The role of pulmonary collectin N-terminal domains in surfactant structure, function and homeostasis, in vivo.

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Running title: Chimeric collectin expression in SP-A⁻ and SP-D⁻ mice
Summary
The N-terminal domains of the lung collectins, surfactant proteins A (SP-A) and D (SP-D), are critical for surfactant phospholipid interactions and surfactant homeostasis, respectively. To further assess the importance of lung collectin N-terminal domains in surfactant structure and function, a chimeric SP-D/SP-A (D/A) gene was constructed by substituting nucleotides encoding amino acids Asn1-Ala7 of rat SP-A with the corresponding N-terminal sequences from rat SP-D, Ala1-Asn25. Recombinant D/A migrated as a 35 kDa band on reducing SDS-PAGE, and as a ladder of disulfide-linked multimers under nonreducing conditions. The recombinant D/A bound and aggregated phosphatidylcholine containing vesicles as effectively as rat SP-A. Mice in which endogenous pulmonary collectins were replaced with D/A were developed by human SP-C promoter driven overexpression of the D/A gene in SP-A−/− and SP-D−/− animals. Analysis of lavage fluid from SP-A−/−,D/A mice revealed that glycosylated, oligomeric D/A was secreted into the airspaces at levels that were comparable to the authentic collectins, and that the N-terminal interchange converted SP-A from a 'bouquet' to a cruciform configuration. Transmission electron microscopy of surfactant from the SP-A−/−,D/A mice revealed atypical tubular myelin containing central "target-like" electron density. Surfactant isolated from SP-A−/−,D/A mice exhibited elevated surface tension both in the presence and absence of plasma inhibitors, but whole lung compliance of the SP-A−/−,D/A animals was not different from the SP-A−/− littermates. Lung specific overexpression of D/A in the SPD−/− mouse resulted in heterooligomer formation with mSP-A, and did not correct the airspace dilation or phospholipidosis that occurs in the absence of SP-D. These studies indicate that the N-terminus of SP-D: 1) can functionally replace the N-
terminus of SP-A for lipid aggregation and tubular myelin formation, but not for surface
tension lowering properties of SP-A, 2) is not sufficient to reverse the structural and
metabolic pulmonary defects in the SP-D<sup>−/−</sup> mouse.
Introduction

Lung surfactant is a mixture of phospholipids, neutral lipids and surfactant proteins (SP-1, 2, 3, and 4), which are secreted into the airspaces by alveolar type II cells and Clara cells of the distal pulmonary epithelium (1). Although the primary function of surfactant is to reduce surface tension, the contribution of each molecular component to surface activity is not completely understood. Surfactant phospholipids form a film at the air-liquid interface which maintains airspace patency by resisting compression as the alveolar radius decreases during expiration. Data from in vitro experiments, gene-targeted animals and naturally occurring mutations in humans indicate that the hydrophobic surfactant proteins, SP-B and SP-C, participate in the assembly and biophysical properties of the surfactant film (2). The hydrophilic surfactant proteins, SP-A and SP-D, have a complex functional profile. The recognition that SP-A and SP-D are structurally homologous to mannose binding protein (MBP) has identified them as members of the collectin family of innate opsonins and directed attention to their host defense properties (3). Like MBP, SP-A and SP-D bind to a wide range of microorganisms, and enhance microbial phagocytosis and killing by alveolar macrophages. These in vitro activities appear to be physiologically relevant, since gene targeted SP-A<sup>−/−</sup> and SP-D<sup>−/−</sup> mice clear microbial infections less effectively than pulmonary collectin sufficient mice (4-7). However, SP-A<sup>−/−</sup> and SP-D<sup>−/−</sup> mice also exhibit abnormalities of surfactant structure, metabolism and function (8-10). Surfactant isolated from SP-A<sup>−/−</sup> mice does not contain the large-aggregate tubular myelin, and has impaired surface activity in the presence of plasma inhibitors (11). SP-D<sup>−/−</sup> mice develop progressive alveolar phospholipidosis and airspace dilation (9,10), associated with increased macrophage production of
metalloproteinases and oxidant species (12). All of these defects are corrected by lung specific expression of the cognate collectin in the SP-A−/− and SP-D−/− mice (13,14).

The structural basis of SP-A and SP-D surfactant functions have been explored by mutagenesis using in vitro and in vivo analyses. The primary structure of both proteins includes an N-terminal segment containing interchain linkages formed by Cys residues, a collagen-like region of gly-x-y repeats, a hydrophobic ‘neck’ domain and a carbohydrate recognition domain (CRD) (15,16). Trimeric association of subunits occurs by the folding of the collagen-like domains into triple helices (17) and coiled-coil bundling of alpha helices in the neck (18). In the fully assembled molecules, the N-terminal sequences and disulfide-bonds of the pulmonary collectins stabilize the parallel arrangement of six trimers that characterizes the “bouquet” structure of SP-A and the radial alignment of four trimers that imparts the cruciform organization to SP-D (19,20). SP-A and SP-D bind to carbohydrate and lipid ligands by their CRDs, but high affinity interactions require oligomeric assembly mediated by N-terminal crosslinking of trimeric arms. This configuration facilitates simultaneous engagement of individual collectin molecules with multiple sites on membranes and microbial surfaces. Deletion of the collagen-like domain from SP-A, which limits oligomeric assembly to simple trimers and hexamers, reduces binding to liposomes but does not block liposome aggregation (21). Deletion of the N-terminal segment of SP-A (22), or selective disruption of interchain disulfide bond formation by Cys6Ser substitution, limits assembly to simple trimers and blocks SP-A mediated liposome aggregation and binding (21). Disruption of interchain disulfide bond formation at the N-terminus of SP-D by Cys15Ser and Cys20Ser
substitutions limits oligomeric assembly to trimerization, and blocks SP-D mediated functions in vitro (23), and in vivo (24). Collectively, these data suggest that the N-terminal segments of SP-A and SP-D are critical for interactions with surfactant phospholipids and microbial ligands.

