CORRECTION OF PULMONARY ABNORMALITIES IN Sftpd⁻/⁻ MICE REQUIRES THE COLLAGENOUS DOMAIN OF SURFACTANT PROTEIN D§
Paul S. Kingma*, Liqian Zhang*, Machiko Ikegami*, Kevan Hartshorn†, Francis X. McCormack‡, and Jeffrey A. Whitsett*

From the *Division of Pulmonary Biology, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio 45229-3039. †Departments of Medicine and Pathology, Boston University School of Medicine, Boston, Massachusetts 02118-3393. ‡Pulmonary/Critical Care Division, University of Cincinnati Medical Center, 231 Bethesda Avenue, PO Box 670564, Cincinnati, OH 45267-0564.

Running title: SP-D collagen domain
Correspondence: Jeffrey A Whitsett, Cincinnati Children’s Hospital Medical Center, Division of Pulmonary Biology, 3333 Burnet Ave., Cincinnati, OH 45229-3039 Tel: 513-636-4830. Fax: 513-636-7868. E-mail: jeff.whitsett@cchmc.org

Surfactant protein D (SP-D) is a member of the collectin family of innate defense proteins. Members of this family share four distinct structural domains: an N-terminal crosslinking domain, a collagenous domain, a neck region, and a carbohydrate recognition domain. In this study the function of the collagenous domain was evaluated by expressing a SP-D collagen deletion mutant protein (rSftpdCDM) in wild type and SP-D null mice (Sftpd⁻/⁻). rSftpdCDM formed disulfide linked trimers that further oligomerized into higher order structures. The mutant protein effectively bound carbohydrate and aggregated bacteria in vitro. While rSftpdCDM did not disrupt pulmonary morphology or surfactant phospholipid levels in wild type mice, the mutant protein failed to rescue the emphysema or enlarged foamy macrophages that are characteristic of Sftpd⁻/⁻ mice. Moreover, rSftpdCDM partitioned with small aggregate surfactant in a manner similar to SP-D, but rSftpdCDM did not correct the abnormal surfactant ultrastructure or phospholipid levels observed in Sftpd⁻/⁻ mice. In contrast, rSftpdCDM completely corrected viral clearance and the abnormal inflammatory response that occurs following pulmonary influenza A challenge in Sftpd⁻/⁻ mice. Our findings indicate that the collagen domain of SP-D is required for assembly of disulfide stabilized oligomers or the innate immune response to viral pathogens. The collagen domain of SP-D is required for the regulation of pulmonary macrophage activation, airspace remodeling, and surfactant lipid homeostasis.

Surfactant protein D (SP-D)¹ is a member of the collectin family of C-type lectins. Members of this family include surfactant protein A (SP-A), SP-D, mannose binding protein (MBP), conglutinin, and CL-43. SP-A and SP-D contribute to the innate immune system of the lung by binding and enhancing the clearance of a variety of viral, bacterial and fungal pathogens (1-3). The collectins are defined by four structural domains shared by all family members: a short amino-terminal crosslinking domain, a triple helical collagenous domain, a neck domain, and a carbohydrate recognition domain (CRD) (4-8). Three neck domains associate to form a triple coiled-coil structure that facilitates the assembly of the remaining domains into a trimer (9). Final assembly of the trimer, thought to be the minimal functional unit of collectins, occurs through disulfide bonds between the cysteine-rich amino-terminal domains (10,11). Trimers further combine into larger multimeric complexes through disulfide stabilized, non-covalent interactions. Although larger structures are commonly observed, SP-D exists predominately as a tetramer of trimeric subunits (dodecamer) assembled into a cruciform structure (10,12).

Animal models of SP-D deficiency have revealed a complex role for SP-D in pulmonary immunity and alveolar homeostasis. Mice with a targeted deletion of the Sftpd gene (Sftpd⁻/⁻)
survived normally, but developed gradually worsening pulmonary inflammation, emphysema, and surfactant phospholipid accumulations (13,14). Sftpd<sup>−/−</sup> mice accumulate apoptotic alveolar macrophages, as well as, enlarged, lipid laden, macrophages that release metalloproteinases and reactive oxygen species (15-19). Uptake and clearance of viral pathogens including influenza A and respiratory syncitial virus were deficient in Sftpd<sup>−/−</sup> mice (20,21). In contrast, clearance of the abnormal macrophage activation, emphysema, or lipid abnormalities in Sftpd<sup>−/−</sup> mice, indicating that the SP-D collagenous domain is critical for normal regulation of lipid homeostasis, macrophage activity, and the structural integrity of peripheral airspaces.

**EXPERIMENTAL PROCEDURES**

**Animal Husbandry**- Mice were handled in accordance with approved protocols through the Institutional Animal Care and use Committee at Cincinnati Children’s Hospital Medical Center. All mice had been maintained in the vivarium in barrier containment facilities. Sentinel mice in the colony were serologically negative for common murine pathogens.

**Generation of Transgenic Mice**- rSftpdCDM cDNA was generated using recombinant PCR to delete a 177 amino acid sequence (Gly26 to Pro202) corresponding to the complete collagen domain from rat SP-D (Fig. 1). The rSftpdCDM cDNA was inserted into the EcoRI site of 3.7hSPC/SV40 expression vector and sequenced (26). The transgene was microinjected into FVB/N oocytes fertilized with Sftpd<sup>−/−</sup> sperm by the Children’s Hospital Transgenic Core facility and founders were identified by transgene specific PCR using upstream primer 5’-GGAGACAAAATCTTCAAGGGCG-3’ and downstream primer 5’-TTCGGATGGTGGCAGCATAG -3’. Transgenic animals were crossed with either Sftpd<sup>−/−</sup> mice or WT mice to generate rSftpdCDMTg+/Sftptd-/− mice or WT mice to generate rSftpdCDMTg+/Sftpd+/+ mice (16).

**Western Blot Analysis**- Animals were weighed, anesthetized by intraperitoneal injection of pentobarbital and exsanguinated. Bronchoalveolar lavage was performed five times with 1 ml of normal saline. Typically, >90% of the instilled volume was recovered. For each lane,
40 µl of the bronchoalveolar lavage fluid (BALF) was dried, reconstituted in 15 µl Laemmli buffer with or without sulfhydryl reduction with β-mercaptoethanol and resolved on 10-20% SDS/Tris/glycine/polyacrylamide gel (Novex, San Diego, CA). After separation and transfer to a nitrocellulose membrane, protein was detected with rabbit anti-mouse SP-D or guinea pig anti-rat SP-A antiserum (Seven Hills Bioreagents, Cincinnati, OH) diluted 1:5000 in Tris-buffered saline (TBS) as previously described (27).

SP-D purification- Previous studies demonstrated that inactivation of the granulocyte-macrophage colony-stimulating factor gene (GM-CSF-) in mice impaired SP-D clearance and increased SP-D levels in BALF several fold (28). Therefore, to increase the amount of starting material for protein purification, rSftpdCDM was purified from rSftpdCDMTg+/Sftptd-/-/GMCSF-- mice. Wild type mouse SP-D was purified from Sftpd+/+/GM-CSF-/- mice that also carried a deletion of the two expressed Sftpa genes in order to minimize the potential of SP-A contamination. A similar Sftpa deletion was not possible in the rSftpdCDMTg+/Sftptd-/-/GMCSF-- mice, however contaminating SP-A was not detectable by silver stain gels in rSftpdCDM preparations.

