# Critical Role of Arg/Lys343 in the Species-Dependent Recognition of Phosphatidylinositol by Pulmonary Surfactant Protein D[†,‡]

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ABSTRACT: Surfactant protein D (SP-D) plays important roles in lung host defense. However, it can also recognize specific host molecules and contributes to surfactant homeostasis. The major known surfactant-associated ligand is phosphatidylinositol (PI). Trimeric neck−carbohydrate recognition domains (NCRDs) of rat and human SP-D exhibited dose-dependent, calcium-dependent, and inositol-sensitive binding to solid-phase PI and to multilamellar PI liposomes. However, the rat protein exhibited a >5-fold higher affinity for solid-phase PI than the human NCRD. In addition, human dodecamers, but not full-length human trimers, efficiently coprecipitated with multilamellar PI liposomes in the presence of calcium. A human NCRD mutant resembling the rat and mouse proteins at position 343 (hR343K) showed much stronger binding to PI. A reciprocal rat mutant with arginine at the position of lysine 343 (rK343R) showed weak binding to PI, even weaker than that of the wild-type human protein. Crystal complexes of the human trimeric NCRD with myoinositol and inositol 1-phosphate showed binding of the equatorial OH groups of the cyclitol ring of the inositol to calcium at the carbohydrate binding site. Myoinositol binding occurred in two major orientations, while inositol 1-phosphate appeared primarily constrained to a single, different orientation. Our studies directly implicate the CRD in PI binding and reveal unexpected species differences in PI recognition that can be largely attributed to the side chain of residue 343. In addition, the studies indicate that oligomerization of trimeric subunits is an important determinant of recognition of PI by human SP-D.

Surfactant protein D is a collagenous C-type lectin (collectin) that plays diverse roles in innate immunity and surfactant homeostasis (*1*, *2*). Although SP-D[1] exhibits specific binding to various simple and complex carbohydrates, it also binds to a variety of molecules that contain lipid domains, including Gram-negative lipopolysaccharides, bacterial glycolipids, and mycobacterial lipoglycans (*3*−*5*). These interactions are believed to contribute to the capacity of SP-D to function as an effector of innate immunity in the lung and various extrapulmonary tissues.

In addition, SP-D interacts with a more restricted range of host ligands. A significant fraction of lung SP-D can be isolated from lung washings in association with surfactant lipids, prompting its early designation as a surfactant-associated protein (*6*). Phosphatidylinositol (PI), an anionic phospholipid component of surfactant, was identified as the primary surfactant-associated ligand in crude surfactant (*7*, *8*). Specific interactions with glucosyl ceramide have also been described (*9*).

Experiments performed shortly after the discovery of SP-D indicated that the protein did not contribute to the biophysical activity of surfactant. Specifically, SP-D could be efficiently extracted from surfactant with competing sugars without perturbing surface tension lowering activity in vitro (*6*). Subsequent studies by other investigators showed that SP-D dodecamers, which are tetramers of trimeric subunits, can mediate a characteristic tubular reorganization of PI-containing lipid mixtures in the presence of calcium and surfactant protein B (*10*). These tubules resemble lipid structures identified in human lipoproteinosis lavage and cultures of isolated type II cells. However, the significance of this ultrastructural organization is uncertain.

Interest in possible roles in surfactant metabolism grew with the finding that SP-D-deficient mice develop an alveolar lipidosis (*11*−*13*) and that this abnormality can be rescued by the transgenic expression of rat SP-D dodecamers in the

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[‡] Atomic coordinates for the crystal structures of this protein are available in the Research Collaboratory for Structural Bioinformatics Protein Data Bank as entry 2ORK for the inositol 1-phosphate complex, entry 2OS9 for the myoinositol complex, and entry 2ORJ for the complex with *N*-acetylmannosamine.

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[1] Abbreviations: SP-D, surfactant protein D; SP-A, surfactant protein A; CRD, carbohydrate recognition domain; myoinositol, inositol (IN); HBSC, Hepes-buffered saline with calcium; HRP, horseradish peroxidase; hNCRD, human neck−CRD; rNCRD, rat neck−CRD.

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Table 1: Apparent Affinity for Myoinositol in Competition Assays[a]

| trimeric NCRD | $I_{50}$ (mM inositol) (mean ± standard error of the mean) | no. of independent experiments |
|---|---|---|
| human wild-type (*17*) | 5.7 ± 1.1 | 10 |
| rat wild-type (*17*) | 0.44 ± 0.05 | 11 |
| human R343K | 0.18 ± 0.04 | 3 |
| human R343A | 1.23 ± 0.18 | 3 |
| rat K343R | 2.7 ± 0.7 | 5 |

[a] The apparent affinity of the proteins for myoinositol was compared in mannan competition experiments, as described in Materials and Methods. The inhibitory activity of the mutants was determined by nonlinear regression analysis and is reported as the concentration of inositol (millimolar) required for 50% inhibition of binding of the fusion protein to mannan ($I_{50}$). Previously published data for the human and wild-type rat proteins are included for comparison with the mutants; the large differences between human and rat are highly significant (*17*).

respiratory epithelium (*14*). Recent studies using conditional knockouts have further confirmed direct roles of SP-D (*15*). The lipidosis, which occurs when animals are deficient from birth, can be dissociated from various other phenotypic abnormalities, including emphysema. The absence of SP-D is accompanied by abnormalities in the ultrastructural organization of surfactant lipids and is associated with the defective reuptake of surfactant by alveolar type II cells in vitro (*16*). Both of these abnormalities can be corrected with purified SP-D dodecamers.