Recently Zhang et al. reported that genetic replacement of endogenous mouse SP-D (mSP-D) with a mutant SP-D containing disrupted interchain disulfide linkages (Cys15Ser, Cys20Ser) failed to correct the alveolar phospholipidosis and airspace dilation that occur in the SP-D−/− mouse (24). In addition, lung specific overexpression of the Cys15Ser, Cys20Ser SP-D in SP-D+/+ mice disrupted oligomeric assembly of the endogenous SP-D and produced airspace dilation and foamy macrophage formation, without phospholipidosis (24). These data suggested that the in vivo activity of SP-D is dependent on its oligomeric structure. The purpose of this study was to examine the role of the N-terminal segment-dependent oligomeric structure of SP-A and SP-D in their functions, in vivo.
Experimental procedures

Animal husbandry-Mice used in experimental procedures were handled in accordance with approved protocols through the Institutional Animal Care and Use Committees at University of Cincinnati School of Medicine, and the Cincinnati Children's Hospital Medical Center. All mice used in experiments were the Swiss Black strain, were maintained under barrier containment in the vivarium facilities, and appeared healthy and free of infection at the time of the study. All comparisons made were among littermates. Sentinel mice in all colonies were serologically negative for common murine pathogens.

Construction of chimeric D/A transgenes- A chimeric SP-D/SP-A (D/A) cDNA containing N-terminal segment of rat SP-D (rSP-D) (Ala1-Asn25) and the collagen-like region, the neck domain and the carbohydrate recognition domain of rat SP-A (rSP-A) (Gly8-Phe228) was generated by overlapping extension PCR using the rSP-A and rSP-D cDNAs as templates (16,25). Nucleotide sequencing of the entire D/A coding region was performed to confirm correct splicing and the absence of spurious mutations (26). The D/A gene was ligated into the unique EcoR1 site of the baculovirus transfer vector, PVL 1392 (22), or the 3.7 kb hSP-C plasmid (21), which contains a 3.7 kb human surfactant protein C promoter. Orientation was confirmed by restriction digestion with Kpn I and Bam HI for the PVL 1392/D/A and hSP-C/D/A constructs, respectively.

Recombinant and native collectin isolation-Recombinant baculoviruses were produced by homologous recombination in Spodoptera frugiperda (Sf-9) cells following cotransfection with linear viral DNA and PVL 1392/cDNA constructs (Baculogold, Pharmingen), as
described (22). Fresh monolayers of $10^7$ Trichoplusia Ni ($T. Ni$) cells were infected with plaque-purified recombinant viruses at a multiplicity of infection of 10, and then incubated with serum-free Excel 400 media (JRH Biosciences) supplemented with antibiotics for 72 h. Recombinant D/A (rec. D/A) or recombinant rat SP-A (rec. rSP-A) were purified from the culture media by adsorption to mannose-Sepharose 6B columns in the presence of 1 mM Ca$^{2+}$ and elution with 2 mM EDTA. The purified recombinant D/A was dialyzed to remove EDTA, and then stored at -20°C. Rat SP-A and SP-D were purified from the bronchoalveolar wash of silica-treated animals using previously published methods (22,27). Small amounts of D/A required for immunoblot analysis were purified from SP-D$^{-/-}$D/A mice by sedimentation of surfactant at 15,000 x g, butanol extraction of the washed surfactant pellet, dialysis of the insoluble proteins, and mannose-Sepharose affinity chromatography. To obtain sufficient D/A for sizing by gel filtration chromatography, SP-A$^{-/-}$D/A mice were lavaged two weeks after intranasal instillation of silica (28). D/A was separated from other lavage proteins and surfactant phospholipids by cosedimentation with the surfactant pellet, repeated washing with 1 mM CaCl$_2$ in 150 mM NaCl, and elution from the pellet with isotonic saline containing 2 mM EDTA. The surfactant lipids were pelleted by high speed centrifugation and the supernatant containing D/A was stored at -20°C.

In vivo replacement of mSP-A and mSP-D by the chimeric D/A collectin- Swiss Black/C129J SP-A$^{-/-}$ and SP-D$^{-/-}$ mice were developed from embryonic stem cells by targeted disruption of the endogenous mouse collectin genes, and maintained by breeding with Swiss Black mice, as previously described (8). Portions of the hSP-C/D/A construct
that were unnecessary for expression of the transgene were removed by digestion with Nde I and Not I. Lung specific overexpression of D/A in SP-A\(^{-/-}\) mice was accomplished by injection of the male pronucleus of fertilized SP-A\(^{-/-}\) mouse eggs with the hSP-C-D/A transgene, followed by uterine implantation in SP-A\(^{-/-}\) females (29). SP-A\(^{-/-},D/A\) progeny identified by PCR were expanded by breeding with SP-A\(^{+/-}\) mice. The D/A transgene was bred into the SP-D\(^{+}\) background by crossing the SP-A\(^{-/-},D/A\) mice with SP-D\(^{+}\) mice, using the genotyping strategies outlined below. Progeny which screened positively for the D/A transgene in the first round were bred in brother/sister matings. Progeny of the second generation which screened positively for the D/A transgene were screened for the gene-targeted mSP-D gene. The SP-D\(^{-/-},D/A\) mice identified by this method were then expanded by crosses with SP-D\(^{+}\) mice.

**DNA analyses**- The D/A transgene and mSP-D genes were identified in the genomic DNA of mice using PCR. Tail clips (0.5-1cm) were digested overnight in buffer containing 50 mM Tris, 100 mM EDTA, 0.5% SDS and 100 µg/ml proteinase K at 55°C and purified using the Wizard genomic DNA kit (Promega, Madison, WI). PCR to identify the D/A transgene was performed using primer set 1 that amplified a region from the distal end of the hSP-C promoter to midportion of the D/A cDNA (5’-CTCAACTCACCAGGTGTGGCTC-3’ and 5’-TTCACAGAAGCCCCATCCAGGTAG-3’). For identification of the mSP-D gene, primer set 2 which amplified the 1.0 kb region spanning the proximal end of mSP-D exon 2 (5’-GCTGCCCTTTCTCTCCATGC-3’) and the distal end of mSP-D intron 2 (5’-TTCCCACCACATTTGGAGTG-3’) was used. No
band is amplified from the mSP-D gene-targeted allele, because the sequences recognized by primer set 2 are ablated by the insertion of the neo targeting cassette.