SP-D or rSftpdCDM containing BALF was applied to a maltosyl-Sepharose (Sigma) column and selectively eluted with manganese as previously described (29). The pooled fractions were diluted 10-fold in 20 mM Tris-HCl, pH 7.4, and 30 mM CaCl2 and applied to a 1 ml bed volume maltosyl-Sepharose column. The column was stripped of lipopolysaccharide with 20 mM Tris-HCl pH 7.4, 20 mM n-octyl-β-d-glucopyranoside, 200 mM NaCl, and 100 µg/ml polymyxin and washed with 20 mM Tris-HCl pH 7.4, 0.5 mM CaCl2, 200 mM NaCl. The protein was eluted with 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA. Under the conditions employed lipopolysaccharide concentration was typically ≤ 0.1 endotoxin units/µg protein.

SP-D Sizing and Detection- The size of rSftpdCDM multimers was determined by gel filtration chromatography using Sepharose CL-6B equilibrated in 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM EDTA, and 0.02% sodium azide. Purified rSftpdCDM was diluted in the same buffer and applied to the column (1.5 cm X 90 cm). rSftpdCDM protein concentration was determined in each fraction using an enzyme-linked immunosorbent assay (ELISA). Plates were washed five times between incubations and all washes and dilutions were carried out with TBS/0.1% Tween 20. A mouse anti-SP-D monoclonal antibody was developed by exposing Sftpd-- mice to purified, full length, human SP-D and selecting cell lines that demonstrated a high affinity for SP-D and minimal cross-reactivity with SP-A (Seven Hills Bioreagents, Cincinnati, OH). ELISA plates were coated with this monoclonal antibody (1µg/ml, 100µl/well) in 0.1 M carbonate buffer, pH 9.6 overnight at 4°C and blocked with 1% BSA for one hour at room temperature. Plates were washed and appropriate dilutions of standards and protein samples were added and incubated for one hour at room temperature. Plates were washed and incubated with rabbit anti-mouse SP-D antiserum (100 µl/well diluted 1/750) for one hour. This was followed by washing and incubation with donkey horseradish peroxidase-conjugated anti-rabbit IgG (100 µl/well diluted 1/10,000) (Jackson Immunoresearch, West Grove, PA) for one hour. After washing plates again, TMB substrate (100 µl/well) (BioFx Laboratories, Owings Mills, MD) was added. The color reaction was stopped after ten minutes with 2 M H2SO4 and plates were read at 450 nm. Typically, this assay results in absorbance changes that are equal, parallel, and linear for mouse SP-D, rSftpdCDM, and human SP-D concentrations between 10 and 150 ng/mL.

Carbohydrate Binding- Direct binding of SP-D to carbohydrate was detected by mixing BALF from 6-8-week old mice with maltosyl-Sepharose linked beads at 4°C for 2 h in 4 mM Tris-HCl, pH 7.4, and 5 mM CaCl2 (23). Binding specificity and calcium dependence were confirmed by the addition of 100 mM maltose and 10 mM EDTA to the binding reaction, respectively. The samples were centrifuged at 10,000 x g for 1 minute and the supernatants were removed. The amount of unbound SP-D in the supernatant was determined by Western blot analysis.

Selective carbohydrate binding was performed as described previously (30). Briefly, microtiter plates were coated with 10µg/ml
mannan at 4 °C overnight, washed, and blocked with 1% bovine serum albumin. BALF was incubated with increasing concentrations of maltose, glucose, galactose, or GlcNAc. Samples were subsequently added to the coated plate and incubated with rabbit anti-SP-D antibody followed by peroxidase conjugated goat anti-rabbit IgG antibody. After washing, o-phenylenediamine was added to each well and the A_{490} nm was measured. The concentration of sugar that inhibited 50% of SP-D binding to the mannan coated plate was defined as the IC_{50}.

**Bacterial Aggregation** - Bacterial aggregation was assessed by measuring light transmission through a bacterial suspension after the addition of SP-D. Purified rSftpDCDM, mouse SP-D, (150 nm based on the monomer molecular weight) or a control reaction that contained protein buffer without SP-D was mixed with 600 µl *Escherichia coli* Y1088 (OD_{700} nm ~1) grown the previous night and resuspended in TBS plus 5 mM CaCl₂ (31). The OD_{700} nm was measured every 2.5 minutes and all values were reported as relative to the absorbance at t=0. The extent of aggregation was determined by the decrease in the optical density of the bacterial suspension. Calcium dependence was confirmed by the inhibition of aggregation in the presence of 10 mM EDTA.

**Lung Morphology** - Lungs from 12-week old mice were fixed at 25 cm of water pressure with 4% paraformaldehyde in phosphate buffered saline (PBS) and processed into paraffin blocks. Sections (5 µm) from each lobe were stained with hematoxylin and eosin. Immunohistochemistry for SP-D was performed at dilutions of 1:200 by using a rabbit polyclonal antibody generated to murine SP-D. Immune complexes were detected using an avidin-biotin-peroxidase technique (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA).

**Metalloproteinase Activity** - Alveolar macrophages (5 X 10⁵) were isolated by centrifuging BALF and cultured for 24 h in AIM-V medium (GIBCO, Carlsbad, CA). Proteinases in the conditioned media were assayed by zymography as described previously (32).

**Phospholipid Analysis** - Saturated phosphatidylcholine (Sat PC) was measured in homogenized lung tissue and BALF from 6-8- week old mice (n = 6-8 mice for each genotype) as previously described (33).

**Isolation of Large and Small Aggregate Surfactant** - Large and small aggregate surfactant lipids were isolated from rSftpDCDM⁷⁺/Sftp⁺⁻ mice (n=6) as previously described (14). Briefly, bronchoalveolar lavage was performed 5 times with 1 ml normal saline. BALF was centrifuged at 40,000 g over a 0.8 M sucrose cushion for 15 minutes. Small aggregate surfactant was collected from the supernatant. Large aggregate surfactant was collected from the interface, diluted with normal saline, and centrifuged again at 40,000 g for 15 minutes. The large aggregate pellet was dissolved in normal saline. An equal volume of each material was dried, diluted in Laemmli buffer and analyzed by Western blot.

Ultrastructure of lipid aggregates was determined in pooled BALF samples (n=5/pool) by electron microscopy from 6-8 week old mice as previously described (14). Briefly, large and small aggregate surfactant was isolated from BALF, fixed with glutaraldehyde, paraformaldehyde and CaCl₂ in 0.1M sodium cacodylate buffer at 4°C and stained with osmium tetroxide, potassium ferrocyanide, and uranyl acetate. After dehydration, they were embedded in Embed812 resin (Electron Microscopy Sciences, Fort Washington, PA) and ultrathin sections (90nm) were obtained. Random electron micrographs were taken and ultrastructure was evaluated.

