Identification and Characterization of a Novel Interaction between Pulmonary Surfactant Protein D and Decorin*

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Surfactant-associated protein D (SP-D) is a collectin that is present in lung surfactant and mucosal surfaces. Although SP-D regulates diverse functions, only a few proteins are known to bind to this collectin. Here we describe the co-purification of decorin, a novel SP-D-binding protein, from amniotic fluid. The human decorin that co-purified with SP-D is a 130–150-kDa proteoglycan, which has a 46-kDa protein core and ~90-kDa dermatan sulfate chain. Both native and recombinant decorin can bind to SP-D that is already bound to malse-agarose matrix, and these SP-D-decorin complexes are dissociated at high salt (0.5–1.0 M NaCl) conditions, releasing the decorin. We further show that SP-D and decorin interact with each other (KD = 4 nM) by two mechanisms. First, the direct binding and competition experiments show that the carbohydrate recognition domain (CRD) of SP-D binds in a calcium dependent-manner to the sulfated N-acetyl galactosamine moiety of the glycosaminoglycan chain. Second, complement component C1q, a complement protein that is known to interact with decorin core protein via its collagen-like region, partially blocks the interaction between decorin and native SP-D. This protein, however, does not block the interaction between decorin and SP-D/n(CRD), a recombinant fragment that lacks the N-terminal and collagen-like regions. Furthermore, the core protein, obtained by chondroitin ABC lyase treatment of decorin, binds SP-D, but not SP-D/n(CRD). These findings suggest that decorin core protein binds the collagen-like region of the SP-D. Concentrations of decorin and SP-D are negatively correlated to each other, in amniotic fluid, implying a functional relevance for SP-D-decorin interaction, in vivo. Collectively, our results show that carbohydrate recognition domains of SP-D interact with the dermatan sulfate moiety of decorin via lectin activity and that the core protein of decorin binds the collagen-like region of SP-D in vitro, and these interactions may be operative in vivo.

*The abbreviations used are: SP, surfactant-associated protein; C1q, complement component 1q; CRD, carbohydrate recognition domain; ELISA, enzyme-linked immunosorbent assay; GAG, glycosaminoglycan (e.g. a sulfated disaccharide polymer [glucuronic or uridic acid and N-acetyl galactosamine 4-sulfate] ); BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid.

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Surfactant protein D (SP-D), a C-type lectin, is present in pulmonary surfactant and several other mucosal surfaces (1). Recent studies show that SP-D modulates multiple functions: innate immunity (2, 3), allergic response (4), expression of matrix metalloproteinases (5, 6), alveolar wall remodeling-embryosoma, fibrosis, and lipid and macrophage homeostasis (7–9). Taken together, these results indicate that SP-D regulates pathway(s) that involve matrix-related proteins. However, the SP-D-binding proteins, which may participate in this pathway, are not known.

In the lung, pulmonary type II cells express and secrete SP-D into the alveolar environment (1, 10). SP-D expression is developmentally regulated, and its concentration increases in amniotic fluid during the last few weeks of pregnancy (10). In adult lungs, the concentration of SP-D varies significantly during allergic and pathological conditions, and therapeutic lung washings and amniotic fluid are routinely used for the purification of SP-D (11–13). Purified SP-D is a glycoprotein and is composed of a short interchain disulfide bond-forming N-terminal domain, a collagen-like region with Gly-X-Y repeats (where X represents any amino acid and Y is often hydroxyproline or hydroxylysine), a hydrophobic neck region, and a C-terminal globular carbohydrate recognition domain (CRD) (14–16). Three CRDs are held together by protein-protein interactions at the hydrophobic neck region (17). The collagen-like region folds into a ~45-nm-long triple helix, and the chains are tethered at the end via the N-terminal segment. Trimeric SP-D subunits (3 × 43 kDa) further assemble as higher order oligomers via interactions at the collagen-like and N-terminal domains. Dodecameric SP-D are X-shaped ~100-nm-long molecules (516 kDa), whereas higher order multimers appear as “fuzzy balls” (16, 18). CRDs of SP-D can interact with glycoproteins (19), carbohydrates (20–22), and lipids (23) present on microbial surfaces, phosphatidylinositol complement of pulmonary surfactant lipid (24), and receptors/binding proteins such as CD14 (25) and gp-340 (26). N-terminal (27) and collagen-like (14) regions of SP-D are glycosylated and are implicated in structural organization and receptor binding. The N-terminal domain is important for disulfide-dependent oligomerization (28), and X-like structural assembly (29). Microfibril-associated protein 4, a fibrinogen-like domain-containing protein with lectin activity, binds to the collagen-like region of SP-D (30); however, its biological function is not clearly established.
Decorin is a member of the small leucine-rich proteoglycans (31) that binds collagens, collagen-like region of C1q (32), and other extracellular matrix components (33). Decorin contains a cysteine-rich N-terminal segment followed by multiple leucine-rich repeats (34, 35), which are involved in protein-protein interactions (36). The decorin peptide (38 kDa) is predicted to fold as a C-shaped corkscrew-like molecule where an amphipathic α-helices and β-sheet forms convex and concave surfaces, respectively (37). The concave side of decorin could accommodate one collagen triple helix, and it is known that the hydroxylsine residue in the collagen is necessary for its interaction with decorin (37, 38). The core protein has three potential Asn-linked carbohydrate attachment sites, which are predicted to be distributed along one side of the molecule, and a Ser-linked sulfated glycosaminoglycan (GAG) chain near the N terminus (39, 40). Asn-linked branched saccharides are highly complex (~6–8 kDa) and are rich in mannosyl residues, whereas the GAG chain (~90 kDa) is primarily composed of dermatan sulfate ([glucuronic or iduronic acid and N-acetyl galactosamine], (39, 40).