Protein analyses- Routine protein concentrations were determined with the bicinchoninic protein assay kit (BCA) (Pierce) using bovine serum albumin as the standard. SP-A and D/A concentrations in lavage were measured by ELISA (14), although D/A levels were semiquantitative because rSP-A (and not D/A) was used as the standard for the ELISA. Surfactant proteins were separated by 8-16% SDS-PAGE and stained with Coomassie blue (30). For immunoblot analyses, protein species were transferred to nitrocellulose membranes and reacted serially with polyclonal rabbit anti-rat SP-A IgG (14) or rabbit anti-rat SP-D IgG (13,14), and horseradish peroxidase (HRP) conjugated anti-rabbit IgG antibody. Blots were developed by HRP/H₂O₂ dependent oxidation of luminol and autoradiography, according to the manufacturer's instructions (ECL, Amersham Pharmacia Biotech). The oligomeric structure of D/A was assessed by chemical crosslinking with disuccinimidyl glutarate (DSG, Pierce), SDS-PAGE under reducing conditions, and staining with Coomassie Blue, as previously described (22). Recombinant D/A mass was estimated under physiologic ionic strength conditions (150 mM NaCl, 10 mM Tris) by Superose 6 gel filtration chromatography using an FPLC column with a bed volume of 10 mm x 300 mm (AKTA, Amersham-Pharmacia). To estimate oligomeric mass of D/A from the lavage of SP-A⁻/⁻D/A mice, D/A isolated as outlined above was analyzed by Superose 6 gel filtration chromatography in running buffer containing 150 mM NaCl and 0.1 mM EDTA. The elution of rec. D/A and lavage D/A was monitored by ELISA.
Lipid binding and aggregation—Binding experiments were performed with multilamellar liposomes produced by vigorous vortexing of a mixture of saturated phosphatidylcholine (Sat PC): egg phosphatidylcholine (PC):phosphatidylglycerol (PG); 9:3:2 in 150 mM NaCl, 0.1 mM EDTA. After incubation of 5 µg/ml D/A or recombinant SP-A with 100 µg/ml liposomes at 23°C for 60 min, the mixtures were centrifuged at 11,000 x g, washed and centrifuged again. SP-A (or D/A) in the supernatant and pellet were measured by ELISA, and fractional binding was calculated according to the equation; % bound = (SP-A pellet/(SP-A pellet + SP-A supernatant) x 100. For aggregation experiments, unilamellar liposomes were produced by sonication of the same lipid mixture as above, as described (22). The vesicles (100 µg/ml) were mixed with 10 µg/ml rec. rSP-A or rec. D/A and equilibrated for three minutes. After addition of 5 mM CaCl₂, aggregation was determined by measuring light scattering (O.D. 400nm) in a spectrophotometer. For both the binding and aggregation experiments, controls with no phospholipids and no CaCl₂ were also performed.

Surfactant isolation and Sat PC measurement—Groups of mice from each genotype were anesthetized by intraperitoneal injection with pentobarbital sodium and exsanguinated by transection of the abdominal aorta. The chest was opened, and the proximal trachea was cannulated with a 20-gauge blunt needle. Alveolar lavage was performed by three cycles of instillation of saline to full lung expansion followed by gentle aspiration, repeated five times (total approximately 5 mls) for each animal (11). Large-aggregate surfactant was isolated from the pooled lavage fluid by centrifugation at 40,000 x g over a 0.8 M sucrose
cushion for 15 min (11). The large-aggregate surfactant then was collected from the interface, diluted with 0.15 M NaCl, and centrifuged again at 40,000 x g for 15 min. The pellet was suspended in normal saline and stored at -20°C. After lavage as above, lung tissue was homogenized in saline. Sat PC was measured by extracting the alveolar lavage sample or lung homogenates with chloroform/methanol (2:1), treatment with OsO₄, separation by alumina column chromatography and phosphorus analysis as described (8).

**Specimen preparation for electron microscopy**-Large-aggregate surfactant samples (four mice/pellet) were fixed for 18 h at 4°C with 2% paraformaldehyde and 4% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, and 1 mM CaCl₂. Pellets were treated with 1% OsO₄ and 1.5% potassium ferrocyanide for 1 h at 23°C, and washed and dehydrated with increasing concentrations of acetone (0 to 100%) and ethanol (0 to 100%). At the 75% ethanol dehydration step, 2% uranyl acetate was added and incubated for 2 to 18 h at 4°C. Fixed and dehydrated pellets were embedded in epoxy resin (Ted Pella, Redding, CA), sectioned and stained with lead citrate and 2% uranyl acetate. Dried specimens were examined and photographed using a JEOL JEM-100CX-II TEM at 8,000-40,000 x nominal magnification (31). At least 4 specimen grids with different sections were systematically examined by EM for each genotype.

**Measurement of TM dimensions**-Dimensions of tubular myelin (TM) figures were measured from magnified images (final magnification of approximately 3x10⁵) captured
on scanned micrographs using Canvas 6 software (Deneba Software, Miami, FL). Clearly identifiable cross sections of TM from 3-5 micrographs at different magnifications (10,000, 20,000 and 40,000) were selected to measure lattice dimensions for each genotype. The smallest side of a randomly selected lattice was considered as side 1, and all the membranes parallel to that side in the row were measured (n=50). Rows of membranes perpendicular to side 1 were measured as side 2 (n=50). All dimensions reported were rounded to the nearest 0.1 nm (1 Å).

**Surface tension measurements**- Surface activity was measured with the captive bubble surfactometer (32), using large-aggregate surfactant pooled from three mice of the same genotype. The concentration of each sample was adjusted to 3 nmol 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 0.93 mg/ml sheep plasma.

**Pulmonary mechanics**- Measurement of respiratory mechanics was performed with a computer controlled small animal ventilator (SAV) (Flexivent; Scireq, Montreal, Canada). Mice were sedated with a combination of xylazine (13 mg/kg i.p.) and ketamine (87 mg/kg i.p.) and then anesthetized with pentobarbital (70-90 mg/kg i.p.). The trachea was cannulated with an 18 gauge metal needle, and mice were ventilated with a quasisinusoidal waveform at a frequency of 160 breaths/min and a tidal volume of 6 ml/kg. Positive end-expiratory pressure (PEEP) was maintained at 2 cm H₂O by attaching a water trap to the expiratory line of the ventilator. Pulmonary mechanics were
measured with an oscillation technique that has been previously described (33). Regular ventilation was stopped and the mouse was allowed to passively expire to relaxation volume while PEEP was maintained. Low-amplitude flow oscillation was delivered to the lung over a 16 sec period of apnea. Tracheal pressure and tracheal volume measured during this maneuver were used to calculate respiratory compliance (33).