**Influenza A Virus** - Experiments utilized influenza A virus strain H₃N₂ A/Phillipines/82 (IAV) and were performed as previously described (34). Briefly, IAV was grown in chorioallantoic fluid of 10-day-old embryonated hen eggs. Virus was purified and stored frozen in PBS until use. Six-week-old (n = 6-8) mice were anesthetized with inhaled isoflurane and inoculated intratracheally with 5 x 10⁵ fluorescent foci in 80 µl PBS. Quantitative IAV cultures were performed 3 days after inoculation. Lungs were removed, homogenized in 2 ml PBS, and aliquots were frozen. IL-6, TNF-α, and IFN-γ concentrations were determined by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN). IAV titers were determined by incubating lung homogenates with Madin-Darby canine kidney monolayers for 7 h at 37 °C. Monolayers were washed, fixed and incubated with monoclonal antibody against IAV.
nucleoprotein followed by a rhodamine-labeled goat anti-mouse IgG. Fluorescent foci were counted and the resulting viral titer was expressed as fluorescent foci/gram lung weight.

SP-D binding to IAV was monitored by hemagglutination inhibition assays (n = 3). Purified mouse SP-D and rSftpdCDM were compared. Sample protein was serially diluted in 96 well round bottom plates with IAV in PBS with 0.5 mM CaCl₂ and 0.5 mM MgCl₂. After incubating the protein/IAV mixture at room temperature for 10 min, fertilized chicken egg erythrocytes were added and the samples were incubated for an additional 2 h. The minimal amount of SP-D or rSftpdCDM needed to fully inhibit agglutination was observed and the number of hemagglutination units inhibited per pmol SP-D or rSftpdCDM was reported.

Data Analysis- Where appropriate, either a representative experiment from one mouse line was shown or results from each line were averaged and data was analyzed by unpaired Student’s t-tests.

RESULTS

**Transgenic Mouse Lines-** rSftpdCDMTg/+ and Sftpd⁻/⁻ transgenic mice were produced by nuclear injection of the rSftpdCDM gene into FVB/N mice and backcrossed to Sftpd⁻/⁻ mice. The rat Sftpd gene is 92% identical (based on nucleotide and mature protein amino acid sequence) to the mouse Sftpd gene and multiple studies indicate mouse and rat SP-D have nearly identical properties. Four founder mice were identified using transgene specific PCR on tail clip DNA. Germ line transmission was demonstrated in all four lines and the transgene was inherited as an autosomal gene following Mendelian inheritance. Survival and breeding were not influenced by the rSftpdCDM⁺/⁻ transgene. Expression and secretion of transgenic protein rSftpdCDM was confirmed in all four mouse lines by Western blot analysis of mouse BALF using a rabbit anti-mouse SP-D antibody (data not shown). Two mouse lines with similar levels of rSftpdCDM protein expression were selected for further breeding and analysis. Experiments were done in parallel with both lines and results were similar.

**Expression of rSftpdCDM Protein-** As determined by ELISA assays, levels of SP-D in BALF from wild type mice were 1.2 µg/ml compared to rSftpdCDM levels of 11 µg/ml in rSftpdCDM⁺/⁻ mice. Rabbit anti-mouse SP-D antibody was used to detect SP-D and rSftpdCDM in BALF (Fig. 2). The monomeric form of the mutant protein migrated under reducing conditions at the predicted molecular mass of 22-kDa. rSftpdCDM migrated slightly slower than the 60-kDa standard under non-reducing conditions indicating that the disulfide linkages normally observed between monomeric chains in the wild type N-terminal domain also formed in the mutant protein. To determine the ability of the rSftpdCDM to form high order complexes, the protein was analyzed by Sepharose column chromatography. Under these conditions, the majority of rSftpdCDM migrated between apoferritin and B-amylase (molecular weights of 443-kDa and 200-kDa, respectively) at a position equaling 270-kDa, which is the molecular weight expected if adding the N-terminal domain to the CRD trimer promoted non-covalent interactions between four trimeric subunits (dodecamer). Smaller peaks were observed at the expected positions of molecules composed of 1, 2, 30, and more trimers suggesting the purified mutant protein consists of a heterogeneous population of multimers. While the mutant protein effectively self assembled to higher order structures, it did not form intermediate molecular weight heteropolymers of mutant and wild type protein when expressed in a wild type SP-D background (data not shown).

**R*SftpdCDM Binds Carbohydrate-** To compare the carbohydrate binding properties of wild type SP-D and rSftpdCDM, binding to maltoosyl-Sepharose was evaluated (Fig. 3). Protein from both wild type and mutant BALF bound maltose. Binding was inhibited by the addition of EDTA, indicating that rSftpdCDM maintained calcium dependent lectin activity. In addition, binding to maltoosyl-sepharose was reversed by the addition of free maltose confirming the specificity of maltose interactions.

The saccharide binding preference of SP-D and rSftpdCDM was assessed using BALF and inhibition of binding to yeast mannan by competing saccharides (Table I). Under the conditions utilized, a higher binding affinity for the competing saccharide was indicated by a lower concentration of saccharide needed to disrupt
protein-mannan interactions. Maltose was the preferred binding substrate of the carbohydrates tested with an IC₅₀ of 1 mM with BALF from wild type mice, consistent with results previously reported (27,30). The order of binding preference was maltose>glucose>galactose>GlcNAc for both SP-D and rSftpCDM. While the mutant and wild type proteins had similar relative saccharide binding preferences, the IC₅₀ values observed with rSftpCDM were lower than SP-D. Assuming there are no compositional differences in the mutant and wild type BALF that might influence binding activity, these results suggest that the mutant protein may have a higher carbohydrate binding affinity.

rSftpCDM Aggregates Bacteria-

Previous studies demonstrated that while mutant trimeric forms of SP-D (rSftpser15, 20 and trimeric CRDs) bind carbohydrates, they do not effectively aggregate infectious particles (11,31). Similar results were observed in earlier work with a SP-D collagen deletion mutant (35). However, unlike the current rSftpCDM, the earlier collagen deletion mutant did not form multimers. In addition, studies with chimeric collectins containing domains of SP-D, MBP, and conglutinin suggest that the relatively large collagen domain of SP-D increases bacterial aggregation activity (25). To determine if rSftpCDM induced bacterial aggregation, light transmission through a suspension of *Escherichia coli* was monitored after the addition of purified rSftpCDM (Fig. 4). The drop in absorbance in the rSftpCDM reaction indicates that the multimeric mutant protein effectively aggregates bacteria. Aggregation by rSftpCDM was completely inhibited by the addition of EDTA, confirming the calcium dependence for protein function. When compared to a reaction that contained an equal molar concentration of wild type SP-D based on the molecular weight of the monomer chain, the aggregation activity of rSftpCDM was slightly better than the wild type protein. Thus, bacterial aggregation is not dependent on appropriate spacing of the CRD by the collagenous domain of SP-D.