SP-D consists of four distinct structural domains: an amino-terminal cross-linking domain, a triple-helical collagen domain, a neck domain that consists of a trimeric coiled coil, and the lectin or carbohydrate recognition domain (CRD). In recent studies, we observed species differences in the affinity of trimeric human, rat, and mouse SP-D neck−CRD (NCRD) for various soluble and solid-phase ligands (*17*). To our surprise, myoinositol bound with a considerably higher affinity to rat and mouse NCRDs than to the homologous human protein (Table 1). This prompted a comparison of binding to PI using N-terminally tagged NCRD fusion proteins. Here we describe major differences in the binding of trimeric rat and human NCRDs to PI and demonstrate specific roles of a nonconserved residue flanking the carbohydrate binding site in PI binding.

## MATERIALS AND METHODS

L-α-Phosphatidylinositol (P-0639, sodium salt, soybean) was initially obtained from Sigma-Aldrich (St. Louis, MO). However, most recent binding studies used the corresponding PI (catalog no. 840044P) from Avanti Polar Lipids (Alabaster, AL). Fatty acid-free BSA with low immunoglobulin (catalog no. BAH66-0050) was from Equitech-Bio, Inc. (Kerrville, TX). All mono- and disaccharides were the D-anomers and of the highest purity available from Sigma-Aldrich. Myoinositol and mannan were also from Sigma. D-Inositol 1-phosphate was enzymatically prepared by hydrolysis of phosphatidylinositol (Avanti Polar Lipids) with recombinant *Bacillus thuringiensis* phospholipase C; the material was purified by chromatography on an AG1-X8 anion exchange column (*18*).

*Expression of Fusion Constructs.* A bacterial expression system was used to generate panels of N-terminally tagged, trimeric human and rat neck−CRD (NCRD) fusion proteins (*17*, *19*). Site-directed mutagenesis was performed using a QuikChange II XL site-directed mutagenesis kit (Stratagene, LA Jolla, CA) and the human or rat SP-D neck−CRD DNAs in the pET-30a+ vector as a template (*17*). All sequences were verified by automated DNA sequencing of the complete coding sequence of the fusion protein.

*Isolation of Recombinant Proteins.* All wild-type and mutant trimeric neck−CRD domains were expressed in RosettaBlue competent cells (Novagen, Madison, WI), isolated from inclusion bodies, and then refolded and oligomerized prior to chelation chromatography (*17*, *19*, *20*). To ensure comparable oligomerization, trimers were further purified by gel filtration chromatography. All proteins migrated as a single major band via SDS−PAGE and migrated more slowly upon reduction, consistent with normal folding in the native protein that yields the intrachain disulfide bonds (data not shown).

Protein concentrations were determined using the bicinchoninic (BCA) protein assay kit (Pierce, Rockford, IL) and BSA as a standard. Endotoxin was quantified using a QCL-1000 chromogenic assay kit (Cambrex Corp., East Rutherford, NJ). Maximum concentrations for the current preparations ranged from 0.03 to 0.2 pg/μg of purified protein.

Human and rat dodecamers, and human trimers, were purified by sequential saccharide affinity and gel filtration chromatography as previously described (*21*, *22*). Endotoxin levels were less than 2.5 pg/μg of purified protein.

*Mannan Binding and Competition Assays.* The binding of trimeric fusion proteins to surface-adsorbed mannan was assessed using 96-well plates and an S-protein−HRP detection system (*17*, *19*). By contrast with our earlier studies, the blocking/binding buffers used 1% (w/v) fatty acid-free, low-IgG BSA. This was implemented to preclude potential confounding interactions with immunoglobulins (*23*). The low level of background absorbance in the absence of fusion protein was subtracted to give the total level of binding. Where indicated, the level of nonspecific binding, which was defined as binding in the presence of EDTA or excess inositol competitor, was determined. Plastic-adsorbed fusion proteins gave equivalent dose-dependent signals in a control assay, indicating identical accessibility of the N-terminal tags (*19*).

*Solid-Phase PI Binding Assays.* Solid-phase, direct PI binding assays were performed using modifications of the mannan binding protocol and assays described by Kuroki and co-workers (*8*). PI was dissolved in chloroform and methanol (1:4) at a concentration of 100 μg/mL. Aliquots (50 μL) were transferred to the wells of 96-well plates (Costar 3590, Corning, Inc.) and air-dried overnight at room temperature in a chemical hood or dried under nitrogen. The freshly prepared films were rehydrated overnight at 4 °C with 100 μL of ELISA coat buffer, washed three times in Tween-free wash buffer containing 5 mM calcium, and blocked for 2 h at room temperature with blocking/binding buffer containing 5 mM calcium and 1% (w/v) fatty acid-free BSA. Fusion proteins were added at the indicated final concentration in blocking/binding buffer in the presence or absence of myoinositol (25−100 mM, depending on the experiment). Plates were incubated for 60 min at room temperature prior to being washed as described above. Bound fusion proteins were detected using an S-protein−HRP conjugate (*19*).

*Preparation of Multilamellar PI Liposomes.* Multilamellar PI liposomes were prepared essentially as described by

Kuroki and co-workers (*8*). PI was dissolved at a concentration of 1 mg/mL as described above, dried to a film under nitrogen in a depyrogenated glass tube, and rehydrated for 1 h at 37 °C in 125 $\mu$L in TBS [140 mM NaCl and 20 mM Tris-HCl (pH 7.4)] with intermittent vigorous agitation using a vortex mixer. The 10 mM stock was used for competition or precipitation assays.