This ubiquitous proteoglycan is secreted by type II cells (41–43) and chondrocytes (40) and detected in alveolar macrophages (44) and extracellular matrix (45). Decorin expression in lung is developmentally regulated, and in adult lung, it is confined to alveolar regions (45). Decorin binds and regulates the concentration of transforming growth factor-β (33). Increased concentrations of transforming growth factor-β result in cell proliferation and eventual pulmonary fibrosis (46), but transient expression of decorin in lung alleviates the disease symptoms (47, 48). Decorin can also induce the expression of matrix metalloproteases 1, 2, and 14 (49), and conversely, matrix metalloproteases 2, 3, and 7 can cleave decorin under certain conditions (49, 50). SP-D-deficient mice (7, 51) and patients with low concentrations of SP-D (12) exhibit lung phenotypes that are associated with inflammation and tissue remodeling (e.g. fibrosis, emphysema). Therefore, decorin may regulate some of the phenotypes observed in SP-D gene deficiency.

In this report, we describe the co-purification of decorin and SP-D from human amniotic fluid. For the first time, we show that SP-D interacts with decorin and that this interaction involves two different types of binding. The globular domain of SP-D binds carbohydrate moieties of decorin, whereas the decorin core protein binds the collagen-like region of SP-D. We also identified that the sulfation of GalNAc is critical for the high affinity interaction between SP-D and this carbohydrate moiety. SP-D and decorin concentrations are inversely related in amniotic fluid samples, and this association may be related to inflammation and/or airway remodeling in vivo.

EXPERIMENTAL PROCEDURES

Chemicals—All of the enzymes and buffer salts were purchased from Sigma unless otherwise stated. Recombinant human decorin expressed in insect cells, using the baculovirus system, was purchased and stored at −20 °C in phosphate-buffered saline with bovine serum albumin (R&D Systems, Abingdon, UK). Affinity-purified anti-recombinant decorin monoclonal antibody or polyclonal antibody raised in goat was also purchased from R&D Systems. Dermatan sulfate and high molecular weight heparin were a gift from Dr. Barbara Mulloy (National Institute of Biological Standards and Control, South Mimms, Potters Bar, UK). Chondroitin 4-sulfate and chondroitin 6-sulfate were purchased from Calbiochem.

Purification of SP-A, SP-D, and C1q—We purified SP-A from the surfactant pellet that was obtained from therapeutic lung washings of individuals with alveolar proteinosis, by butanol extraction, mannoseagarose affinity, and gel filtration chromatography, as described previously (29). SP-D was purified from supernatants of lung washings by maltose-agarose affinity, selective manganese elution, and gel filtration chromatography as described previously (13). We purified C1q from human plasma samples (HD Supplies) with minor modifications to the previously published procedure (52). In this procedure, we used DEAE-Sepharose and SP-Sepharose and Superose 6 instead of DEAE-Sepha- dex A-50, CM-cellulose 32, and Bio-gel A-5 M columns, respectively. Recombinant human SP-D that contain only the neck/CRD was expressed in Pichia pastoris and purified by SP-Sepharose ion exchange, maltose-agarose affinity, and Superose 6 gel filtration chromatography as described previously (17).

Calculation of the Molecular Mass of Native SP-D—Native SP-D was purified from alveolar proteinosis lung washings eluted in the void volume of the Superose 6 gel filtration column as described previously (13). To estimate the molecular mass of the protein used in this study, we examined the oligomeric state of the negatively stained SP-D by transmission electron microscopy (data not shown). The SP-D appeared as pentamers, as described previously (13). The pH was adjusted to 7.4, and the suspension was gently stirred for 1 h at 23 °C or 18 h at 4 °C. The SP-D-maltose-agarose and SP-D-binding protein complexes were collected by centrifugation at 5000 × g for 10 min or filtered through a sintered glass filter and packed into a 10 × 1.6-cm column. The complexes were washed extensively with 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 10 mM CaCl2, 0.02% (w/v) Na2EDTA buffer. The proteins that interacted with the complexes or matrices were eluted with 1 M NaCl in the wash buffer. The eluate was saved for decorin purification. SP-D was eluted from the column using 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 10 mM CaCl2, 0.02% (w/v) Na2EDTA buffer. Protein eluted between 3- and 5-ml volume was pooled and concentrated using a 10-kDa molecular mass cut-off Amicon membrane filter under pressure and gentle stirring or an ultrafree centrifugation filter (Millipore, Watford, UK). The buffer of the concentrated sample was changed to 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 2 mM EDTA, and applied to a Superose 6 (30 × 1.6-cm) gel filtration column. Fractions (0.5–1.0 ml) were collected and stored at 4 °C until further analysis.