Lung Morphology-

Deletion of the mouse *SftpD* gene caused several distinct alterations in lung morphology, including emphysema and accumulations of enlarged foamy macrophages (13,16-18). To determine if the mutant protein influenced these findings, pulmonary structure was assessed in wild type and *SftpD* mice expressing rSftpCDM (Fig. 5). Immunostaining revealed marked expression of rSftpCDM in alveolar type II cells and bronchiolar epithelial cells. However, rSftpCDM did not alter the lung morphology in 12 week-old wild type mice. Moreover, the mutant protein did not correct the abnormal morphology typically observed in *SftpD* mice. Enlarged, foamy macrophage accumulations and emphysema were detected readily in both transgenic mouse lines expressing rSftpCDM in a *SftpD* background. Therefore, while targeted expression of the full length rat *SftpD* gene in *SftpD* mice fully rescues the *SftpD* phenotype (36), expression of rSftpCDM does not correct the abnormal lung morphology in *SftpD* mice.

**MMP-9 and MMP-2 Activity-** Proteinase activity gels were used to assess MMP-9 and MMP-2 activity in media containing alveolar macrophages from wild type, *SftpD*, and rSftpCDM mice (Fig. 6). While minimal MMP-9 and MMP-2 activity was observed in wild type samples, metalloproteinase activity was markedly elevated in both *SftpD*, and rSftpCDM mice indicating that rSftpCDM does not correct the increased metalloproteinase production by alveolar macrophages from *SftpD* mice (32).

Lung Phospholipids and Surfactant Structure-

SP-D selectively interacts with small aggregate surfactant in adult mice and regulates the uptake and catabolism of surfactant lipids by alveolar type II cells (13,14). Consequently, mice lacking SP-D have two- to five-fold higher surfactant pool sizes (13,16). Moreover, surfactant isolated from *SftpD* mice has abnormally large aggregate lipid structures and small aggregates consisting of atypical multilamellated forms (14). To evaluate if rSftpCDM corrected surfactant phospholipids pool sizes, Sat PC in BALF and lung homogenates were assessed in wild type and *SftpD* mice with and without rSftpCDM (Fig. 7). Expression of the mutant protein did not alter alveolar, tissue, or total Sat PC levels in wild type mice and it did not correct the elevated Sat PC levels in *SftpD* mice. In addition, to determine if rSftpCDM corrected the abnormal surfactant ultrastructure characteristic of *SftpD* mice, large and small
aggregate surfactant was examined by electron microscopy (Fig. 8). The large aggregate fraction from Sftpd−/− and rSftpdCDM+/+/Sftpd−/− mice contained abnormal, large lamellated lipid structures. The small aggregate fraction in wild type mice consisted of single layer sheets or vesicles, while atypical multilayered structures predominated in the small aggregate fraction from Sftpd−/− and rSftpdCDM+/+/Sftpd−/− mice. Despite the failure of rSftpdCDM to correct surfactant lipid structure, analysis of large and small aggregate surfactant from rSftpdCDM+/+/Sftpd+/+ mice revealed that rSftpdCDM partitioned with small aggregate surfactant in a manner that was similar to SP-D (Fig. 9). This is in contrast to SP-A which segregated primarily with large aggregate surfactant. Therefore, these results demonstrate that the collagen domain of SP-D is not required for the selective partitioning of SP-D with small aggregate surfactant, but it is required for normal surfactant ultrastructure that in turn influences uptake by alveolar type II cells.

Correction of Influenza A Infection by rSftpdCDM- Previous in vitro studies demonstrated that SP-D binds influenza A virus (IAV) and enhanced IAV binding and uptake by neutrophils (31,37,38). Decreased viral clearance and enhanced inflammation was observed in Sftpd−/− mice exposed to intratracheal IAV (21). As determined by hemagglutination inhibition assays, rSftpdCDM effectively bound IAV and inhibited IAV mediated hemagglutination (Fig. 10). Moreover, hemagglutination inhibition activity of rSftpdCDM was ~2-fold greater than that observed with an equal molar (based on the molecular weight of a SP-D or rSftpdCDM monomer) amount of wild type SP-D. To further evaluate the anti IAV activity of rSftpdCDM, IAV was administered to mouse lungs intratracheally and the viral titers and cytokine response (Fig. 11) were measured 3 days later. In contrast to Sftpd−/− mice, no detectable IAV was recovered from the wild type or rSftpdCDM+/+/Sftpd−/− lung homogenates. In addition, the increased IL-6, TNFα, and IFNγ levels observed in IAV challenged Sftpd−/− mice were restored to wild type levels in rSftpdCDM+/+/Sftpd−/− mice. Taken together these results demonstrate that rSftpdCDM completely corrects viral binding, clearance, and inflammatory responses observed in Sftpd−/− mice.

**DISCUSSION**

SP-D plays multiple complex roles in pulmonary physiology including binding and clearing of infectious pathogens, regulation of the innate host defense system, airspace remodeling, and surfactant phospholipid metabolism (1-3). In the present study, the function of the SP-D collagen domain was evaluated by expressing a SP-D collagen deletion mutant in the lungs of wild type and Sftpd−/− mice.

Deletion of the collagen domain resulted in the secretion of an approximately 22 kDa protein that migrated as a trimer under non-reducing conditions. These findings are consistent with in vitro studies describing an SP-D collagen deletion mutant that was expressed in CHO cells by Ogasawara and Voelker (35,39). In contrast to these earlier studies, the majority of the present rSftpdCDM formed higher order complexes of four trimeric subunits (dodecamer) when expressed in the mouse respiratory system. The reason for this disparity is unclear, but given the close proximity of the regions deleted, it likely reflects differences in the protein expression systems utilized. In addition, the finding that adding the N-terminus to the trimeric CRD facilitates dodecamer formation supports previous results that indicate the structural predilection for dodecamers is contained within the N-terminal domain (40).

Ogasawara and Voelker demonstrated that the collagen deletion mutant bound mannose-sepharose and phosphatidylinositol with affinities comparable to wild type SP-D, but binding to mannosyl bovine serum albumin and glucosylceramide was somewhat diminished. Although specific binding constants were not determined in the present work, when comparing equal molar quantities of SP-D and rSftpdCDM, the mutant protein binding activity for virus, bacteria, and carbohydrate was consistently equal to or greater than wild type SP-D demonstrating that deletion of the collagen domain does not inhibit substrate binding.

Despite the considerable binding affinity of rSftpdCDM, expression of the mutant protein failed to correct the aberrant alveolar macrophage activity characteristic of Sftpd−/− mice (13,16-18). Foamy macrophages, increased secretion of metalloproteinases, and emphysema persisted despite expression of high levels of rSftpdCDM.
In contrast, influenza virus binding, clearance and the associated cytokine response mediated by rSftpdCDM were similar to or better than observed in wild type mice. Similar findings of abnormal baseline macrophage activation in the setting of a normal response to influenza virus were also described in studies utilizing a SP-D conglutinin (SftpdCong) fusion protein consisting of the N-terminus and collagen domains of rat SP-D and the neck and CRD of conglutinin (27). Several interesting considerations regarding SP-D are raised from these observations. First, even though the rSftpdCDMTg+/Sftpd−/− alveolar macrophages have an abnormal histological appearance and baseline level of activity, they still are able to mount an effective and relatively normal inflammatory response to the infectious pathogen, influenza A. Second, although limited by the fact that only one infectious pathogen was tested, the normal cytokine response observed following influenza A viral infection suggests that the elevated baseline macrophage activity in Sftpd−/− mice is not due to the inability to clear a persistent low level infection. Finally, the divergence of macrophage baseline activity and the inflammatory response to viral infection in rSftpdCDM+/Sftpd−/− mice suggests that SP-D regulates these processes through two independent pathways.