Histology-Mouse lungs (12 weeks old) were fixed at 25 cm of water pressure with 4% paraformaldehyde in PBS, and processed into paraffin blocks. Seven micrometer sections from each lobe were stained with hematoxylin and eosin and examined under light the microscope.

Statistics-Comparisons between the SP-A^{−/−} and SP-A^{−/−,D/A} mouse lines, and the SP-D^{−/−} and SP-D^{−/−,D/A} mouse lines were made using a two tailed t-test. The variables included SP-A levels, Sat PC levels, tubular myelin dimensions, lung compliance and surface tension. Data were expressed as mean ± S.E. unless otherwise noted, and p values of less than 0.05 were considered significant.
Results

*Expression and characterization of recombinant D/A-A chimeric ‘D/A’ collectin,* composed of the N-terminal segment from rSP-D and the collagen-like region, neck and CRD from rSP-A, was generated to assess the importance of N-terminal oligomeric determinants on collectin functions. The synthesis, processing and functional activity of the D/A was assessed *in vitro* prior to expression in mice. Recombinant D/A synthesized in insect cells was secreted into the culture media at an average concentration of 12.5 ± 1.1 µg/ml/6 x 10⁶ cells, and 94.6 ± 1.1% of the protein bound to the mannose-Sepharose affinity column, indicating that trafficking through the secretory pathway and carbohydrate binding activity were preserved. The rec. D/A migrated as a broad 35 kDa band under reducing conditions and as a ladder of disulfide-dependent oligomers under non-reducing conditions (Fig. 1). By comparison, rec. rSP-A was slightly smaller under reducing conditions, and less extensively crosslinked by interchain disulfide bonds under non-reducing conditions. Noncovalent interactions that also contribute to the assembly of the D/A oligomer were analyzed by DSG crosslinking and the results are shown in Fig. 1, lanes e and f. Treatment of the rec. D/A with the non-reducible crosslinker DSG followed by size-fractionation on reducing SDS-PAGE resulted in the appearance of a series of at least 8-9 distinct bands, indicating that the same number of polypeptide chains were closely associated in the largest oligomeric forms of the protein, most likely as three trimers. In comparison, the same analysis of rec. rSP-A revealed assembly from approximately six subunits. The mass of D/A was estimated using a Superose 6 gel filtration column and SP-A ELISA analysis (Fig. 2). The D/A eluted as a single broad peak in a position centered between molecular mass standards ferritin (440 kDa) and
aldolase (158 kDa), very close to catalase (232 kDa). The elution profile was similar to that of rec. rSP-A (Fig. 2). The data are consistent with assembly from approximately six 35 kDa subunits for both proteins, although substantial amounts of smaller and larger oligomers were also present, and the elongated structure of D/A complicates mass estimates based on the Stokes radius of more globular standards. The rec. D/A bound and aggregated Sat Pc containing vesicles in a calcium-dependent manner (Fig. 3). As shown in Fig. 3A, 57.7 ± 4.0% of D/A cosedimented with the multilamellar liposomes upon centrifugation, compared to 53.7 ± 1.4% of the rec. rSP-A. Liposome aggregation induced by rec. rSP-A, rSP-A and D/A all reached approximately the same endpoint for light scattering at six minutes, but the kinetics were different (Fig. 3B). The initial rate of aggregation for the rec. D/A (3.2 ± 2.0 x 10^{-5} a.u./sec) was greater than that of rec. rSP-A (1.3± 0.3 x 10^{-3} a.u./sec, p<0.01), but less than that of rSP-A (6.3 ± 0.5 x 10^{-3} a.u./sec, p < 0.01) (Fig. 3 B, inset). As expected, the rSP-D did not aggregate the Sat PC containing vesicles. Collectively, these data indicate that rec. D/A was secreted from eucaryotic cells, assembled into disulfide-linked oligomers, and bound to carbohydrate and phospholipid ligands.

Development of SP-A^{-/-},D/A and SP-D^{-/-},D/A mouse lines- Mouse SP-A or SP-D were replaced with D/A by human SP-C promoter directed expression of the D/A transgene in the distal lung epithelium of collectin deficient mice (Fig. 4). The SP-A^{-/-} and SP-D^{-/-} gene targeted mice were developed as previously described (8,9). The hSP-C/D/A construct was injected into fertilized SP-A^{-/-} oocytes, which were then transferred to SP-A^{-/-} surrogate mothers. Three SP-A^{-/-},D/A founder lines were identified using the transgene
specific primer set 1 by the presence of a 1.0 kb band on PCR analysis of tail clips from the pups (Fig. 4, Panel C), and two were found to transmit the transgene to progeny. The highest producing line was expanded by further breeding with SP-A^{−/−} mice. For the study of D/A function in the SP-D^{−/−} background, the SP-A^{−/−}, D/A mice were bred with the SP-D^{−/−} mice (fig. 4, Panel D). Transgene positive progeny from the first generation were identified by the presence of a 1.0 kb PCR band using primer set 1, and then bred again with the SP-D^{−/−} line. Transgene positive F2 mice that were homozygous for the gene-targeted mSP-D allele were identified by the presence of a 1.0 kb band using transgene specific primer set 1, and the absence of a 0.6 kb band using the endogenous mSP-D specific primer set 2. The SP-D^{−/−}, D/A mice were further expanded by breeding with SP-D^{−/−} mice. The SP-D^{−/−}, D/A mice are SP-A^{+/+}, since the SP-D locus is very closely linked to the SP-A locus on mouse chromosome 14 (34). An immunoblot analysis of D/A in whole lavage fractions from SP-A^{−/−}, D/A mice was performed with a polyclonal anti-rSP-A IgG (fig. 5A), and from the SP-D^{−/−}, D/A lines with both polyclonal anti-rSP-D and anti-rSP-A antibodies (fig. 5B). Under reducing conditions, mSP-A from wild type Swiss Black (SP-A^{+/+}) mice migrated as a doublet at 32 and 36 kDa, consistent with variable asparagine-linked glycosylation of the N-terminal segment and CRD, as has previously been described for rat SP-A (35). In contrast, the D/A from the SP-A^{−/−}, D/A line appeared as a narrow band at 35 kDa (fig. 5A) under reducing conditions. The diminished polymorphism of D/A compared to mSP-A under reducing conditions is most likely due to the absence of the N-terminal glycosylation site in the chimeric protein. Under nonreducing conditions, the anti-rSP-A IgG revealed that both mSP-A from SP-A^{+/+} mice and D/A from SP-A^{−/−}, D/A mice formed extensive arrays of disulfide-linked multimers.
There were no immunoreactive species detected with the anti-rSP-A IgG in the SP-A⁻/⁻ littermates (not shown). Assessment of D/A expression in the SP-D⁻/⁻,D/A mice (Fig. 5B) was primarily based on the recognition of SP-D sequences of D/A by anti-rSP-D IgG, because the presence of endogenous mSP-A complicates the analysis using anti-rSP-A IgG. The anti-rSP-D IgG reacted with a single 43 kDa band in SP-D⁺/⁺ lavage proteins separated under reducing conditions, and the absence of immunoreactive species at 32-36 kDa demonstrates the lack of crossreactivity with mSP-A. In the SP-D⁻/⁻,D/A lavage, the anti-rSP-D IgG bound to a reduced 35 kDa species consistent with D/A in the transgene positive animals, which was not present in the transgene negative (SP-D⁻/⁻) littermates. Under nonreducing conditions, the anti-rSP-D IgG exhibited strong binding to a >110 kDa band consistent with oligomeric SP-D in SP-D⁺/⁺ lavage. For reasons that were initially unclear, however, the anti-rSP-D IgG was only weakly reactive for protein species from the SP-D⁻/⁻,D/A mice that were separated under nonreducing conditions (not shown). To enhance the signal, D/A was purified and concentrated from the lavage of SP-D⁻/⁻,D/A animals by sedimentation of the surfactant pellet, butanol extraction and mannose Sepharose affinity chromatography. Immunoblot analysis with anti-rSP-D IgG revealed disulfide dependent assembly of D/A in the SP-D⁻/⁻ background, but the pattern was different than in SP-A⁻/⁻,D/A mice, and the extent of oligomerization was reduced. The nonreduced molecular species that reacted with the anti-rSP-D IgG were also recognized by the anti-rSP-A IgG, as were a ladder of bands consistent with mSP-A. To further assess oligomeric assembly, gel filtration analysis of D/A isolated from the lavage of six silica treated SP-A⁻/⁻,D/A mice was performed. D/A eluted as a narrow peak centered near the peak for blue dextran (Fig. 6), and much closer to the elution position of rat SP-D
than rat SP-A. Collectively, these data indicate that D/A is expressed and secreted into the airspaces of SP-A$^{-/-,D/A}$ mice as disulfide-linked homooligomers that are similar in Stokes radius to rat SP-D. In the SP-D$^{-/-,D/A}$ mice, D/A and mSP-A most likely form disulfide-linked heterooligomers, which may account for the observed differences in the levels of D/A immunoreactivity under reducing and nonreducing conditions.

