immobilized on 4% beaded agarose (Sigma) overnight at 4 °C in TCB (20 mM Tris, pH 7.4, 10 mM CaCl₂). After washing three times with TCB, equal fractions of the maltoyl-Sepharose beads were incubated with 0, 2, 5, 10, and 25 μM maltose in TCB overnight at 4 °C. The maltoyl-Sepharose beads then were suspended in 15 μl of reduced Laemmli sample buffer (Bio-Rad) and resolved on a 10–20% SDS-glycine-polyacrylamide gel. Western analysis with rabbit anti-mouse SP-D antiserum was performed to detect the SP-Ds that bound to the maltoyl-Sepharose beads.

**Phospholipid Analysis—**Bronchoalveolar lavage was performed five times with saline on each lung for 8–10-week-old mice. Lung tissue was homogenized in saline after lavage. The amount of saturated phosphatidylcholine (Sat PC) in BALF and lung homogenate was measured as described previously (25). Animals from each genotype were analyzed, and the differences between genotypes were evaluated by ANOVA Fisher analysis. Differences of p < 0.05 were considered significant.

**Lung Morphology—**Mouse lungs (12 weeks old) were fixed at 25 cm of water pressure with 4% paraformaldehyde in phosphate-buffered saline and processed into paraffin blocks. Seven-micrometer sections from each lobe were stained with hematoxylin and eosin. Morphometric measurements on the ratio of alveolar parenchyma to airspace areas were performed as described previously (36). Animals (4–6) from each genotype were analyzed in each mouse line, and the differences between genotypes were evaluated by ANOVA Fisher analysis. Differences of p < 0.05 were considered significant.

**Immunostaining of endogenous mSP-D and transgenic RrSP-Dser15/20 was performed in lung tissue using an avidin-biotin-peroxidase technique (Vectastain Elite ABC kit, Vector Laboratories). The rabbit anti-SP-D antibody was generated against purified mouse SP-D and affinity absorbed against lung homogenates from SP-D−/− mice (8). Immunostaining was blocked completely by pre-incubation with purified mouse SP-D.

**RESULTS**

**Transgenic Mouse Lines**—Eleven founder mice were identified by Southern blot analysis of tail-clip DNA using 1.3-kb RrSP-Dser15/20 cDNA as a probe (data not shown). Germ line transmission was demonstrated in eight founders using trans-
the oligomeric form of SP-D migrates slower than 200 kDa (MW KD). The monomeric form of SP-D migrates at 43 kDa, and gene-specific PCR on offspring tail DNA (data not shown). The expression of transgenic protein was confirmed in these eight mouse lines by Western blot analysis of mouse BALF using a rabbit anti-mouse SP-D antibody (data not shown). Two mouse lines, one with moderate (line 52) and the other with high (line 75) levels of RrSP-Dser15/20 protein expression were selected for further breeding and analysis.

Expression of RrSP-Dser15/20 Protein—Mouse and rat SP-Ds are 92% identical, and the rabbit anti-mouse SP-D antibody recognized both endogenous mSP-D and transgenic RrSP-Dser15/20 protein by Western blot and immunohistochemistry. However, RrSP-Dser15/20 protein did not form interchain disulfide bonds because of the substitution of serine for cysteine at positions 15 and 20 (16). Therefore, under non-reducing conditions on SDS gels, endogenous mSP-D was detected as an oligomer, whereas RrSP-Dser15/20 protein migrated as a monomer. In line 52 mice, the concentration of the mutant protein in BALF was similar to that of endogenous mSP-D (Fig. 2A, density volume ratio between RrSP-Dser15/20 in lane 3 and mSP-D in lane 2 is 2:1). The monomeric form of SP-D was detected in transgenic mice of both wild-type (mSP-D+/+) and null (mSP-D−/−) backgrounds (Fig. 2A, lanes 1 and 3). No oligomeric forms of SP-D were detected in the null (mSP-D−/−) background (Fig. 2A, lanes 3 and 4). In wild-type mice the larger oligomeric form of mSP-D was decreased consistently in the transgenic mouse (RrSP-Dser15/20+, mSP-D+/+) compared with nontransgenic mice (mSP-D+/+) (Fig. 2A, lanes 1 and 2). In mice from line 75, the concentration of RrSP-Dser15/20 was increased markedly compared with that of the endogenous mSP-D (Fig. 2B, density volume ratio between RrSP-Dser15/20 in lane 3 and mSP-D in lane 2 is 24). The slower mobility oligomeric forms of mSP-D typical of wild-type mice were not detectable in line 75 transgenic mice (RrSP-Dser15/20+, mSP-D+/+) or in mSP-D−/− null mice (Fig. 2B, lanes 1, 3, and 4).