Radioiodination of SP-D and Its Binding to Decorin—A volume of 500 μl of native SP-D (50 μg/ml) in 5 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 2 mM EDTA buffer was placed in an Iodogen bead (400 ng) precoated Eppendorf tube. An aliquot of 5 μl of Na125I (0.5 μCi; Amer sham Biosciences) was added to the tube, mixed, and incubated at 20 °C for 10 min on ice. The reaction mixture was applied to a Sephadex G25 PD-10 desalting column (Amersham Biosciences) that was pre-equilibrated with 1% (w/v) BSA followed by 20 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA buffer. Protein eluted between 3- and 5-ml volume was pooled and stored at 4 °C. The specific activity of this SP-D preparation was 6.1 × 105 cpm/μg of protein.

In SP-D binding experiments, recombinant decorin (0.0–5.0 μg/well) was immobilized in the wells of 96-well plates (NUNC-Maxi-Sorp) in 0.1 mM sodium bicarbonate buffer (pH 9.6) at 4 °C for 18 h. The washed wells were blocked with 1% (w/v) BSA and incubated with a fixed amount of 125I-SP-D (0.1 μg/ml) in 20 mM Tris (pH 7.4), 150 mM NaCl, 5 mM CaCl2, 0.02% (w/v) BSA, and 0.02% (v/v) Tween 20 buffer for 3–4 h at 37 °C. The wells were washed with the binding buffer, and the bound 125I-SP-D was recovered after incubating the wells with 100 mM NaOH, 5 mM EDTA, 1% (w/v) SDS buffer for at least 30 min at 23 °C. Radioactivity of 50-μl aliquots was determined by a mini–γ-counter (Mini-Assay 6-20; Mini-Instruments, Essex, UK), and the decorin-specific binding was calculated by subtracting the radioactivity detected in the BSA-coated blank wells from the decorin-containing wells. To determine the affinity between the SP-D and decorin, 0.5 μg of recombinant decorin was immobilized in each well of a 96-well plate, blocked with BSA. 125I-SP-D (0.0–1.0 μg/ml) diluted in binding buffer was allowed to bind decorin present in these wells. Decorin-specific binding of radioactive protein was detected as above.

Deglycosylation of Decorin—Native decorin (100 μg) purified from amniotic fluid was digested with 1 unit of chondroitin ABC lyase in 40 mM Tris-HCl, 40 mM sodium acetate, 10 mM EDTA, 0.1 mM phenylmeth- ylamethylene fluoride, 0.36 mM pepstatin buffer (pH 8.0) for 18 h at 37 °C.
Decorin from the reactions was repurified on a Superose 6 (30 × 1.6 cm) gel filtration column in TSE buffer.

**Biotinylation of Carbohydrate Ligands—**Yeast mannan (1 mg/ml) was reacted with either 0.5, 1.5, or 4.5 mM NaIO4 in 100 mM sodium acetate buffer (pH 5.5) for 30 min on ice, and the reaction was stopped with 15 mM glycerol. The mixture was dialyzed in 100 mM sodium acetate buffer (pH 5.5) for 18 h at 4°C and reacted with 5 mM biotin-LC-hydrazide (Pierce) for 2 h at 23°C in the same buffer. The mixture was dialyzed for 18 h at 4°C to remove nonreacted biotin and stored at the same temperature. The GAGs were biotinylated using a modification of the method previously described (53). A volume of 20 μl of 250 mM biotin-LC-hydrazide in Me2SO was added to 1 ml of 6 mg/ml solutions of dermatan sulfate, chondroitin 4-sulfate, or chondroitin 6-sulfate in 100 mM MES (pH 5.5) buffer. Subsequently, 15 μl of 12.5, 25, or 50 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 100 mM MES (pH 5.5) was added, and the mixture was rotated at room temperature for 18 h. Samples were then dialyzed extensively against distilled water and stored at 4°C.