Previous studies by Gardai et al. described simultaneous inhibitory and stimulatory roles for SP-D in macrophage regulation that were proposed to be mediated through two competing signaling cascades (41). In the first, SP-D inhibited NFκB and subsequent immune cell activation through binding of the CRD to the signal inhibitory regulatory protein α (SIRPα). In the second, binding to SIRPα was inhibited by the presence of an infectious particle within the CRD, thereby allowing interactions between the collagenous domain or N-terminus of SP-D and the macrophage activating receptor calreticulin/CD91. This model is supported by evidence from the collagen deletion mutant described by Ogasawara and Voelker, as well as, data derived from a recombinant fragment of SP-D consisting of only a trimeric CRD that indicate both proteins inhibited alveolar macrophage activation, presumably through interactions with SIRPα (41). However, this model also predicts that the fully functional CRD of rSftpdCDM in the present study would bind SIRPα and inhibit macrophage activation. Moreover, rSftpdCDM mediated stimulation of macrophage activation through calreticulin/CD91 would be limited by the absence of a collagen domain. Therefore, the model proposed by Gardai et al. might predict that the rSftpdCDM+/Sftpd−/− mouse would display an alveolar macrophage phenotype that is predominantly anti-inflammatory. The enlarged foamy macrophages, elevated metalloproteinases, and emphysema indicate that rSftpdCDM+/Sftpd−/− mice are in a pro-inflammatory state at baseline and seemingly contradict the model proposed by Gardai et al. As with any mutational study, the current findings may be a result of an unanticipated change in the structure of the CRD, neck or N-terminal domains of rSftpdCDM as a result of the collagenous domain deletion. This qualification notwithstanding, an alternative explanation exists that may resolve this discrepancy. The present study suggests that SP-D regulates activation of the pulmonary immune system through two independent pathways. The first would control activation of alveolar macrophages in the presence of infectious particles and might involve the competing activities of SIRPα and calreticulin/CD91. Activation of these receptors would be appropriately balanced by the CRD and oligomerized N-terminus of rSftpdCDM and would explain the wild type response to influenza virus exhibited by the rSftpdCDM+/Sftpd−/− mice. A second pathway would control the baseline level of alveolar macrophage activation in the lung and in the absence of appropriate regulation elicit the phenotype of enlarged foamy macrophages, increased metalloproteinases, and emphysema. The results of the present study suggest that rSftpdCDM does not effectively activate this pathway. Similar aberrant macrophage activation was reported with SftpdCong, rSftpdSer15, 20, and rSftpa/d (22,23,27). Therefore, while the receptors and signaling molecules that mediate this pathway are unknown, SP-D mediated regulation of baseline macrophage activity, alveolar remodeling and surfactant homeostasis requires a multimeric SP-D containing the collagen domain.

The failure of rSftpdCDM to correct the enlarged foamy macrophages, emphysema, and pulmonary phospholipid accumulations observed
in Sftpd⁻/⁻ mice is in contrast to earlier work with a recombinant fragment of SP-D consisting of a trimeric CRD and neck domain, which partially corrected the abnormal macrophages and surfactant pool sizes in Sftpd⁻/⁻ mice (15). The reason why a single trimeric CRD would partially resolve the abnormalities that an oligomerized trimeric CRD fails to correct is uncertain, but it may reflect unanticipated changes that sometimes occur in mutant proteins or differences in the concentration of the mutant SP-D’s utilized in each study. Alternatively, this inconsistency may suggest a complex interplay between the N-terminal domain and the CRD in macrophage regulation and control of phospholipid pool sizes.

Although alveolar macrophages and type II epithelial cells equally contribute to surfactant phospholipid catabolism, previous studies demonstrated that SP-D regulates surfactant pool size by enhancing surfactant uptake by alveolar type II cells (14). Specifically, SP-D maintains normal surfactant ultrastructure by selectively interacting with small aggregate surfactant, thereby facilitating surfactant uptake by type II cells. The lipid binding specificity of collectins is mediated by the CRD in vitro (42-45) and intranasal administration of trimeric CRDs decreased intra-alveolar lipid accumulations in Sftpd⁻/⁻ mice (15,24). In contrast, the SP-D CRD and neck domain in the rSftpa/d fusion protein failed to correct surfactant lipid ultrastructure or lower the phospholipid levels when expressed in Sftpd⁻/⁻ mice (22). In the present study, the addition of the SP-D N-terminus to the CRD failed to correct surfactant ultrastructure or increased phospholipid levels. Therefore, normal surfactant ultrastructure and the uptake of surfactant by type II cells depend on the collagen domain.

Although rSftpa/d included the neck and CRD of SP-D, analysis of large and small aggregate surfactant lipids demonstrated that rSftpa/d partitioned with large aggregate surfactant in a manner similar to SP-A (22). In contrast, rSftpdCDM, which included the neck, CRD, and N-terminal domains of SP-D, partitioned with small aggregate surfactant like the full length SP-D protein. Taken together, these studies suggest that while phospholipid binding in vitro may be mediated by the CRD, the structural features of SP-D (and SP-A) that influence partitioning between small versus large aggregate surfactant are contained within the N-terminal domain.

In summary, our study provides further support for the importance of the distinct molecular domains of SP-D in mediating the complex functions of this protein. The finding that the collagen deletion mutant fails to correct the abnormal lung morphology or surfactant lipid homeostasis of Sftpd⁻/⁻ mice supports a role of the collagen domain that is beyond proper spacing of trimeric subunits for bacterial aggregation. Assimilating the results of this study with those of prior reports on SP-D reveals that while our understanding of SP-D is improving, inconsistencies still exist. The CRD is critical for binding to lipopolysaccharide, viruses, bacteria, fungus, and lipids. Nonetheless, SP-D interactions with small aggregate surfactant and uptake of surfactant by alveolar type II cells in vivo do not depend on the CRD alone. The collagen domain is required for surfactant lipid structure and metabolism, but it is not needed to effectively aggregate bacteria or to suppress inflammatory responses to influenza A virus. The N-terminal domain is critical for partitioning with large versus small aggregate surfactant lipids in adult mice and interactions between interchain N-terminal domains are essential for oligomerization, which in turn, influences CRD binding affinity, phospholipid catabolism, and the inflammatory response to infectious pathogens. Finally, at the time of this writing, all SP-D mutant proteins that delete or substitute even a single domain of SP-D fail to fully correct the enlarged, foamy macrophages that are characteristic of Sftpd⁻/⁻ mice suggesting that this function of SP-D is dependent on a full length, multimeric protein.