**Surface tension lowering properties of surfactant isolated from transgenic mice** - To assess the role of the N-terminal domain of SP-A in surfactant function, large surfactant aggregates were isolated on discontinuous sucrose gradients and directly applied to the air-liquid interface of a captive bubble surfactometer. Changes in the dimensions of the bubble over a 300 second interval were measured and used to determine the equilibrium surface tension (Fig. 7) (14). The experiments were performed in the presence and absence of sheep plasma, to model the surfactant inhibition caused by proteinaceous pulmonary edema associated with lung injury. The surface properties of surfactant isolated from SP-A$^{-/-}$ mice which were engineered to overexpress rat SP-A in the lung (SP-A$^{-/-,rSP-A}$), published previously (14), were included for comparison. Large-aggregate surfactant isolated from D/A mice had higher equilibrium surface tensions than surfactant from SP-A$^{-/-}$ littermates or SP-A$^{-/-,rSP-A}$ mice (Fig. 7A). In the presence of plasma, surfactant from the SP-A$^{-/-,D/A}$ animals did not achieve the low equilibrium surface tensions reached by surfactant from SP-A$^{-/-,rSP-A}$ animals, and was even more susceptible to protein inhibition than surfactant from SP-A$^{-/-}$ mice (Fig. 7B) (11). The minimum surface tension achieved after cycling the bubble through 5 oscillations of maximum and minimum (65% volume reduction) radius was markedly elevated in the SP-A$^{-/-,D/A}$
surfactant compared to the SP-A<sup>−/−</sup> or SP-A<sup>−/−,rSP-A</sup> controls, and neither the SP-A<sup>−/−</sup> or the SP-A<sup>−/−,D/A</sup> surfactant achieved surface tensions that were comparable to the SP-A<sup>−/−,rSP-A</sup> surfactant when cycled in the presence of plasma (Fig. 8). To assess surfactant function in vivo, pulmonary mechanics were measured. The whole lung compliance of SP-A<sup>−/−,D/A</sup> mice was not significantly different than SP-A<sup>−/−</sup> littermates (2.00 ± 0.05 vs. 1.97 ± 0.16 ml/cmH<sub>2</sub>O-kg, n= 4 each genotype). Thus, the N-terminal region of SP-D cannot replace the N-terminal region of SP-A for maintenance of low surface tension in the presence or absence of surfactant inhibitors.

Role of the N-terminal region of SP-A in tubular myelin formation—SP-A<sup>−/−</sup> mice do not make the surfactant aggregate TM (8). To assess the role of the N-terminal domain of SP-A in the formation of TM, we examined the ultrastructure of large-aggregate surfactant from SP-A<sup>−/−,D/A</sup> mice (Fig. 9). Lipid lattices were abundant in the SP-A<sup>−/−,D/A</sup> mice but surfactant from the SP-A<sup>−/−</sup> littermates consisted only of lipid sheets without TM formation, as described previously (not shown) (8). Target-like electron densities in the center of each TM square were found in the SP-A<sup>−/−,D/A</sup> mice, a finding distinct from SP-A<sup>+/+</sup> or SP-A<sup>−/−,rSP-A</sup> animals. TM lattices in surfactant from SP-A<sup>−/−,D/A</sup> mice appeared as rectangles with side dimensions of 42.3 ± 5.9 nm and 50.2 ± 6.1 nm (mean ± S.D., side 1 and side 2, respectively) (p < 0.001). By comparison, TM lattices in surfactant from SP-A<sup>−/−,rSP-A</sup> mice were previously reported to have nearly equal side dimensions of 52.8 ± 9.1 nm and 52.2 ± 6.4 nm (side 1 and side 2, respectively)(p < 0.67)(14). The dimensions of TM lattices of SP-A<sup>−/−, D/A</sup> mice were different than those of SP-A<sup>−/−, rSP-A</sup> mice (p<0.05).
These data indicate that N-terminal domain of SP-D can functionally replace the N-terminus of SP-A for formation of tubular myelin.