**Enzyme-linked Immunosorbent Assay (ELISA)**—Two types of ELISA were used in this study. In the first type, recombinant or native decorin that was purified from amniotic fluid was immobilized on 96-well plates (NUNC-Maxi-Sorp) in 0.1% sodium carbonate (pH 9.6) buffer for 18 h at 4°C. Washed wells were blocked with 2–5% (w/v) BSA and incubated with SP-D in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.02% (v/v) Tween 20, 2 mM CaCl2, or 1 mM EDTA. After washing the unbound proteins, biotinylated anti-human SP-D(NC/CRD) antibody (10 μg/ml) was incubated for 1–2 h. The SP-D-antibody complexes were detected using horseradish peroxidase-conjugated streptavidin system (see manufacturer’s instructions (Bio-Rad). The absorbance (405 or 450 nm) of individual wells was measured by a Spectrophotometer (Multiscan Ascent, Labsystems; Fisher). In the second type of ELISA, native SP-A, SP-D, and anti-goat or anti-mouse horseradish peroxidase-conjugated secondary antibody system or biotinylated anti-decorin primary antibody and horseradish peroxidase-conjugated streptavidin system (see “Results” and the legends to Figs. 1–3, 6, 7, and 9–11 for further details on ELISA).

**Binding of Recombinant Decorin to SP-D-Maltose-Agarose Matrix—**Fresh maltose-agarose (1 ml) was washed with 20 mM Tris-HCl buffer (pH 7.4), 1 M NaCl, 10 mM EDTA and equilibrated with 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM CaCl2, binding buffer. Native SP-D (50 μg) or control binding buffer was mixed with 500 μl of maltose-agarose and incubated for 1 h at 23°C. The SP-D-maltose-agarose complexes were washed three times in 1 ml of binding buffer to remove any free SP-D. Recombinant human decorin (1 μg) was mixed with 50 μl of SP-D-maltose-agarose or control maltose-agarose complexes and incubated for at least 23°C. Unbound decorin was removed by washing the resin three times with 1 ml of binding buffer, and the complexed decorin was eluted by incubating the resin three times in 100 μl of binding buffer that contained either 50, 100, 150, 250, 500, 750, or 1000 mM NaCl. The amounts of decorin and SP-D present in the eluate were determined by ELISA, as described above. Specific release of decorin was calculated after subtracting the background values obtained for the matrix alone.

Surface Plasmon Resonance Analysis—Mannan was immobilized on a streptavidin-coated BIAcore chip (SA-chip, BIAcore 2000, Herts, UK) by flowing biotinylated mannan-containing solution (1 mg/ml) in 100 mM NaOAc (pH 5.5) buffer at 5 μl/min for 5–10 min. Leaving flow cell 1 as blank, the next three flow cells were used for immobilization of mannan that was biotinylated after treating the saccharide with either 0.5, 1.5, or 4.5 mM NaIO4. Free streptavidin in all flow cells was blocked with biotin, and the chip was normalized with 50% (v/v) glycerol distilled water and stored at 4°C.

To show that SP-D binds to biotinylated mannan, we flowed SP-D (0–20 μg/ml) over all flow cells at 10 μl/min for 4 min in 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM CaCl2, 0.005% (v/v) surfactant P-20, 0.02% (w/v) Na2S2O3, 0.05% (w/v) HSC-buffer at 25°C. The complexes were allowed to disassociate for at least 5 min, and bound proteins were then removed with two pulses of 5 μl of 0.1% (w/v) SDS solution. The flow cells were re-equilibrated by washing them with 20 μl of 10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.005% (w/v) surfactant P-20, 0.02% (w/v) Na2S2O3, 0.05% (w/v) HSC-buffer and 20 μl of HSC-buffer. To determine the relative affinities of SP-D binding to dermatan sulfate and other saccharides, we conducted competition experiments. Competing saccharides were dissolved in HSC buffer, mixed with SP-D (4 μg/ml) at different concentrations (0–32 mM), and flowed over the mannan chip. SP-D binding to immobilized mannan for each saccharide concentration was calculated, assuming a 100% binding of SP-D in the absence of any competitor. The 50% inhibition concentration (IC50) of each competitor was calculated from the dose–response curves. Each data point represents the mean ± S.D. (n = 3).

**RESULTS**

Co-purification of SP-D and Decorin from Amniotic Fluid—To identify SP-D-binding proteins, we systematically examined the proteins that could interact with human SP-D-maltose-agarose matrix. Typically, 1–2 liters of clarified amniotic fluid was mixed with maltose-agarose in the presence of 10 mM calcium chloride to allow preferential binding of SP-D or SP-D complexes to the saccharide ligand. Nonspecifically bound proteins were removed by extensive washing of the complexes with 150 mM NaCl-containing binding buffer (at least 5 column volumes). The proteins that bound to the SP-D-containing matrix were released by washing the column with 1 mM NaCl in binding buffer and further separated by gel filtration chromatography (Figs. 1, A and B). The major protein isolated by this method migrated between the void volume (5000 kDa) and 200-kDa globular protein molecular weight standards. When separated by SDS-PAGE under reducing conditions, the
putative SP-D-binding protein corresponded to a molecular mass of \(-130\) kDa (Fig. 1B). N-terminal amino acid sequencing resulted in the sequence \(\text{DEASIGPVEVPDDR}\), which matched (100\%) with the N-terminal sequence of the mature chain of human decorin (54). This result suggested that decorin is one of the major proteins in amniotic fluid that can interact with SP-D-containing maltose-agarose matrix and can be released under high ionic strength conditions.