*PI Solution-Phase Competition Assay.* Plates were coated with mannan as described above, washed, and then blocked for 1 h at room temperature with blocking/binding buffer containing 10 mM calcium. The calcium concentration was increased to ensure sufficient free calcium for SP-D activity in the presence of the liposomes. Human or rat NCRD fusion proteins (10 $\mu$g/mL) were preincubated in calcium-containing buffer for 15 min at room temperature with serial dilutions of 10 mM PI liposomes, prior to being transferred to the plate. The plate was incubated for 60 min at room temperature and washed three times with wash buffer, and binding of the fusion proteins was assessed as described above. A parallel competition was performed using serial dilutions of myoinositol.

*PI Precipitation Assay.* Interactions with PI were also examined using an assay similar to that described by Ogaswara and Voelker (*24*). Proteins were preincubated for 15 min at room temperature in the presence of 10 mM calcium and in the absence or presence of 50 mM myo-inositol. An equal volume of a suspension of calcified PI liposomes was added, and the mixture was incubated for 60 min at room temperature with intermittent agitation using a vortex mixer. The insoluble lipids were collected by centrifugation (21000*g* for 10 min at 23 °C). For some preliminary experiments, the reaction mixtures were chilled on ice prior to centrifugation at 4 °C, but this had no qualitative effect on the results. The supernatant was combined with nonreducing 6× sample buffer and denatured by boiling, and proteins were resolved via SDS−PAGE on a 10 or 12% gel. Proteins were visualized by staining with Bio-Safe Coomassie stain (Bio-Rad). In some control experiments, the pellets were washed three times in the presence of calcium, solubilized in a small volume of TBS containing 20 mM EDTA, and transferred to a new tube prior to the addition of 6× sample buffer. The latter precaution reduced the contribution of proteins nonspecifically adsorbed to the walls of the reaction tube. Stained and thoroughly destained gels of supernatants were scanned within the linear range of band intensity using a flatbed scanner with a transparency adapter. Densitometric analysis was performed using ImageQuant TL (Amersham-Pharmacia).

*Data Analysis for Binding Assays.* All values are given as the mean ± standard error of the mean (SEM) of at least three independent assays using at least two independent preparations of recombinant protein. Binding data were analyzed using Sigmaplot 9.0 (SPSS Inc., Chicago, IL). Apparent dissociation constants were calculated by nonlinear regression of the saturation binding curves assuming a molecular mass of 66 kDa per trimeric fusion protein.

*Crystallographic Analysis.* Human trimeric NCRDs were purified, and crystals were prepared as previously described (*20*). Myoinositol and inositol 1-phosphate were soaked into crystals by dissolving the ligand in mother liquor and transferring a crystal into the resulting solution for a period of 1−2 h. Inositol was incubated at a concentration of 30 mM. Complexes with I-1-P were obtained at 20 mM. After being soaked, the crystal was flash-frozen in a 100 K stream of nitrogen gas at the beamline. All data were collected at beamline X8C at the National Synchrotron Light Source (Brookhaven National Laboratory, Upton, NY). Data were processed using DENZO and SCALEPACK (*25*). Data collection statistics are shown in Results. Model building and structure refinement were performed using O (*26*), CNS (*27*), and REFMAC5 (*28*).

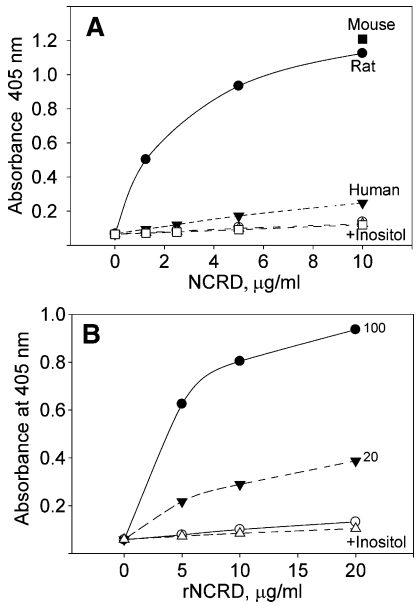


FIGURE 1: Rat and mouse NCRD fusion proteins preferentially bind to PI. The dose-dependent binding of NCRD fusion proteins to solid-phase PI was examined as described in Materials and Methods. Specificity was assessed by performing parallel incubations in the presence of 50 mM inositol. Lines were smoothed but not fitted. (A) Wells were coated with 50 $\mu$L of 100 $\mu$g/mL Sigma PI. The binding of human (▼) and rat (●) NCRDs is compared. Binding of the mouse NCRD [10 $\mu$g/mL (■)] is also shown. Binding of all three proteins was largely abrogated by inositol (white symbols). (B) The binding of the rat NCRD is compared at coating concentrations of 20 (▼) and 100 $\mu$g/mL (●). Binding at both concentrations was largely abrogated by 50 mM inositol (white symbols). Similar differences in maximal binding were obtained in numerous experiments using both Sigma and Avanti PI. Binding affinities for saturation binding experiments are summarized in Table 2.