**FOOTNOTES**

§Supported by NIH HL56387 (JAW), HL63329 (MI), HL68861 (FXM), NHLBI HL60931 (KH)

¹The abbreviations used are: SP-D, surfactant protein D; rSftpdCDM, surfactant protein D collagen deletion mutant protein; Sftpd⁻/⁻, surfactant protein D null mice; SP-A, surfactant protein A; MBP, mannose binding protein; CRD, carbohydrate recognition domain; TNF, tumor necrosis factor; IL,
interleukin; BALF, bronchoalveolar lavage fluid; TBS, tris buffered saline; GlcNAc, N-acetyl glucosamine; PBS, phosphate buffered saline; IAV, influenza A virus; ELISA, enzyme linked immunosorbent assay; MMP, matrix metalloproteinase; Sat PC, saturated phosphatidylcholine; SIRP, signal inhibitory protein; NF, nuclear factor.

ACKNOWLEDGEMENT

We are grateful to Mitchell R. White for invaluable advice and technical assistance in influenza A viral assays and to Sarah K. Yoshimura for critical reading of the manuscript.

REFERENCES

1. Hawgood, S., and Poulain, F. R. (2001) *Annu Rev Physiol* **63**, 495-519.
2. Crouch, E., and Wright, J. R. (2001) *Annu Rev Physiol* **63**, 521-554.
3. Whitsett, J. A. (2005) *Biol Neonate* **88**, 175-180.
4. Rust, K., Grosso, L., Zhang, V., Chang, D., Persson, A., Longmore, W., Cai, G. Z., and Crouch, E. (1991) *Arch Biochem Biophys* **290**, 116-126.
5. Shimizu, H., Fisher, J. H., Papst, P., Benson, B., Lau, K., Mason, R. J., and Voelker, D. R. (1992) *J Biol Chem* **267**, 1853-1857.
6. Crouch, E., Rust, K., Veile, R., Donis-Keller, H., and Grosso, L. (1993) *J Biol Chem* **268**, 2976-2983.
7. Lu, J., Wiedemann, H., Holmskov, U., Thiel, S., Timpl, R., and Reid, K. B. (1993) *Eur J Biochem* **215**, 793-799.
8. van de Wetering, J. K., van Golde, L. M., and Batenburg, J. J. (2004) *Eur J Biochem* **271**, 1229-1249.
9. Zhang, P., McAlinden, A., Li, S., Schumacher, T., Wang, H., Hu, S., Sandell, L., and Crouch, E. (2001) *J Biol Chem* **276**, 15808-15813.
10. Crouch, E., Persson, A., Chang, D., and Heuser, J. (1994) *J Biol Chem* **269**, 17311-17319.
11. Brown-Augsburger, P., Hartshorn, K., Chang, D., Rust, K., Fliszar, C., Welgus, H. G., and Crouch, E. C. (1996) *J Biol Chem* **271**, 13724-13730.
12. Crouch, E., Chang, D., Rust, K., Persson, A., and Heuser, J. (1994) *J Biol Chem* **269**, 15808-15813.
13. Ikegami, M., Whitsett, J. A., Jobe, A., Ross, G., Fisher, J., and Korfhagen, T. (2000) *Am J Physiol Lung Cell Mol Physiol* **279**, L468-476.
14. Ikegami, M., Na, C. L., Korfhagen, T. R., and Whitsett, J. A. (2005) *Am J Physiol Lung Cell Mol Physiol* **288**, L552-561.
15. Clark, H., Palaniyar, N., Strong, P., Edmondson, J., Hawgood, S., and Reid, K. B. (2002) *J Immunol* **169**, 2892-2899.
16. Korfhagen, T. R., Sheftelyevich, V., Burhans, M. S., Bruno, M. D., Ross, G. F., Wert, S. E., Stahlman, M. T., Jobe, A. H., Ikegami, M., Whitsett, J. A., and Fisher, J. H. (1998) *J Biol Chem* **273**, 28438-28443.
17. Botas, C., Poulain, F., Akiyama, J., Brown, C., Allen, L., Goerke, J., Clements, J., Carlson, E., Gillespie, A. M., Epstein, C., and Hawgood, S. (1998) *Proc Natl Acad Sci USA* **95**, 11869-11874.
18. Wert, S., Jones, T., Korfhagen, T., Fisher, J., and Whitsett, J. (2000) *Chest* **117**, 248S.
19. LeVine, A. M., Whitsett, J. A., Gwozdz, J. A., Richardson, T. R., Fisher, J. H., Burhans, M. S., and Korfhagen, T. R. (2000) *J Immunol* **165**, 3934-3940.
20. LeVine, A. M., Elliott, J., Whitsett, J. A., Srikiatkhachorn, A., Crouch, E., DeSilva, N., and Korfhagen, T. (2004) *Am J Respir Cell Mol Biol* **31**, 193-199.

21. LeVine, A. M., Whitsett, J. A., Hartshorn, K. L., Crouch, E. C., and Korfhagen, T. R. (2001) *J Immunol* **167**, 5868-5873.

22. Zhang, L., Ikegami, M., Korfhagen, T. R., McCormack, F. X., Yoshida, M., Senior, R. M., Shipley, J. M., Shapiro, S. D., and Whitsett, J. A. (2006) *Am J Physiol Lung Cell Mol Physiol*.

23. Zhang, L., Ikegami, M., Crouch, E. C., Korfhagen, T. R., and Whitsett, J. A. (2001) *J Biol Chem* **276**, 19214-19219.

24. Clark, H., Palaniyar, N., Hawgood, S., and Reid, K. B. (2003) *Ann N Y Acad Sci* **1010**, 113-116.

25. Hartshorn, K. L., White, M. R., and Crouch, E. C. (2002) *Infect Immun* **70**, 6129-6139.

26. Wert, S. E., Glasser, S. W., Korfhagen, T. R., and Whitsett, J. A. (1993) *Dev Biol* **156**, 426-443.

27. Zhang, L., Hartshorn, K. L., Crouch, E. C., Ikegami, M., and Whitsett, J. A. (2002) *J Biol Chem* **277**, 22453-22459.

28. Reed, J. A., Ikegami, M., Robb, L., Begley, C. G., Ross, G., and Whitsett, J. A. (2000) *Am J Physiol Lung Cell Mol Physiol* **278**, L1164-1171.

29. Wert, S. E., Yoshida, M., Korfhagen, T. R., and Whitsett, J. A. (1993) *Dev Biol* **156**, 426-443.

30. Hartshorn, K. L., Crouch, E., White, M. R., Colamussi, M. L., Kakkanatt, A., Tauber, B., Shepherd, V., and Sastry, K. N. (1998) *J Immunol Methods* **220**, 139-149.

31. Lim, B. L., Wang, J. Y., Holmskov, U., Hoppe, H. J., and Reid, K. B. (1994) *Biochem Biophys Res Commun* **202**, 1674-1680.

32. Wert, S. E., Yoshida, M., Korfhagen, T. R., and Whitsett, J. A. (2000) *Proc Natl Acad Sci U S A* **97**, 5972-5977.

33. Hartshorn, K. L., Crouch, E., White, M. R., Colamussi, M. L., Kakkanatt, A., Tauber, B., Shepherd, V., and Sastry, K. N. (1998) *Am J Physiol* **274**, L958-969.