Effect of N-terminal interchange on the association of SP-A with large-aggregate surfactant—SP-A has been reported to stabilize large surfactant aggregate forms which contain TM and are more surface active than small surfactant aggregates (36). Surfactant aggregates were separated into large and small forms by centrifugation on discontinuous sucrose gradients. For wild type Swiss Black animals, 21 ± 2% of SP-A in lavage was associated with large surfactant aggregates, and the large- to small-aggregate SP-A ratio was 0.27 (n = 3 pooled sets of 3 mice). For SP-A\(^{-/-,D/A}\) mice, 39 ± 1% of D/A was associated with large surfactant aggregates and the large- to small-aggregate ratio was 0.63 (n = 3 pooled sets of 3 mice). The large-aggregate SP-A content and large- to small-aggregate ratio was different for SP-A\(^{+/+}\) and SP-A\(^{-/-,D/A}\) mice (p<0.01 for both values). These results suggest that replacement of the N-terminal segment of SP-A with that from SP-D enhances the association of SP-A with the large-aggregate forms of surfactant.

Effect of D/A expression on saturated PC pool sizes and lung histology in SP-D\(^{-/-}\) mice—To examine the role of the N-terminal domain of SP-D in surfactant homeostasis, the pool sizes of Sat PC in the SP-D\(^{-/-,D/A}\) mice (n = 13) and their transgene negative littermates (n = 17) were compared. Expression of D/A in the SP-D\(^{-/-}\) background had no effect on the Sat PC pool sizes in lavage (21 ± 1 and 22 ± 1 μmol/kg, SP-D\(^{-/-,D/A}\) and SP-D\(^{-/-}\) littermates, respectively) (p = .39) or the lung homogenate (42 ± 2 and 42 ± 3 μmol/kg, SP-D\(^{-/-,D/A}\) and SP-D\(^{-/-}\) littermates, respectively) (p = .95). The D/A level in the
lavage of the SP-A⁻/⁻,D/A mice measured by ELISA was 13.4 ± 0.5 µg SP-A/kg (n = 9), comparable to the level of SP-A in the lavage of wild type mice (5.9 ± 0.6 µg SP-A/kg, n = 9) (Fig. 5). As expected, D/A levels in the SP-A⁻/⁻ littermates were undetectable. Although endogenous SP-A precludes ELISA measurement of D/A levels in the SP-D⁻/⁻,D/A mice, they would be expected to be similar to the parental SP-A⁻/⁻,D/A mice. On histologic examination, both the SP-D⁻/⁻,D/A mice and the SP-D⁻/⁻ littermates exhibited similar degrees of airspace dilation and surfactant phospholipid accumulation (9) (not shown). We conclude that the N-terminus of SP-D is not sufficient to correct the disrupted lipid homeostasis or emphysema that are characteristic of murine SP-D deficiency.
Discussion

The data presented here indicate that the N-terminal domain of SP-D can functionally replace the N-terminal domain of SP-A for liposome aggregation and tubular myelin formation, but not for protection of the surface activity of surfactant from inhibition by plasma proteins. The atypical morphological appearance of TM in the SP-A-/-,D/A mouse supports a primary role for the N-terminal domain of SP-A in the organization of tubular myelin. Lung specific expression of D/A did not correct the emphysema and phospholipidosis observed in SP-D-/- deficiency (24), indicating that the N-terminal segment of SP-D is not sufficient for maintenance of normal pulmonary homeostasis.

We have previously proposed a model in which SP-A bridges liposomes by CRD-mediated binding to the membrane interface and SP-A-SP-A interactions at the N-terminus, based on evidence that point mutations of the CRD and deletion of the N-terminal segment independently block liposome aggregation by the protein (37). To further examine the structural basis of SP-A/lipid interactions, we produced a chimeric protein composed of the N-terminus of SP-D and the collagen-like region, neck region and carbohydrate recognition domain of SP-A. The SP-D/SP-A N-terminal interchange was predicted to result in an radial arrangement of D/A trimers, rather than the parallel, ‘flower-bouquet’ configuration that is characteristic of SP-A. Our hypothesis was that the extended conformation of D/A would facilitate bridging of liposomes, and that the property of SP-A to aggregate Sat PC containing vesicles would be preserved and perhaps enhanced. The rec. D/A chimera formed a more extensive ladder of disulfide-dependent oligomers than rec. rSP-A, probably because the N-terminus of SP-D contains
2 N-terminal Cys available for interchain disulfide formation, compared to 1 or 2 N-terminal Cys in the rat SP-A isoforms that result from alternative N-terminal processing (38). Gel filtration analysis indicated that the oligomeric mass of the rec. D/A was somewhat heterogenous, but the predominant species comigrated with rec. rSP-A at approximately 230 kDa consistent with assembly from about six monomers. The rec. D/A retained authentic collectin activity including calcium-dependent binding to carbohydrates and phospholipids. The D/A also aggregated phospholipid vesicles, with a greater initial rate of aggregation than rec. SP-A, indicating that the interchange of N-terminal domains enhanced the ability of SP-A to interact with liposomes.

The recent availability of genetically engineered animal models has facilitated the study of pulmonary collectin structure and function, in vivo (14,24). Because the SP-A N-terminal segment is important for self-association and phospholipid interactions in vitro, we postulated that it plays a primary role in TM structure, in vivo (22). SP-A is known to be required for TM formation in vitro (39) and in vivo (8,14), and is located in the corners of the TM lattice, with the N-terminal regions of the molecule extending toward the center (40). Successful in vitro synthesis and processing of rec. D/A suggested that the D/A gene product would be expressed and properly folded in vivo. The SP-A⁻/⁻ mouse was used as a model for in vivo surfactant studies since it produces surfactant that is devoid of TM and easily inhibited by plasma proteins (8,11). D/A was efficiently expressed and secreted into the airspaces of SP-A⁻/⁻,D/A transgenic mice, and formed disulfide-linked oligomers that were similar in Stokes radius to SP-D, based on gel filtration analysis of D/A in lavage fluid. These data strongly suggest that SP-D/SP-A N-
terminal interchange converted SP-A from a ‘bouquet’ to the ‘cruciform’ structure that is characteristic of SP-D. The extent of oligomeric assembly was much more extensive for D/A expressed in the mice than in the in vitro system, most like due to incomplete post translational modification of the collagen-like region and interchain disulfide bond formation known to occur in collectins expressed in insect cells (22). Lung specific overexpression of the D/A in SP-A−/− mice generated novel TM forms. The dimensions of the lattice and presence of central electron density seen in the TM from the SP-A−/−,D/A mouse were consistent with a model in which individual D/A molecules extend trimeric subunits to each of the corners of TM squares from a central radiating hub (41), and suggest that the oligomeric structure of SP-A exerts a dominant influence on TM morphology. In addition, the D/A was enriched in the large surfactant aggregate fraction of surfactant from SP-A−/−,D/A mice compared with the content of mSP-A in large-aggregate surfactant from SP-A+/+ mice. This finding is consistent with our in vitro data that the SP-D/SP-A N-terminal interchange enhanced the association of SP-A with phospholipids, perhaps by presenting the trimeric CRDs to liposomal interfaces in a configuration that is sterically favorable for simultaneous binding.