RrSP-Dser15/20 Binds Maltosyl-Sepharose—To assess whether the mutant SP-D maintained lectin activity, binding of wild-type SP-D and RrSP-Dser15/20 to maltosyl-Sepharose was assessed. Both wild-type and mutant SP-D in the mouse BALF maintained the ability to bind to maltose in vitro (Fig. 3). Increasing concentrations of maltose inhibited binding of wild-type and mutant SP-D to the maltosyl-Sepharose beads (Fig. 3). The affinity of wild-type SP-D and mutant SP-D to maltosyl-Sepharose was similar.

RrSP-Dser15/20 Does Not Correct Lung Phospholipids—Sat PC in BALF and lung homogenates was assessed in transgenic (RrSP-Dser15/20+, mSP-D+/+ and RrSP-Dser15/20−, mSP-D−/) and null (mSP-D−/) and wild-type (mSP-D+/+) mice. After normalization for body weight, there were no statistically significant differences in lung phospholipid levels between lines 52 and 75 mice. Therefore, data from lines 52 and 75 mice were pooled together and presented in Fig. 4. Although statistically significant increased Sat PC pool sizes were observed in mSP-D−/− mice compared with wild-type mice (Fig. 4, compare bars 1 and 2 with bars 3 and 4, respectively), RrSP-Dser15/20 did not correct Sat PC pool sizes in SP-D null mice (Fig. 4, bars 3 and 4) and did not perturb Sat PC levels in wild-type mice (Fig. 4, bars 1 and 2).

Lung Morphology—Expression of the RrSP-Dser15/20 trans-
SP-D Activity in Vivo Depends on its Oligomerization

**FIG. 4.** Saturated phosphatidylcholine pool sizes. Alveolar tissue and total lung Sat PC concentrations were determined in wild-type (mSP-D+/+), null (mSP-D−/−), and transgenic (RrSP-Dser15/20+, mSP-D+/− and RrSP-Dser15/20+, mSP-D−/−) mice and normalized for body weight. Data from lines 52 and 75 were not different and therefore were pooled. Values are mean ± S.E. ANOVA Fisher analysis showed that Sat PC in mice from the mSP-D−/− background were significantly lower than in mice with mSP-D+/− background (p < 0.0001). There were no statistically significant differences in Sat PC in RrSP-Dser15/20+ and mSP-D−/− mice whether in mSP-D+/+ (wild-type) or mSP-D−/− (null) backgrounds.

**DISCUSSION**

The expression of a mutant RrSP-Dser15/20 protein was directed to bronchiolar and alveolar epithelial cells with the human SP-C promoter in wild-type and SP-D−/− null mice. Mutations of the two amino-terminal cysteine residues resulted in the secretion of a protein that bound to maltose and migrated exclusively as monomers on SDS-polyacrylamide gel electrophoresis in the absence of reduction. These findings are consistent with the results described by Brown-Augsburger et al. (15) for transfected CHO-K1 cells. Given that interchain bonds are required for the stability of dodecamers at 37 °C in vitro (16), the absence of disulfide cross-linked oligomers is consistent with the absence of dodecamers in vivo. Because expression of the RrSP-Dser15/20 mutant protein failed to correct phospholipid accumulation, foamy macrophage production

**Fig. 5.** Lung histology. Lungs were fixed and stained with hematoxylin and eosin. A, lungs from wild-type mice; B, RrSP-Dser15/20 (line 52) in wild-type background; C, RrSP-Dser15/20 (line 75) in wild-type background; D, SP-D−/− mice; E, RrSP-Dser15/20 (line 52) in SP-D−/− background; F, RrSP-Dser15/20 (line 75) in SP-D−/− background. The arrows point to enlarged foamy alveolar macrophages. The arrowheads point to normal macrophages.
from line 75 mice. Parenchyma/airspace ratios were similar in RrSP-
were compared by ANOVA Fisher analysis. The
upper panel
represents data from line 52 mice, and the lower panel represents data from line 75 mice. Parenchyma/airspace ratios were decreased significantly in RrSP-Dser15/20+, mSP-D+/+ mice compared with mSP-D+/− mice in line 52. In line 75 mice, parenchyma/airspace ratios were decreased significantly in RrSP-Dser15/20+, mSP-D+/+ mice compared with wild-type mice (*, p = 0.0114). Parenchyma/airspace ratios were decreased in both RrSP-Dser15/20+, mSP-D+/− and mSP-D+/− mice, reflecting emphysema in both mice.