## RESULTS

*Rat SP-D NCRDs Show Stronger Binding to Solid-Phase PI than Human NCRDs.* We initially compared the binding of identical, N-terminally tagged, trimeric human and rat NCRDs to solid-phase PI. In previous studies, we observed similar, specific dose-dependent binding of the wild-type rat and human NCRDs to mannan, a complex fungal polysaccharide (*17*). However, there were marked differences in the binding of rat, mouse, and human NCRDs to PI. The maximal binding and apparent affinity were significantly greater for the rat and mouse proteins (Figure 1A and Table 2). Binding to PI varied with NCRD dose and was blocked with excess myoinositol (inositol, Figure 1). Binding was also blocked by maltose, the prototypical carbohydrate competitor (data not shown). Binding was also dependent on the coating concentration of PI (Figure 1B). Binding of all three proteins was inhibited with inositol (IN); however, the $I_{50}$ (millimolar) was substantially lower for the rat

Table 2: Apparent Affinity for Phosphatidylinositol[a]

| trimeric NCRD | apparent $K_d$ (M) (mean ± the standard error of the mean) | no. of independent experiments |
|---|---|---|
| human wild-type | $(2.75 \pm 0.19) \times 10^{-7}$ | 2 |
| rat wild-type | $(5.06 \pm 0.6) \times 10^{-8}$ | 7 |
| mouse wild-type | $(2.64 \pm 0.73) \times 10^{-8}$ | 3 |
| human R343K | $(2.5 \pm 0.5) \times 10^{-8}$ | 4 |
| human R343A | $(3.12 \pm 0.68) \times 10^{-7}$ | 4 |
| rat K343R | $1.16 \pm 10^{-6}$ | 1 |

[a] Binding of NCRD fusion proteins (0−40 μg/mL) to solid-phase PI was examined in dose−response experiments. Although there was no significant nonspecific binding, the level of binding in the presence of inositol was usually subtracted from the total level of binding. Binding of the human protein and rat K343R was often too weak to obtain meaningful data. Each of the indicated independent experiments showed evidence of saturation and a good fit by nonlinear regression analysis. Because each CRD has an identical site and because three CRDs are probably required for detectable binding, the molecules are considered functionally univalent for the purpose of this analysis. Data were fitted using a single-site model assuming a trimer mass of 66 kDa. Most of these experiments used Avanti PI. However, comparable differences were observed with the Sigma lipid. Note that the $K_d$ values for rat and mouse wild-type and human R343K differ by nearly a log order from the human protein and human R343A. Given the large differences and small variance, no statistical analysis was performed.

(Table 1) and mouse proteins (*17*). There was minimal binding of rat or human NCRDs to phosphatidylglycerol, and the low level of binding that was observed was not inhibited by inositol, suggesting headgroup specificity for the interaction (data not shown).

*Rat SP-D NCRDs Exhibit Stronger Binding to PI Liposomes than Human NCRDs.* To confirm binding of PI to aggregated lipids in a solution phase, we implemented a mannan competition assay using multilamellar PI liposomes as a competitor. For these and subsequent experiments, we focused on comparisons between the human and rat protein. The liposomes caused dose-dependent inhibition of binding of both NCRDs to mannan (Figure 2). However, $I_{50}$ was much lower for the rat NCRD, consistent with differences in the solid-phase binding assay. Binding of the proteins was blocked by myoinositol, indicating specificity.

*NCRDs Differentially Coprecipitate with Aggregates of PI Liposomes.* Given the above, we used modifications of a published liposome precipitation assay to further examine the species-dependent differences in PI binding (*24*). Multilamellar PI liposomes were grossly aggregated in the presence of physiological calcium and sedimented under unit gravity. Precipitation was blocked by EDTA but unaffected by inositol (data not shown). Under these conditions, no significant amount of PI remained in the solution phase, as assessed by SDS−PAGE and staining with Coomassie blue. Specifically, the solubilized pellet, but not the supernatant, exhibited a discrete band migrating near the dye front.

As shown in Figure 3, rat NCRDs (rNCRD) specifically coprecipitated with the aggregated PI liposomes. In the absence of competing inositol, there was little NCRD in the supernatant and protein was readily identified in the pellet. By contrast, in the presence of competing inositol, the amount of protein in the supernatant was increased and the amount recovered with the PI pellet greatly decreased. Although human NCRDs exhibited qualitatively similar behavior, the extent of coprecipitation was lower, with larger amounts of soluble protein in the absence of competitor. In the absence of PI, there was no evidence of protein precipitation (data not shown). Like the rat protein, the trimeric mouse NCRD was efficiently precipitated with the liposomes (data not shown).

Gels were analyzed by densitometry of bands stained within the linear range. The amount of protein remaining in the supernatant was assessed in the absence and presence of competing inositol. Peak densities were integrated, and the percent of protein precipitating with the liposomes was calculated (mean ± the standard error of the mean) for three independent experiments. While approximately two-thirds of the rat NCRD precipitated (71 ± 10%), there was less than 20% precipitation of the human NCRD (16 ± 2%); the differences were reproducible and significant ($p < 0.05$).

*Rat and Human Dodecamers Exhibit Similar Binding to PI.* Recombinant rat SP-D is almost exclusively assembled as dodecamers (*29*). By contrast, human SP-D is secreted as a more heterogeneous mixture of trimeric subunits, hexamers, dodecamers, and higher-order multimers depending on genotype (*30*). Accordingly, we used the precipitation assay to compare the behaviors of the NCRDs with the full-length rat and human molecules, as well as human trimers. As shown in Figure 4, rat and human dodecamers exhibited similar, inositol-sensitive precipitation with the aggregated PI liposomes. Comparable findings were obtained in six independent experiments. In preliminary experiments, we observed similar binding of rat and human dodecamers to PI-coated wells, as assessed by SDS−PAGE and densitometry of the bound proteins (data not shown).

By contrast, the full-length human trimer was incompletely precipitated (Figure 4). Although we observed some variability in the amount of unprecipitated trimer, the extent of specific binding was reproducibly lower than that observed for the full-length molecules. Because some preparations of trimer exhibit detectable agglutinating activity (data not shown), we attribute such variability to differences in protein aggregation or the presence of minor hexameric contaminants.