Experiments using model surfactant phospholipids (42,43) and surfactant isolated from SP-A−/+ mice (11) have demonstrated that the surface activity of surfactant is more resistant to plasma inhibitors in the presence of SP-A. Overexpression of SP-A in the lungs of SP-A−/+ mice, but not a truncated SP-A containing a deletion of the collagen-like domain, restored wild type (SP-A+/+) surfactant function (14). This result suggests that the determinants for oligomeric assembly that reside within the collagen-like domain are
important for the protective effects of SP-A on surfactant surface activity. To determine the contribution of the SP-A N-terminal segment to surfactant function, we isolated large surfactant aggregates from the SP-A^{−/−,D/A} mouse. The surface activity of the D/A containing surfactant was impaired both in the presence and the absence of plasma inhibitors. The results indicate that the N-terminal segment of SP-A is essential for the interactions of SP-A with surfactant that promote adsorption to the interface, help to refine the monolayer during cycling, and protect the surface active properties of the monolayer from inhibition by plasma proteins. The D/A-induced surfactant dysfunction may be due to the enhanced association of D/A with large-aggregate surfactant or the atypical configuration that D/A confers on TM. While the surface tension disrupting effects of D/A were readily observed in isolated surfactant, they were not apparent in the measured whole lung compliance of the SP-A^{−/−,D/A} mice, which was indistinguishable from SP-A^{+/+} controls. These results, which are similar to prior studies which revealed no correlation between in vitro surface activity of isolated surfactant and pulmonary mechanics in the animal (14,44), suggest that the surfactant is more susceptible to inhibition at the dilute concentrations that are tested in vitro than at physiologic phospholipid concentrations in the airspace. The role of SP-A in maintaining low alveolar surface tensions in vivo remains unclear. Although available lung injury studies in SP-A^{−/−} mice do not yet support a primary physiologic role for SP-A in surfactant biophysical function, multiple reports of SP-As protective effects on surfactant surface tension in in vitro studies (42,43) and in other SP-A animal models (45) warrant additional investigation in SP-A^{−/−} models under a variety of experimental conditions.
The SP-D\textsuperscript{−/−} mouse develops time-dependent airspace dilation and phospholipidosis. These abnormalities are corrected by lung specific expression of rSP-D (13), but not by expression of a trimeric mutant SP-D containing disrupted interchain disulfide bonds at the N-terminus (RrSP-D\text{ser15/20}) (24). Overexpression of the RrSP-D\text{ser15/20} in wild type mice (SP-D\textsuperscript{+/+}) disrupted oligomeric assembly of the endogenous protein and resulted in foamy macrophage formation and airspace dilation without phospholipidosis (24). These data suggest that the N-terminal segment dependent oligomeric structure of SP-D is critical for the maintenance of pulmonary homeostasis. To further examine the role of the N-terminus of SP-D \textit{in vivo}, we bred the D/A transgene into the SP-D\textsuperscript{−/−} background. The D/A was expressed into the airspace, and formed disulfide-linked hetero-oligomers with mSP-A. We found that expression of the SP-D N-terminal domain in the context of a chimeric pulmonary collectin did not correct the emphysema or phospholipidosis in SP-D\textsuperscript{+/−} mice.

In summary, the N-terminal domain of SP-A is an important determinant of oligomeric assembly, the extent of association of the protein with surfactant aggregates, the structure of TM and the resistance of isolated surfactant to protein inhibitors. The N-terminal domain of SP-D is not sufficient for the maintenance of lung structure and phospholipid homeostasis by SP-D. Collectively, the data highlight the importance of the oligomeric structure of the pulmonary collectins in their \textit{in vivo} functions.
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Footnotes

1. SP-, surfactant protein; MBP, mannose binding protein; CRD, carbohydrate recognition domain; D/A, chimeric collectin containing the SP-D N-terminal segment and C-terminal domains from SP-A; mSP-A, mouse SP-A; rSP-A, rat SP-A; rec. rSP-A, recombinant rat SP-A; mSP-D, mouse SP-D; rSP-D, rat SP-D; rec. rSP-D, recombinant rat SP-D, Sat PC, saturated phosphatidylcholine; PG, phosphatidylglycerol; TM, tubular myelin; PBS, phosphate buffered saline
Figure legends

Figure 1. Electrophoresis of recombinant proteins. The rec. D/A (lanes a,c,e) and rec. rSP-A (lanes b,d,f) produced in insect cells were purified by mannose-Sepharose affinity chromatography, resolved on SDS-PAGE gels under reducing (lanes a,b,e,f), and nonreducing (lanes c and d) conditions, and stained with Coomassie blue. Proteins in lanes e and f were crosslinked with DSG prior to electrophoresis.

Figure 2. Gel filtration chromatography of recombinant D/A. Recombinant D/A (square) or rec. rSP-A (circle) were loaded on a Superose 6 column equilibrated and eluted with 150 mM NaCl, 10 mM Tris buffer. The D/A or SP-A content of the eluate was determined by SP-A ELISA. The elution positions of molecular mass standards thyroglobulin (a), ferritin (b), catalase (c), aldolase (d) and ovalbumin (e) are shown.