FIG. 6. Effects of mutant SP-D on the relative proportions of lung parenchyma to airspace areas. Parenchyma/airspace ratios were determined by morphometric analysis. Values are mean ± S.E. and were compared by ANOVA Fisher analysis. The upper panel represents data from line 52 mice, and the lower panel represents data from line 75 mice. Parenchyma/airspace ratios were similar in RrSP-Dser15/20+/+ mice compared with mSP-D+/− mice in line 52. In line 75 mice, parenchyma/airspace ratios were decreased significantly in RrSP-Dser15/20+, mSP-D+/+ mice compared with wild-type mice (*, p = 0.0114). Parenchyma/airspace ratios were decreased in both RrSP-Dser15/20+, mSP-D+/− and mSP-D+/− mice, reflecting emphysema in both mice.

In both transgenic mouse lines, expression of RrSP-Dser15/20 failed to rescue the histological and biochemical phenotypes of SP-D−/− mice. The characteristic phenotype of mSP-D null mice, with the increased pool size of alveolar phospholipids, abnormal foamy macrophages, and emphysema, persisted despite high levels of RrSP-Dser15/20 proteins. In contrast, a previous study demonstrated that expression of the normal rSP-D at high levels rescued all aspects of the pulmonary abnormalities seen in mSP-D−/− null mice using the same human SP-C promoter used in this study (28). Although the findings suggest that multivalency of trimeric subunits is required, it remains possible that cysteines 15 and 20 or more subtle local conformational perturbations render the protein defective with respect to its ability to rescue the SP-D null phenotype. In this regard, thermal stability of the collagen domain of RrSP-Dser15/20 is decreased, presumably secondary to the absence of interchain disulfide cross-links (16).

In RrSP-Dser15/20 transgenic mouse line 75, in which normal disulfide cross-linked oligomers of SP-D were undetectable by Western blot analysis, lung morphology was similar to that characteristic of mSP-D−/− null mice, with enlarged foamy macrophages and emphysema. The expression of RrSP-Dser15/20 protein did not disrupt lung morphology in line 52, in which the mutant protein was expressed at levels that did not eliminate the formation of disulfide cross-linked oligomers of the endogenous mSP-D. These results suggest that overexpression of chains lacking the capacity to participate in interchain disulfide bonds may interfere with the function of SP-D, causing a dominant negative effect through formation of heteropolymers of wild-type mouse and mutant rat chains that are unable to participate in the formation of stable dodecamers. Given that the mouse and rat proteins are identical in length and domain structure, it is likely that folding of the carboxy-terminal CRDs and neck domains of the heteropolymers is unaltered. Indeed, the lectin activity of the mutant SP-D was retained, supporting the likelihood that the folding of the CRDs was maintained. However, it is unclear whether differences in functions of the mutant SP-D molecules are related to activities determined by the serine at positions 15 and 20 or to the effect of the mutant protein on oligomerization of the wild-type SP-D, leaving the lung relatively deficient in functional disulfide-linked oligomeric forms. Alternatively, heteropolymers of SP-D could be degraded, resulting in selective secretion of mutant SP-D. Although the lungs of wild-type mice expressing the mutant SP-D at high levels (line 75) contained numerous foamy macrophages and developed emphysema, pulmonary Sat PC concentrations were not increased significantly, suggesting that some function of the endogenous mSP-D was maintained. It was possible that a very small amount of appropriately assembled endogenous mSP-D was present in the BALF but was undetectable by Western blot. It remains possible that various activities of SP-D may be concentration-dependent. For example, the concentrations of SP-D inducing neutrophil chemotaxis (20) are 100–1,000 times lower than those required for aggregating particles (16).

In summary, the present findings strongly support the con-
cept that the dodecameric structure of SP-D is essential for some of its homeostatic functions in vivo. Increased expression of the mutant SP-D in wild-type mice decreased the concentration of the normal disulfide cross-linked oligomeric forms of mouse SP-D and caused emphysema and foamy macrophage accumulation in the absence of alterations in alveolar phospholipid concentrations. Thus, some aspects of the SP-D null phenotype, namely the pulmonary emphysema and production of foamy alveolar macrophages, are not likely caused by increased surfactant phospholipids. Although the human lung contains a predominance of highly oligomerized SP-D, trimeric species have been identified in the lungs of some patients with alveolar proteinosis (37), and less highly aggregated SP-D forms may be generated by proteolytic degradation in the setting of infection or lung injury. Polymorphisms in nucleotide sequences of the collagen domain of the serum mannose binding lectin altered or lung injury. Polymorphisms in nucleotide sequences of the collagen domain of the serum mannose binding lectin altered

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