*A Human Mutant with Lysine at Position 343 Shows Stronger Binding to PI.* Previous studies have shown that arginine 343 (Arg343) plays roles in modulating glucose recognition by human SP-D. Specifically, the nonconservative substitution of valine for arginine (R343V) was reported to increase the apparent glucose affinity while interfering with the normal discrimination between glucose and *N*-acetylglucosamine (*31*). We recently demonstrated preferred recognition of maltose, glucose, and inositol by trimeric rat and mouse NCRDs, as compared to the corresponding human protein (*17*). Notably, rat and mouse (as well as all other known SP-Ds) have lysine at position 343.

Accordingly, we examined the binding activity of a mutant with a "conservative" exchange of lysine for arginine at position 343. The trimeric human mutant (hR343K) was indistinguishable from the human protein by SDS−PAGE in the absence and presence of reduction and predominantly eluted as a trimer on gel filtration (data not shown). As shown in Figure 5 (hR343K), the protein showed much stronger binding to PI. The level of specific binding was the same as or greater than that of the normal wild-type rat protein and was specifically and completely inhibited by inositol. In saturation binding experiments, the apparent binding affinity

Table 3: Crystallographic Data[a]

| | myoinositol | inositol 1-phosphate | *N*-acetylmannosamine |
|---|---|---|---|
| wavelength (Å) | 1.1 | 1.1 | 1.1 |
| resolution range (Å) | 28−1.7 | 38−1.9 | 27−1.8 |
| no. of measured reflections | 654729 | 643901 | 725745 |
| no. of unique reflections | 72901 | 51917 | 61345 |
| completeness (%) [overall (final shell)] | 94.7 (87.7) | 92.7 (72.6) | 98.0 (96.2) |
| $\langle I/\sigma I \rangle$ (final shell) | 22.4 (4.0) | 46.2 (8.4) | 25.8 (9.7) |
| $R_{merge}$ (%) (final shell) | 0.04 (0.28) | 0.04 (0.07) | 0.045 (0.17) |
| refinement | | | |
| $R_{free}$ | 0.223 | 0.226 | 0.205 |
| $R_{cryst}$ | 0.195 | 0.186 | 0.178 |
| no. of atoms | | | |
| protein | 3454 | 3457 | 3462 |
| water | 515 | 517 | 524 |
| ions | 9 | 9 | 9 |
| ligand | 36 | 32 | 45 |
| total | 4014 | 4015 | 4040 |
| average $B$ (Å$^2$) | | | |
| main chain | 24.1 | 19.0 | 21.2 |
| side chain | 26.0 | 21.0 | 23.2 |
| ligand | 24.7 | 29.6 | 23.4 |
| all atoms | 26.2 | 21.3 | 23.3 |
| rms deviation | | | |
| bonds (Å) | 0.012 | 0.013 | 0.012 |
| angles (deg) | 1.21 | 1.25 | 1.25 |

[a] Ramachandran plots of the structures that are described showed all residues falling within the most favorable or allowed regions.

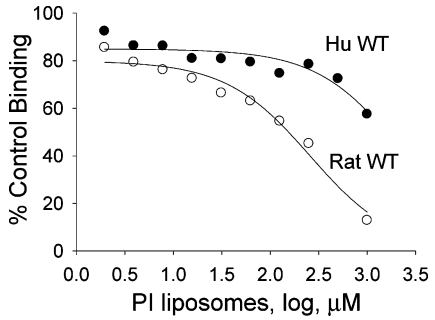


FIGURE 2: PI liposomes preferentially compete for the binding of rat NCRDs to solid-phase mannan. Competition assays were performed using soluble Sigma PI and mannan-coated plates as described in Materials and Methods. Rat and human NCRDs exhibit comparable dose-dependent binding to solid-phase mannan, as shown in previous studies (*17*) and Figure 5.

increased to levels resembling that of the wild-type rat protein (Table 2). The mutation was also accompanied by a significant increase in the apparent affinity for inositol (Table 1). As predicted by the other assays, the mutation increased the level of precipitation with the liposomes, as compared to that of the wild-type human protein. Specifically, the protein was preferentially recovered with the pellet but was primarily recovered in the supernatant in the presence of competing inositol (Figure 3, hR343K). The level of precipitation was 64 ± 5%, as assessed by densitometry, comparable to that of the wild-type rat NCRD. Interestingly, hR343K showed minimal binding to mannan (Figure 5), despite binding to maltosyl-BSA. This was confirmed using two independent preparations of mutant NCRD.

*A Rat NCRD Mutant with Arginine at Position 343 Shows Weaker Binding to PI.* To assess the role of this specific residue in the preferred recognition of PI by rat NCRDs, we examined a rat mutant with a reciprocal exchange of arginine for lysine at position 343. As indicated in Materials and Methods, the rat mutant (rK343R) was indistinguishable from the rat protein by SDS−PAGE in the absence and presence of reduction and predominantly eluted as a trimer on gel filtration. However, the level of binding to PI and apparent affinity were markedly decreased, to levels at least as low as those observed for the human protein (Figure 5 and Table 2). This was accompanied by a nearly 6-fold decrease in the $I_{50}$ for inositol (Table 1). The mutant showed specific, calcium- and dose-dependent binding to mannan, at levels comparable to those of the rat and human proteins (Figure 5). This suggests that effects on PI binding are not secondary to a decrease in calcium binding affinity. As predicted by the other assays, the substitution greatly reduced the level of precipitation with the PI liposomes (Figure 3). The level of precipitation was only 21 ± 7%, as assessed by densitometry, and not significantly different from that observed for the wild-type human NCRD.