Figure 3. Binding and aggregation of phospholipid liposomes by D/A. (Panel A) Multilamellar liposomes composed of Sat Pc:egg PC:PG, 9:3:2 were incubated with rec. rSP-A or D/A, and then spun at 11,000 x g. Rec. rSP-A or D/A in pellet and supernatant fractions were determined by SP-A ELISA. Controls in which CaCl₂ or liposomes were omitted are shown. Data are n = 3, mean ± S.E.. (Panel B) Unilamellar liposomes were incubated with rSP-A, rec. rSP-A, D/A or rSP-D in a quartz cuvette at 20°C. After equilibration for 2 min., 5 mM CaCl₂ was added and light scattering was measured at 400 nm for 6 additional min. Data are n = 3, mean ± S.E. (Inset, Panel B). Initial rate of aggregation for each protein is shown. Data are n = 3, mean ± S.E., * = p < 0.05.
Figure 4. Panel A) The D/A transgene construct containing the 3.7 kb human SP-C promoter (open box), the chimeric SP-D (striped box)/SP-A (shaded box) cDNA in a unique Eco RI site, and SV40 t intron and poly A sequences (black box) are shown. Primer set 1 (Pr1) was used to identify the D/A transgene. Pr1 amplifies a 1.0 kb fragment from the 3’ end of the hSP-C promoter and to the midpoint of the D/A cDNA, Panel B) SP-D gene locus used for genotyping animals. Primer set 2 (Pr2) amplifies a 0.6 kb fragment from endogenous mSP-D gene, spanning the proximal end of exon 2 (open box) to the distal end of intron 3, near exon 3 (striped box). The pgkneo targeting construct ablates this locus, and PCR with Pr2 does not amplify a band from the null allele. Panel C) SP-A−/− (lanes 1 and 2) and SP-A−/−,D/A littermates (lanes 3,4,5) were distinguished by PCR using Pr1. Panel D) SP-D−/−,D/A, SP-D−/−,D/A and SP-D+/− mice were distinguished by PCR amplification using both Pr1 and Pr2.

Figure 5. Immunoblot analysis of bronchoalveolar lavage fluid from transgenic mice. Alveolar lavage was performed on wild type animals and D/A transgene positive and negative animals in the SP-A+/− background (Panel A) or SP-D+/− background (Panel B). Electrophoresis was performed under reducing and nonreducing conditions on alveolar wash proteins (wild-type, SP-A−/− background and SP-D−/− background, reducing conditions) or butanol-extracted and affinity purified D/A (SP-D−/− background, nonreducing conditions only) using 8-16% SDS-PAGE gels. After transfer to nitrocellulose, the membranes were incubated with anti-rSP-A (A) or anti-rSP-D (D) antibodies, as indicated, and developed using alkaline phosphatase conjugated anti-rabbit IgG and enhanced chemiluminescence.
Figure 6-Gel filtration chromatography of D/A from SP-A\(^{-/-}\),D/A mice. D/A isolated from silica-treated SP-A\(^{-/-}\),D/A mice was loaded on a Superose 6 column equilibrated with 150 mM NaCl, 10 mM Tris, 0.1 mM EDTA buffer. The D/A content of the eluate was determined by SP-A ELISA. The elution positions of rat SP-D (a), blue dextran (b), and rat SP-A (c) are shown.

Figure 7-Equilibrium surface tension of surfactant isolated from SP-A transgenic mice. Large aggregate surfactant isolated from SP-A\(^{-/-}\) or SP-A\(^{-/-},\)D/A mice was applied to the air-liquid interface of the captive bubble surfactometer in the absence (Panel A) and presence (Panel B) of plasma. Change in the shape of the static bubble from rounded to discoid was monitored over time, and used to calculate equilibrium surface tension. Previously published data from SP-A\(^{-/-},\)rSP-A mice are shown for comparison (14). Values are mean ± S.E., n = 3-4.

Figure 8-Minimum surface tension of surfactant isolated from SP-A transgenic mice. The minimum surface tension achieved by cycling surfactant isolated from SP-A\(^{-/-}\) or SP-A\(^{-/-},\)D/A mice in the surfactometer in the presence and absence of plasma inhibitors is shown. Previously published data from SP-A\(^{-/-},\)rSP-A mice are included for comparison (14). Values are mean ± S.E., n = 3-4. *p < 0.05 for SP-A\(^{-/-}\) vs. SP-A\(^{-/-},\)D/A mice.

Figure 9-Surfactant aggregate structure in SP-A\(^{-/-},\)D/A transgenic mice. Large-aggregate surfactant that was isolated from SP-A\(^{-/-},\)D/A mice was examined by transmission electron
microscopy (Panels A-D). Tubular myelin forms with an atypical ‘stack of boxes’ appearance (arrows), and central ‘target-like’ electron density (arrowheads) are shown. Scale bars indicate 200 nm.
Fig. 2 Palaniyar et al.
Figure 3

A. Bar graph showing the % protein bound to lipid with different conditions: lipid, CaCl2, rec. rSP-A, and D/A.

B. Graph showing the initial aggregate A.U. (400 nm) with different conditions: rec. rSP-A, rSP-A, rSP-D, D/A, and rSP-D. The graph also includes data points for initial aggregate (A.U./sec x 10^-3).
Fig. 5. Palaniyar et al.

A.  

| mSP-A | +/+ | -/- | +/+ | -/- | -/- | +/+ | -/- | -/- | mSP-D |
|------|-----|-----|-----|-----|-----|-----|-----|-----|------|
| D/A  | -   | +   | -   | +   | +   | -   | +   | +   | D/A  |
| antibody | A | A | A | A | A | A | A | A | antibody |
| Reducing | | | | | | | | | Non-reducing |

| kDa |
|-----|
| 110 |
| 90  |
| 51  |
| 35  |

B.  

| mSP-A | +/+ | -/- | +/+ | -/- | -/- | +/+ | -/- | -/- | mSP-D |
|------|-----|-----|-----|-----|-----|-----|-----|-----|------|
| D/A  | -   | +   | -   | +   | +   | -   | +   | +   | D/A  |
| antibody | D | D | D | D | D | A | A | A | antibody |
| Reducing | | | | | | | | | Non-reducing |

| kDa |
|-----|
| 110 |
| 90  |
| 51  |
| 35  |
Fig. 8 Palaniyar et al

SP-A-/-, D/A + plasma

- plasma
+ plasma

Surface tension (mN/m)

SP-A-/-, rSP-A
SP-A-/-
SP-A-/-, D/A

0 5 10 15 20 25
The role of pulmonary collectin N-terminal domains in surfactant structure, function and homeostasis, in vivo

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