**Decorin Present in Amniotic Fluid Binds SP-D**—In addition to SP-D, several other proteins that are present in the amniotic fluid, including SP-A, can interact with maltose-agarose in the presence of calcium. Hence, to confirm that the proteins isolated by this procedure can interact directly with SP-D, we immobilized the proteins eluted from gel filtration chromatography (50 \(\mu\)l from each fraction) on an ELISA plate and detected their binding to SP-D using native SP-D and an antibody raised against recombinant human SP-D(n/CRD). The fractions that contained purified proteins interacted with SP-D in a concentration-dependent manner (Fig. 1C). These results provide confirmatory evidence that purified SP-D directly binds decorin.

**Recombinant Human Decorin Binds SP-D-Maltose-Agarose Complexes**—To further investigate whether the SP-D bound to maltose-agarose can bind to decorin, we mixed SP-D or buffer with maltose-agarose matrix in the presence of calcium and washed away the unbound proteins. Recombinant human decorin was mixed with these complexes, incubated, washed, and eluted with binding buffer containing varying concentrations of NaCl. High concentrations of NaCl (0.5–1.0 M) specifically released decorin from the SP-D-containing matrix (Fig. 2A). SP-D, however, was not released from the matrix under any of these conditions. The ability of SP-D to bind maltose-agarose matrix at a high ionic strength conditions has been recognized previously (13, 17) and may be attributable to the additional affinity of this lectin to the agarose matrix. These results show that decorin bound specifically to SP-D-containing maltose-agarose matrix and was released at high ionic strength conditions.

**SP-D Binds Recombinant Decorin Immobilized to Solid Support**—To determine whether SP-D present in solution can bind to decorin attached to a solid support, we immobilized a fixed amount (1 \(\mu\)g/well) of recombinant human decorin on ELISA plates. SP-D (0.0–5.0 \(\mu\)g/ml) was allowed to bind to decorin, and the bound protein was detected by biotinylated anti-SP-D(n/CRD) antibody. The results show that SP-D bound to immobilized decorin in a concentration- and calcium ion-dependent manner (Fig. 2B). Hence, SP-D can bind both native and recombinant human decorin, regardless of whether the SP-D or the decorin is immobilized onto solid supports.

**SP-D Binding to Recombinant Decorin Requires Calcium Ions**—To determine the cations necessary for the interaction between SP-D and decorin, we immobilized decorin (0.5 \(\mu\)g/well) on ELISA plates and allowed it to bind to SP-D (2.5 \(\mu\)g/ml) in the presence of 1 M divalent cations. SP-D-decorin interaction was detected in the presence of calcium but not in the presence of EDTA, magnesium, or manganese (Fig. 3A; p < 0.05). When present together with calcium, manganese inhibited the interaction between SP-D and decorin. To determine the concentration of calcium ions necessary for optimal SP-D-
A.  

B.  

Interaction between SP-D and Decorin

Fig. 4. Binding of native SP-D to biotinylated GAGs. Indicated amounts of immobilized SP-A (A) and SP-D (B) were allowed to bind to a fixed concentration of biotinylated GAGs (125 ng/ml) in the presence of 5 mM CaCl₂.  

We also used other GAGs such as hyaluronan and heparin in the competition experiments to determine whether these saccharides are also substrates for SP-D. Hyaluronan (IC₅₀ = 2 mM) and high molecular weight heparin competed effectively with mannan for SP-D binding (Fig. 5C). The relative affinities of SP-D to bind dermatan sulfate, chondroitin 4-sulfate, and chondroitin 6-sulfate are similar to those determined for the binding of SP-D to the biotinylated GAGs (Fig. 4). The dermatan sulfate and the related GAGs competed for SP-D binding more effectively than manose, mannnose, and GlcNAc (Fig. 5C).

We also used other GAGs such as hyaluronan and heparin in the competition experiments to determine whether these saccharides are also substrates for SP-D. Hyaluronan (IC₅₀ = 2 mM) and high molecular weight heparin competed effectively with mannan for SP-D binding (Fig. 5D). Low molecular weight heparin, however, showed little effect on SP-D binding to mannan. Further experiments are under way to determine the importance of these interactions. These results collectively indicate that SP-D binds dermatan sulfate with a higher affinity than manose and other related GAGs.

Interaction between Different Domains of SP-D and Decorin—To further investigate whether decorin can interact with the other lung collectin, we conducted solid phase assays. SP-A (0.0–0.5 μg) was immobilized on ELISA plates and allowed to bind to recombinant decorin (5 μg/ml) in calcium-containing buffer. Decorin that interacted with SP-A was detected by biotinylated anti-decorin antibody and a streptavidin-horseradish peroxidase system. No significant binding between SP-A and decorin was detected under these experimental conditions (Fig. 6A). These results suggest that SP-A may not interact with decorin.