*PI Binding Is Favored by a Lysine Side Chain at Position 343.* A control mutant NCRD with an alanine substitution was examined to further assess the contribution of the side chain of residue 343. The mutant (hR343A) bound to mannan at least as effectively as the wild-type protein. However, binding to PI was similar to that of the wild-type human protein (Figure 5 and Table 2). Although the substitution increased the apparent affinity for inositol, effects were significantly weaker than that observed for hR343K (Table 1). The alanine mutant exhibited a similar increase in affinity for several other saccharide competitors, including maltose (data not shown).

*Crystal Structure of the Human NCRD Complexed with Inositol.* To understand the mechanism of interaction of inositol and PI, the crystal structure of the enterokinase-cleaved human trimeric NCRD (*19*) was determined as a complex with myoinositol (Figure 6A,B). Electron density corresponding to bound myoinositol was well-represented

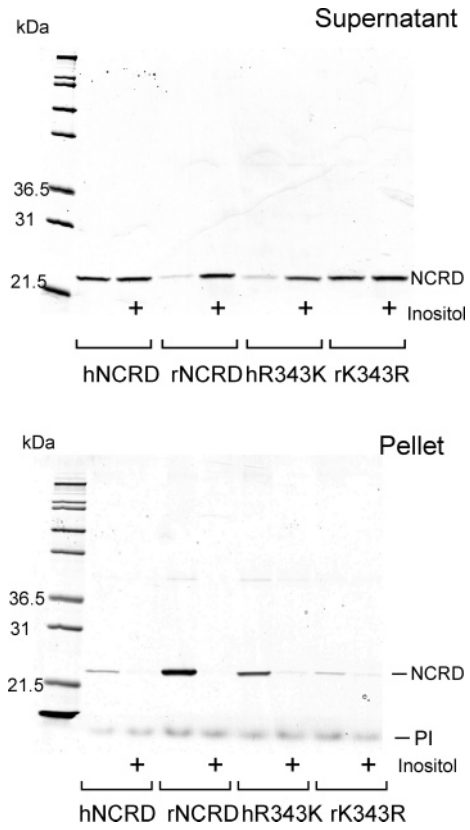


FIGURE 3: NCRDs can coprecipitate with PI liposomes in the presence of calcium. Multilamellar PI liposomes were prepared and precipitation assays performed in the presence of 10 mM calcium, as described in Materials and Methods. For this experiment, NCRD fusion proteins (12 $\mu$L of a 200 $\mu$g/mL stock) were incubated with 24 $\mu$L of 2 mM Avanti PI in the presence of 12 $\mu$L of competing inositol or buffer. Proteins in the supernatant and pellet were resolved by SDS−PAGE in the absence of reduction and visualized by protein staining. The top panel shows the supernatant, while the bottom panel shows the pellet. The position of migration of the NCRDs is indicated by the arrow. PI can be seen migrating near the dye front. All proteins were predominantly recovered in the supernatant in the presence of competing inositol. Wild-type rat (rNCRD) and human NCRDs (hNCRD) are shown in the four lanes at the left. The human R343K and rat K343R mutants are also shown. The rat NCRD and human R343K efficiently bound to PI liposomes, allowing greater recovery in the pellet in the absence of competing inositol.

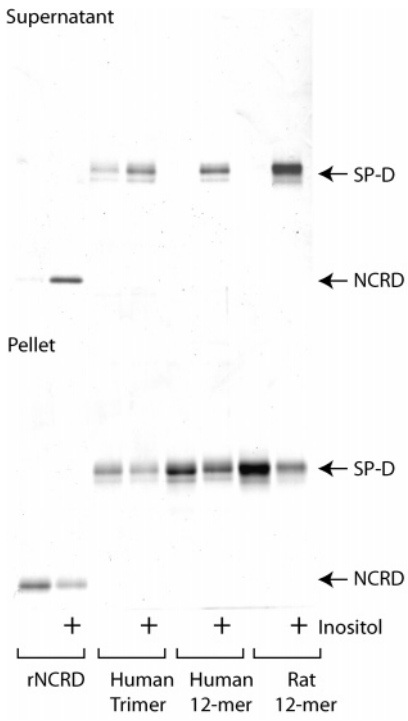


FIGURE 4: Human and rat dodecamers bind to PI liposomes. Binding assays were performed as described in the legend of Figure 3, except that full-length recombinant proteins (12 $\mu$L of a 200 $\mu$g/mL stock) were examined. Proteins remaining in the postincubation assay supernatants (top) or pellets (bottom) were resolved by SDS−PAGE in the presence of reduction. Notably, both human and rat dodecamers bound to the liposomes. However, a significant proportion of the human trimer resembled the human NCRD with respect to incomplete binding in the absence of competing inositol. For this experiment, pellets were solubilized directly in sample buffer; protein observed in the presence of maltose is attributed to a nonspecific binding to the tube (see Materials and Methods).

in each of the three subunits of the NCRD. The position of the ring was similar to that of the first sugar ring of known carbohydrate ligands (*20*, *32*) (Figure 6C). The distribution of electron density corresponding to the OH substituents of the cyclitol ring, particularly the single axial OH group at C2, suggests that there are two major orientations for the inositol molecule. Both orientations bind to calcium ion 1 (*20*, *32*) via OH groups at C1 and C6 (Figure 6A,B). The difference in the two orientations is a simple 180° rotation around an axis connecting the midpoints of the C3−C4 and C1−C6 bonds. In each orientation, a cyclitol OH group is within 3.0 Å of the guanidyl moiety of R343. However, the latter is oriented such that hydrogen bonding appears to be unfavorable. The R343 side chain appears to be held in place by a hydrogen bond to the side chain of E333. However, as noted previously (*28*), the guanidyl group can also adopt a slightly altered position in the A subunit, causing the distance to E333 to exceed hydrogen bonding length.