34. Wert, S. E., Yoshida, M., LeVine, A. M., Ikegami, M., Jones, T., Ross, G. F., Fisher, J. H., Korfhagen, T. R., and Whitsett, J. A. (2000) *Proc Natl Acad Sci U S A* **97**, 5972-5977.

35. Ogasawara, Y., and Voelker, D. R. (1995) *J Biol Chem* **270**, 19052-19058.

36. Hartshorn, K. L., Crouch, E., White, M. R., Colamussi, M. L., Kakkanatt, A., Tauber, B., Shepherd, V., and Sastry, K. N. (1998) *Am J Physiol* **274**, L958-969.

37. Wert, S. E., Yoshida, M., LeVine, A. M., Ikegami, M., Jones, T., Ross, G. F., Fisher, J. H., Korfhagen, T. R., and Whitsett, J. A. (2000) *Proc Natl Acad Sci U S A* **97**, 5972-5977.

38. Hartshorn, K. L., Crouch, E., White, M. R., Colamussi, M. L., Kakkanatt, A., Tauber, B., Shepherd, V., and Sastry, K. N. (1998) *Am J Physiol* **274**, L958-969.

39. Hartshorn, K. L., Crouch, E., White, M. R., Colamussi, M. L., Kakkanatt, A., Tauber, B., Shepherd, V., and Sastry, K. N. (1998) *Am J Physiol* **274**, L958-969.

40. Hartshorn, K. L., Crouch, E., White, M. R., Colamussi, M. L., Kakkanatt, A., Tauber, B., Shepherd, V., and Sastry, K. N. (1998) *Am J Physiol* **274**, L958-969.

41. Gardai, S. J., Xiao, Y. Q., Dickinson, M., Nick, J. A., Voelker, D. R., Greene, K. E., and Henson, P. M. (2003) *Cell* **115**, 13-23.

42. Ogasawara, Y., McCormack, F. X., Mason, R. J., and Voelker, D. R. (1994) *J Biol Chem* **269**, 29785-29792.
43. Kishore, U., Wang, J. Y., Hoppe, H. J., and Reid, K. B. (1996) *Biochem J* **318** (Pt 2), 505-511.
44. Sano, H., Kuroki, Y., Honma, T., Ogasawara, Y., Sohma, H., Voelker, D. R., and Akino, T. (1998) *J Biol Chem* **273**, 4783-4789.
45. DeSilva, N. S., Ofek, I., and Crouch, E. C. (2003) *Am J Respir Cell Mol Biol* **29**, 757-770.
FIG. 1. **Schematic representation of the rSftpdCDM transgene.** The rSftpdCDM cDNA was generated using recombinant PCR to delete the 177 amino acid sequence of the collagen domain from rat SP-D. The rSftpdCDM cDNA was inserted into the EcoRI site of 3.7hSPC/SV40 expression vector and sequenced. The transgene was identified by transgene specific PCR primers used to generate the transgenic mice.

FIG. 2. **Analysis of bronchoalveolar lavage fluid and purified protein from mice expressing rSftpdCDM.** A. BALF from wild type (lanes 1 and 3) and rSftpdCDM<sup>Tg</sup>+/Sftpd<sup>-/-</sup> (lanes 2, and 4) mice were resolved on SDS-PAGE under reducing (Reduced) conditions. Disulfide linked trimers were resolved under non reducing (Non Reduced) conditions. Protein was detected with rabbit anti-mouse SP-D antibody. Protein levels were determined by ELISA and expressed as relative to the peak fraction. The arrows indicate the peak position of eluted standards: a, blue dextran; b, thyroglobulin; c, apoferritin; d, β-amylase; e, alcohol dehydrogenase; f, albumin; g, carbonic anhydrase.

FIG. 3. **SP-D and rSftpdCDM proteins bind maltosyl-Sepharose.** BALF from rSftpdCDM<sup>Tg</sup>+/Sftpd<sup>+/+</sup> mice was incubated with maltosyl-Sepharose beads followed by centrifugation to pellet the beads. The resulting supernatant was evaluated by SDS-PAGE and Western analysis. Binding of endogenous SP-D (SP-D) or mutant rSftpdCDM (CDM) was indicated by the absence of protein in the supernatant. BALF was incubated with (+) or without (-) beads, or with beads and EDTA (EDTA) or excess free maltose (Maltose).

FIG. 4. **rSftpdCDM aggregates Escherichia coli.** Bacterial suspensions (n = 3) were incubated with 150 nM purified rSftpdCDM (CDM, open triangles) or wild type SP-D (SP-D, closed circles). Absorbance values were recorded at the indicated times before (t = 0) and after addition of the indicated protein and reported as relative to the absorbance value at t = 0. Aggregation was indicated by the clustering of bacterial particles and a subsequent drop in absorbance. Aggregation with rSftpdCDM was completely reversed by the addition of EDTA to the reaction mixture (EDTA, open circles). Control (closed circles), protein buffer alone. Standard errors were <10% for all time points.

FIG. 5. **rSftpdCDM does not correct lung morphology in Sftpd<sup>-/-</sup> mice.** Lungs were fixed and stained with hematoxylin and eosin. A, lungs from wild type mice; B, rSftpdCDM<sup>Tg</sup>+/ in a wild type background; C, immunostaining of SP-D in wild type mice; D, Sftpd<sup>-/-</sup> mice; E, rSftpdCDM<sup>Tg</sup>+/ in Sftpd<sup>-/-</sup> background; F, immunostaining of rSfpdCDM in a Sftpd<sup>-/-</sup> background. Arrowheads point to enlarged, foamy macrophages. Arrows point to SP-D immunostaining in type II cells and bronchiolar epithelial cells. Bars represent 100 μm. Insets show same tissue under higher magnification.

FIG. 6. **rSftpdCDM does not correct increased metalloproteinase activity in Sftpd<sup>-/-</sup> mice.** Proteinase activity in conditioned media from alveolar macrophages isolated from wild type (WT), rSftpdCDM<sup>Tg</sup>+/Sftpd<sup>-/-</sup>, and Sftpd<sup>-/-</sup> mice was evaluated on zymogram gels. BALF was diluted as shown and MMP-2 and MMP-9 activity was indicated by a clear band at 72 and 92 kDa, respectively.

FIG. 7. **Increased lung saturated phosphatidylcholine in rSftpdCDM<sup>Tg</sup>/Sftpd<sup>-/-</sup> mice.** Alveolar, tissue and total Sat PC levels were determined in wild type (Sftpd<sup>+/+</sup>) and SP-D null (Sftpd<sup>-/-</sup>) mice with (open bars) or without (closed bars) the rSftpdCDM transgene (n = 6-8 mice for each genotype). Values were normalized for body weight. Sat PC levels in Sftpd<sup>-/-</sup> mice were significantly (p≤0.05) higher than wild type controls. The addition of rSftpdCDM did not significantly (p=0.48, 0.47, 0.40 for alveolar, tissue and total, respectively) correct this increase. Error bars indicate standard error.
**FIG. 8.** *rSftpdCDM does not correct surfactant ultrastructure in Sftpdc−/− mice.* Ultrastructure of large aggregate (LA) and small aggregate (SA) surfactant isolated from wild type (*Sftpdc+/+CDM−*), *Sftpdc−/−* (*Sftpdc−/−CDM−*) and *rSftpdCDMTg+/Sftpdc−/−* (*Sftpdc−/−CDM+/−*) mice. Closed arrowheads point to normal large aggregate lamellar bodies and small aggregate single layer sheets and vesicles. Open arrowheads point to abnormal large lipid structures in large aggregate surfactant. Arrows point to atypical multilayered structures in small aggregate surfactant. Photomicrographs are representative of samples pooled from 3 mice of each genotype.