To determine whether decorin can interact with the other lung lectin, we compared the binding of the recombinant decorin to C1q and SP-D. Decorin bound to native SP-D in a concentration-dependent manner, which is comparable with that of C1q binding to decorin (Figs. 6B and 7A). To determine whether C1q can compete with SP-D for decorin binding, we immobilized a fixed amount (0.2 μg) of native SP-D or SP-D(n/CRD) on ELISA plates and incubated it with recombinant decorin (5 μg/ml) in the presence of varying concentrations of C1q in the binding buffer. C1q competed with native SP-D, but not SP-D(n/CRD), for decorin binding, which reached a plateau near 53% (Fig. 7B). Therefore, C1q and SP-D may share a common type of interaction with decorin, which is present in the collagen-like region of SP-D. In addition, C1q only competed partially, which suggests that SP-D-decorin interaction takes place via more than one type of interaction. These results are consistent with decorin binding to the collagen-like region of SP-D and the CRDs of SP-D binding to carbohydrate moieties of decorin.

To investigate further the interaction between the globular domains of SP-D and the sulfated carbohydrate moiety, we immobilized SP-D(n/CRD) on an ELISA plate and competed its binding to recombinant decorin with increasing concentrations of dermatan sulfate. We detected decorin-specific binding to SP-D by a monoclonal antibody raised against the core protein. This GAG effectively competed with decorin for binding with SP-D(n/CRD), and the 50% inhibition concentration (IC₅₀) of dermatan sulfate was 1.2 mM (Fig. 8). This result establishes
that the carbohydrate recognition domain of SP-D binds to the dermatan sulfate moiety of the decorin via its lectin activity.

**Decorin Core Protein Binds to SP-D**—To further verify that the core protein can bind to SP-D, we removed the dermatan sulfate moiety from decorin by chondroitin ABC lyase treatment and allowed it to bind to SP-D in an ELISA. The removal of dermatan sulfate found on decorin by digestion converted the heterogeneous high molecular mass proteoglycan (>200 kDa) to a small uniform-sized molecule that migrated between alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa) globular proteins on gel filtration chromatography (data not shown). The decorin core migrated as a 46-kDa protein on SDS-PAGE under reducing conditions (Fig. 9A). When we tested the ability of native SP-D to bind these proteins, SP-D bound to decorin -2-fold more efficiently than to the core protein (Fig. 9B; p < 0.05). Although the SP-D(n/CRD), which lacks the N terminus and the collagen-like regions present in whole SP-D, bound to native proteoglycan, its interaction with the decorin core was undetectable (p < 0.05). Hence, native SP-D that contains the collagen-like regions can interact with the decorin core protein, albeit less effectively than with the intact proteoglycan. Furthermore, in the absence of the collagen-like region, the SP-D does not bind to the core protein. Taken together, these results indicate that the globular domains of SP-D bind primarily to the GAG chain of the proteoglycan, whereas the decorin core protein interacts with the collagen-like region of the lectin.

**Binding Affinity between SP-D and Decorin**—To determine the affinity between SP-D and the proteoglycan, we radiolabeled SP-D and allowed it to bind to the immobilized recombinant decorin (0.0–5.0 µg). SP-D (0.1 µg/ml) bound to decorin in a saturable and concentration-dependent manner (Fig. 10A).

When a fixed amount of decorin (0.5 µg) was allowed to bind to increasing concentrations of 125I-SP-D (0.0–1.0 µg/ml), the protein bound specifically to decorin (Fig. 10B). Scatchard plot analysis showed that the affinity (K<sub>d</sub>) between these two molecules is 4.34 nM (Fig. 10C). These results suggest that decorin and SP-D can bind efficiently.

**Decorin and SP-D Concentrations in Amniotic Fluid**—To find out whether any relationship exists between SP-D and decorin concentrations in the amniotic fluid, we determined the concentrations of these proteins by ELISA. We immobilized the proteins present in amniotic fluid on an ELISA plate and quantified the concentration of decorin and SP-D using biotinylated anti-recombinant human decorin or anti-recombinant human SP-D(n/CRD), respectively. The concentrations of decorin in the samples varied considerably (4.41–79.71 ng/ml) with a mean ± S.D. of 35.25 ± 20.97 ng/ml. Like decorin, SP-D concentrations also varied in the amniotic fluid (18.1–640.0 ng/ml with a mean ± S.D. of 182.4 ± 176.8 ng/ml). Correlation analysis showed that decorin and SP-D concentrations are negatively correlated with each other (decorin = -17.185 ln[SP-D] + 115.92; correlation co-efficient, r² = 0.6028) (Fig. 11).