*Crystal Structure of the Inositol 1-Phosphate Complex.* Because inositol is substituted at the C1 position in PI, it would not be able to bind in either of the orientations observed for myoinositol. Accordingly, we also examined binding of the hNCRD to inositol 1-phosphate (I-1-P). As shown in Figure 6D, I-1-P primarily bound in one orientation, different from those observed for myoinositol. The inositol ring bound to calcium ion 1 via the OH groups at C4 and C5, with the phosphate extending away from the shallow binding pocket. As for myoinositol, a cyclitol ring OH group (at C6) is close to R343 but appears to be unfavorably oriented for hydrogen bonding. The orientation of the phosphate seems to be maintained by steric constraints and by electrostatic interaction (4 Å) with the guanidyl group of R343 (Figure 6D). The electron density of the phosphate is slightly weaker than for the rest of the ligand, which suggests that some radiolysis of the phosphate could have occurred during X-ray exposure.

Electron density for the whole inositol 1-phosphate molecule was well-represented in the B and C subunits, but only parts of the ligand structure were observed in the A subunit. Intersubunit variation in binding complicates the structural analysis but is consistent with previous findings concerning the binding of maltose (*20*, *32*). Ligands that protrude from the pocket and/or lack secondary interactions at the binding surface can bind differently in each of the three CRDs. This is attributed primarily to interference

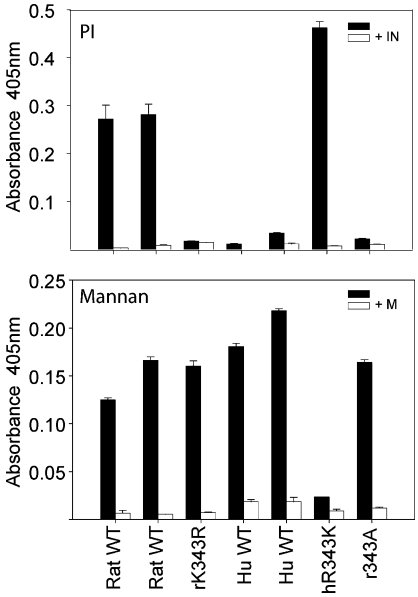


FIGURE 5: Wild-type and mutant NCRDs exhibit differential binding to PI and mannan in solid-phase assays. Equivalent amounts of wild-type and mutant human and rat NCRDs (10 $\mu$g/mL) were examined in solid-phase PI and mannan binding assays, as described in Materials and Methods. The histogram shows the mean and standard error for three independent dilutions of each protein. In addition, two entirely different preparations of the wild-type proteins are shown.

resulting from close packing of adjacent NCRD molecules in the crystal. In the structure presented here, the close approach of packing molecules mainly affects the CRD binding site in the A subunit, interfering with the binding of inositol 1-phosphate, while there is more open access above the site in the B and C subunits.

## DISCUSSION

Previous studies suggested that binding of rat SP-D to PI involves interactions with the inositol moiety (*7*, *8*). Myo-inositol is an effective competitive inhibitor of the lectin activity of SP-D, and early studies using purified natural rat SP-D showed that it was at least as potent as maltose, the prototypical competing sugar (*7*). Because inositol is a sugarlike cyclic alcohol with vicinal hydroxyl groups, Persson and co-workers speculated that inositol coordinates with calcium in a manner resembling the carbohydrate ligands of C-type lectins (*7*). Consistent with this suggestion, we have demonstrated specific, inositol and saccharide-sensitive, calcium-dependent binding of human, rat, and mouse NCRDs to PI. Single-site substitutions at position 343 within the CRD reciprocally altered the binding of human and rat NCRDs, conclusively demonstrating contributions of the lectin domain and residues near calcium ion 1. This conclusion is consistent with the crystal structures of the human NCRD complexed with inositol and inositol 1-phosphate. Both ligands coordinated with calcium ion 1 and residues coordinating with this calcium ion.

It was previously suggested that inositol might bind to SP-D in multiple orientations using various pairings of vicinal hydroxyl groups (*27*). In our studies, it appears that myo-inositol binds predominantly in two orientations, both of which use C1 and C6−OH groups to bind to calcium ion 1. In inositol 1-phosphate, the binding to calcium ion 1 appears to be exclusively mediated by the equatorial C4 and C5−OH groups. It seems likely that this is the dominant mode of interaction with inositol in PI.

Previous studies using rat surfactant protein A−rat SP-D chimeras indicated the importance of the 25 C-terminal residues of rat SP-D for PI binding and strongly implicated the small loop between Cys331 and Cys345 (*33*, *34*). Our findings provide strong support of this inference. On the basis of the available data, we conclude that species differences in PI binding largely result from differences in affinity for inositol, which are in turn influenced by the side chain of residue 343. Human R343K exhibited enhanced binding to PI and increased affinity for inositol, resembling the wild-type rat protein. By contrast, rat K343R showed weaker binding to PI and decreased affinity for inositol. Notably, hR343A exhibited only a slight decrease in affinity for inositol. This indicates that a basic residue is not necessary for inositol binding in the context of the human NCRD. We speculate that the presence of a lysine side chain at this position enhances inositol binding via more favorable direct interactions with inositol, or through altered interactions with Glu333 or other hydrophilic residues in the vicinity of calcium ion 1. Additional insights will undoubtedly come from crystallographic analysis of rat SP-D or the rK343R and hR343K mutants.