**FIG. 9.** *rSftpdCDM partitions with small aggregate surfactant.* BALF was isolated from *rSftpdCDMTg+/Sftpdc+/+* mice and surfactant lipids were separated into small (SA) and large (LA) aggregate fractions by centrifugation. A. SP-D (*SP-D*) and *rSftpdCDM* (*CDM*) were detected in the BALF prior to lipid isolation (BAL) and in small aggregate lipids by Western blot with anti-SP-D antibody. B. Identical samples were analyzed with anti-SP-A antibody. SP-A (*SP-A*) was detected in BALF and in large aggregate surfactant.

**FIG. 10.** *rSftpdCDM binds Influenza A virus.* Binding to influenza A virus was determined by hemagglutination inhibition assays (n = 3). The number of hemagglutination units inhibited per pmol of *rSftpdCDM* (*CDM*) or wild type SP-D (*SP-D*) is shown. Error bars indicate standard error.

**FIG. 11.** *rSftpdCDM corrects response to Influenza A virus in Sftpdc−/− mice.* IL-6, TNFα, and IFNγ levels, as well as, influenza A viral titers and were determined in lung homogenates three days after intratracheal installation of virus into wild type (*WT*), *rSftpdCDMTg+/Sftpdc−* (*CDM*), or *Sftpdc−/−* (*Sftpdc−/−*) mice (n = 6-8 mice for each genotype). Viral titers are expressed as relative values with *Sftpdc−/−* mice normalized to 100%. Inflammatory cytokine levels and viral titers were significantly higher (P<0.05) in *Sftpdc−/−* mice when compared to wild type or *rSftpdCDMTg+/Sftpdc−* mice. Error bars indicate standard error.
TABLE I

*Binding of SP-D and rSftpdCDM to Carbohydrates*

BALF from wild type and *rSftpdCDM*<sup>Tg<sup>+/Sftpd<sup>−/−</sup> mice was incubated in mannan coated ELISA plates with increasing concentrations of competing carbohydrate. The saccharide concentration required to inhibit 50% of the binding (IC<sub>50</sub>) to mannan is reported (n=2).

| Saccharide (IC<sub>50</sub> mM) |
|-----------------------------|
| Maltose | Glucose | Galactose | GlcNAc |
|-----------------------------|
| SP-D  | 1 | 8 | 20 | >100 |
| rSftpdCDM | 0.5 | 1 | 5 | 20 |
Fig. 1
Fig. 2
Fig. 3
Relative Absorbance (700nm)

|     | Control | EDTA   |
|-----|---------|--------|
| Time (m) |        |        |
| 0.25 |         |        |
| 0.5  |         |        |
| 0.75 |         |        |
| 1    |         |        |

Fig. 4
Fig. 5
| Dilution | WT | SftpΔCDM+ | SftpΔCDM- |
|----------|----|-----------|-----------|
|          | 1  | 1:2       | 1:4       |
| MMP9     | 1  | 1:2       | 1:4       | -97kD     |
| MMP2     | 1  | 1:2       | 1:4       | -68kD     |

Fig. 6
Sat PC µmole/kg

Alveolar

Tissue

Total

---

- -

Sftpd$^{+/+}$

---

+ +

Sftpd$^{-/-}$

---

CDM

Fig. 7
Fig. 8
Fig. 10
Fig. 11

- IL-6 (pg/ml)
- IFN-γ (pg/ml)
- TNF-α (pg/ml)
- Viral titer (%)
Correction of pulmonary abnormalities in Sftpd^{-/-} mice requires the collagenous domain of surfactant protein D
Paul S. Kingma, Liqian Zhang, Machiko Ikegami, Kevan Hartshorn, Francis X. McCormack and Jeffrey A. Whitsett

J. Biol. Chem. published online June 20, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M600651200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts
Interaction of the mammalian endosomal sorting complex required for transport (ESCRT) III protein hSnf7-1 with itself, membranes, and the AAA⁺ ATPase SKD1.

Yuan Lin, Lisa A. Kimpler, Teresa V. Naismith, Joshua M. Lauer, and Phyllis I. Hanson

The plasmid used to express FLAG-hSnf7N (residues 1–116) in this paper had an unintended missense mutation changing serine at residue 2 to cysteine. We found that this cysteine was palmitoylated. Changing it back to serine decreased the amount of hSnf7N associated with membranes from all for the mutant fragment containing cysteine to approximately half for the wild-type fragment containing serine. The images of FLAG-hSnf7N in Figs. 7B and 8 represent average cells expressing mutant (Cys-2) hSnf7N, whereas for wild-type (Ser-2) hSnf7N, these images correspond to cells expressing high levels of protein. All other plasmids are as indicated, and the conclusions of the paper remain unchanged.

Role of the N-terminal domain of the human DMC1 protein in octamer formation and DNA binding.

Takashi Kinebuchi, Wataru Kagawa, Hitoshi Kurumizaka, and Shigeyuki Yokoyama

Due to an inadvertent error, the wrong image was presented in Fig. 4C. Fig. 4C should appear as shown below. The figure legend and text remain unchanged.

Conditional deletion of hypothalamic Y2 receptors reverts gonadectomy-induced bone loss in adult mice.

Susan J. Allison, Paul Baldock, Amanda Sainsbury, Ronaldo Enriquez, Nicola J. Lee, En-Ju Deborah Lin, Matthias Klugmann, Matthew During, John A. Eisman, Mei Li, Lydia C. Pan, Herbert Herzog, and Edith M. Gardiner

Dr. Klugmann’s name was misspelled in the author line. The correct spelling is shown above.

The first structure from the SOUL/HBP family of heme-binding proteins, murine P22HBP.

Jorge S. Dias, Anjos L. Macedo, Gloria C. Ferreira, Francis C. Peterson, Brian F. Volkman, and Brian J. Goodfellow

Column 2, first line: The concentrations should read “(4.0 μM) or hemin (3.5 μM). . .”
PTP-PEST couples membrane protrusion and tail retraction via VAV2 and p190RhoGAP.

Sarita K. Sastry, Zenon Rajfur, Betty P. Liu, Jean-Francois Cote, Michel L. Tremblay, and Keith Burridge

In Fig. 5, panel D was inadvertently omitted and is shown below. The figure legend is correct as it appears.
Correction of pulmonary abnormalities in Sftpd−/− mice requires the collagenous domain of surfactant protein D.
Paul S. Kingma, Liqian Zhang, Machiko Ikekami, Kevan Hartshorn, Francis X. McCormack, and Jeffrey A. Whitsett

Fig. 5: An incorrect image was used for the panel A inset. The correct image is shown below.