**DISCUSSION**

Our data show that a novel SP-D-binding protein, decorin, interacts with SP-D under a variety of assay conditions in vitro. Binding between these two proteins occurs via two mechanisms: (a) decorin core protein binds to the collagen-like region of SP-D through protein-protein interactions, and (b) the CRDs of SP-D bind to the sulfated carbohydrate moiety of decorin by a lectin-carbohydrate type interaction. Concentrations of SP-D and decorin are inversely related in amniotic fluid samples.
which may suggest the existence of a biologically relevant SP-D-decorin interaction in vivo.

Like bronchoalveolar lavage, amniotic fluid is a rich source of SP-D (11–13, 27); hence, we used amniotic fluid to isolate putative SP-D-binding proteins. We hypothesized that highly oligomerized SP-D and SP-D that is complexed with its binding proteins can bind to maltose-agarose matrix. Since SP-D-maltose-agarose complexes are stable even at 1 M NaCl concentration (13, 17), only SP-D-binding proteins should be released from this complex at high ionic strength conditions. To achieve this aim, we selectively captured SP-D or SP-D-binding protein-SP-D complexes that are present in the amniotic fluid, using a maltose-agarose matrix in the presence of calcium. We eluted the putative SP-D-binding proteins from these complexes by increasing the NaCl concentration (1 M) in the wash

Fig. 6. Human decorin binding to SP-A, SP-D, and SP-D(n/CRD). Native SP-A (A), SP-D (B), and recombinant fragment of SP-D(n/CRD), which has no collagen-like region (C), were immobilized on ELISA plates at the amounts indicated and allowed to bind to decorin (5 μg/ml) in calcium-containing buffer. Binding of decorin to the immobilized proteins was detected by biotinylated anti-decorin antibody. No significant binding was detected between SP-A and decorin. Interaction between SP-D or SP-D(n/CRD) and the proteoglycan was detected primarily in decorin-containing wells (filled symbol) but not in the blank wells (open symbol) (p < 0.05). Each data point represents the mean ± S.D. (n = 3).

Fig. 7. Binding of decorin with C1q and its competition for SP-D-decorin interactions. A, the indicated amounts of C1q were immobilized on an ELISA plate and allowed to bind to decorin (5 μg/ml). Binding of decorin to the immobilized C1q was detected by biotinylated anti-decorin antibody. Significant interaction was detected primarily in decorin-containing wells (filled symbol) but not in the blank wells (open symbol) (p < 0.05). B, a fixed amount (0.2 μg) of SP-D (●) or SP-D(n/CRD) (▲) was immobilized on ELISA plates, and decorin (5 μg/ml) was allowed to bind to these proteins in the presence of the indicated concentrations of C1q as a competitor. Absorbance (450 nm) in the absence of competitor was considered as 100% binding. Each data point represents the mean ± S.D. (n = 3).

Fig. 8. Competition of dermatan sulfate with decorin for binding to recombinant SP-D(n/CRD). Recombinant decorin (5 μg/ml) was allowed to bind to immobilized SP-D(n/CRD) in the presence of the indicated concentrations of dermatan sulfate in calcium-containing buffer. Bound decorin was determined using a monoclonal antibody raised against the recombinant protein. The 50% inhibition concentration (IC50) of dermatan sulfate is 1.2 mM. Each data point represents the mean ± S.D. (n = 3).
buffer and systematically analyzed the proteins present in the eluate. We identified decorin as one of the major SP-D-binding proteins present in amniotic fluid (Figs. 1 and 9) by its N-terminal sequence (33) and molecular weight on SDS-PAGE (55, 56).

To investigate whether decorin interacted with SP-D but not with matrix or other proteins, we examined the interaction between purified SP-D and decorin by several methods. Since SP-A and some minor contaminants co-purify during the maltose-agarose affinity purification scheme of SP-D (13), we verified that SP-D that is already bound to maltose can interact with decorin. Decorin preferentially bound to SP-D-maltose-agarose complexes and is subsequently eluted with increasing NaCl concentration (Figs. 1 and 2). SP-D, however, was not released from the maltose-agarose matrices. This finding is consistent with the ability of SP-D to bind maltose-agarose under high ionic strength conditions in calcium-containing buffers (13, 17). These results support our hypothesis that decorin binds to SP-D-maltose-agarose complexes and is released at 1 M NaCl elution. Since native SP-D has a long collagen-like region and assemblies as oligomers (14, 16, 18), it is conceivable that SP-D simultaneously interacts with both decorin and maltose. Our results further show that SP-D can also bind to decorin that is already bound to a solid support (Figs. 1, 2, 6, and 10). Since both native and recombinant decorin interacted well with SP-D (Figs. 1 and 2), it was clear to us that SP-D and decorin can interact with each other regardless of which protein is immobilized on the solid support. To further show that SP-D interacts not only with native decorin but also with recombinant human decorin via the same mechanism, we studied their interaction by ELISA. The results are similar to those for native protein interactions, hence confirming that SP-D-decorin interactions are genuine.

SP-D interacts with other proteins (19, 25, 26, 30, 57) and ligands (20–24) in a divalent cation-dependent manner. Our results show that SP-D bound to decorin only in the presence of calcium (Figs. 2 and 3). Other cations such as magnesium and manganese failed to support the SP-D-decorin interactions (Fig. 3). This result suggests that SP-D binds decorin via C-type lectin activity. The optimal interaction occurred at physiological concentrations (1.0–5.0 mM) of CaCl₂, indicating the relevance of this binding in vivo. Interestingly, manganese inhibited the calcium-dependent interactions between SP-D and decorin (Fig. 3A), and this is consistent with the inhibitory effect of manganese on the lectin activity of SP-D (13, 58). Therefore, this result is in agreement with the known lectin property of SP-D and supports the presence of such activity in the interaction between SP-D and decorin.

Less than 40% of the mass of decorin is peptide. The remainder is sulfated GAG chain, which is a polymer of disaccharide (mainly IdoUA and GalNAc-4-sulfate) (39, 40, 55). SP-D does not bind to GalNAc (15, 58), and its interaction with GlcA or IdoUA is unknown. Therefore, to determine which component of the dermatan sulfate is responsible for SP-D-ligand interaction, we first compared the efficiency of the interaction between SP-D and biotinylated dermatan sulfate and related GAGs. SP-D binds most efficiently to dermatan sulfate (Fig. 4), suggesting that this sulfated GAG present on decorin is a preferred ligand for SP-D. SP-A, however, did not show significant bind-
ings to any of these GAGs. Since SP-D binds chondroitin 4-sulfate, more effectively than chondroitin 6-sulfate (Figs. 4 and 5), the sulfation at the C-4 position of GalNAc is important for enhanced binding of SP-D to this saccharide. The competition experiments suggest that some of the other GAGs also bind SP-D (Fig. 5). Polyionic ligands including dermatan sulfate are known to interact with collagens (59); hence, this GAG may also interact with the collagen-like region of SP-D to yield a high affinity interaction.

Since the recombinant fragment of SP-D(n/CRD) directly binds to decorin (Figs. 6 and 8) and their interaction can be competed by dermatan sulfate (Fig. 8), the CRD of the SP-D directly binds to dermatan sulfate moiety of decorin. Therefore, we conclude that CRDs of SP-D primarily bind to decorin via the lectin activity by recognizing the dermatan sulfate moiety of decorin.

Decorin also binds to the collagens via protein-protein interactions (33, 38). Although both of the lung lectins, SP-A and SP-D, contain collagen-like regions, only SP-D shows significant binding to decorin (Fig. 6). Our results show that collagen-like region-dependent SP-D-decorin interactions can be competed by C1q (Fig. 7), and decorin core protein can bind SP-D (Fig. 9). Hence, like C1q (32, 55, 60), the collagen-like region of SP-D interacts with decorin. The interaction between decorin core protein and collagen is dependent on hydroxylysine residues (38), and SP-D, but not SP-A, contains such hydroxylysine amino acids within its collagen-like region (14, 15). Therefore, our results are consistent with known properties of decorin.

Interaction between radioiodinated SP-D and decorin shows that they bind to each other with a high affinity (4.34 nm) (Fig. 10). Hence, these two molecules are likely to interact with each other under physiological conditions.

Interestingly, SP-D and decorin concentrations are inversely related in amniotic fluid samples (Fig. 11). Since our results show that SP-D and decorin efficiently interact with each other in vitro, it is conceivable that similar interactions are likely to occur in the intraterine environment. Decorin expression is developmentally regulated and plays an important role in lung morphogenesis (45, 61). This proteoglycan is highly expressed in the early stage of fetal development but is confined to alveolar septa in the mature lung (34, 45). Expression of SP-D in lung is also developmentally regulated but with increasing alveolar concentrations occurring in near term fetuses (10, 11). SP-D and decorin are known to present primarily in the alveolar space and extracellular matrix, respectively. Therefore, the interaction between these two proteins is likely to occur during pulmonary inflammation and/or tissue remodeling. Determination of whether the concentrations of SP-D and decorin are suggestive of inflammatory conditions or the SP-D-decorin interactions play any regulatory role in vivo requires further investigation.

In summary, this paper describes the following. (a) It describes a new method to isolate and purify SP-D-binding proteins, including decorin, from amniotic fluid. This method could conveniently be carried out in combination with SP-D purification. We characterized the interaction between SP-D and decorin in detail. (b) It shows that SP-D and decorin interact with each other via two mechanisms. First, SP-D binds the glycan moiety of decorin via its lectin activity; second, decorin core peptide probably binds the collagen-like region of SP-D via protein-protein interaction. (c) It provides the measurements of concentrations of SP-D and decorin in amniotic fluid and suggests that they may be negatively correlated with each other in vivo.
Interaction between SP-D and Decorin

Identification and Characterization of a Novel Interaction between Pulmonary Surfactant Protein D and Decorin
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