In preliminary experiments, we observed that the affinity of the human NCRD for I-1-P ($I_{50} = 1.4$ mM) is substantially greater than for inositol (Table 1; $I_{50} = 5.7$ mM). By contrast, the affinity of the rat NCRD for inositol 1-phosphate ($I_{50} = 0.43$ mM) is comparable to that of myoinositol (Table 1; $I_{50} = 0.44$ mM). Consistent with the latter finding, computational modeling of hR343K predicted that the shorter side chain of lysine will prevent direct interactions of the $\epsilon$-amino group with the phosphate moiety (data not shown). Thus, the interactions between the phosphate of inositol 1-phosphate and the guanidyl group of R343 may partially offset the intrinsically lower affinity of human SP-D for inositol. However, findings with hR343A indicate that interactions of the R343 side chain with phosphate are not required for the binding of human SP-D to PI and strongly suggest that binding of SP-D to PI is primarily driven by interactions with the inositol moiety.

Although our findings indicate an important role of calcium ion 1 and surrounding residues in PI binding, other secondary interactions are anticipated. Altering the coordination of calcium ion 1 by the simultaneous substitution of Gln for Glu321 and Asp for Asn323 in rat SP-D (QPD) only partially inhibited binding to PI liposomes, despite altered saccharide preferences and loss of binding to glucosyl ceramide and saccharide affinity columns (*24*). Furthermore, SP-D can interact with acyl chains or fatty acids, and there is evidence that fatty acid micelles can inhibit binding to PI (*8*, *35*, *36*). Kishore and co-workers (*37*) reported that the human neck domain can bind to solid-phase PI and other phospholipids. Although this might reflect interactions of exposed hydrophobic residues near the C-terminal end of the neck (*37*), hydrophobic interactions of acyl chains with other regions of the NCRD are likely.

We have shown that residue 343 plays important roles in PI and inositol recognition. However, other nonconserved residues could contribute to differences in ligand recognition by rodent and human SP-D. We recently demonstrated that

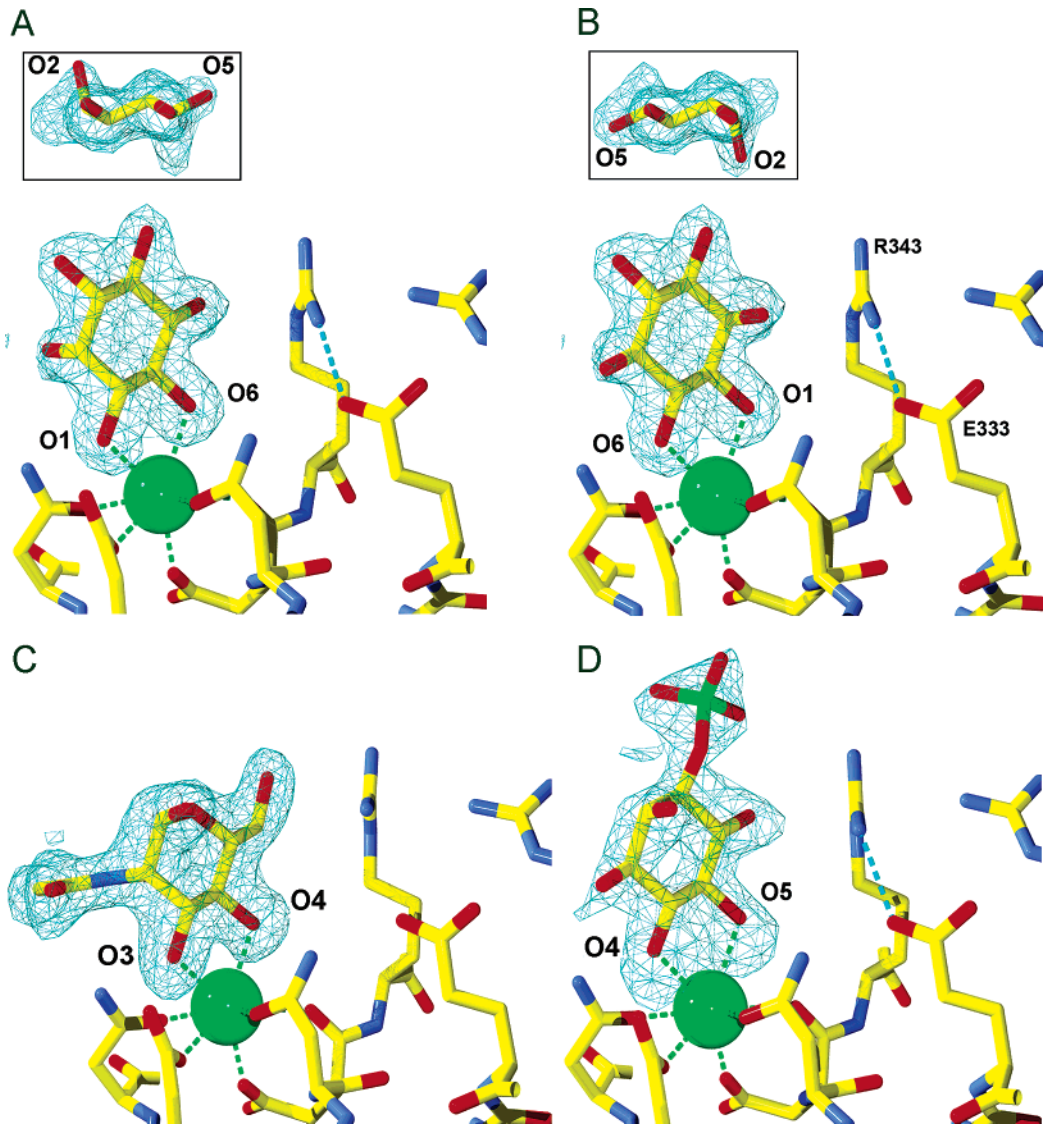


FIGURE 6: Crystallographic complexes of the human trimeric NCRD with myoinositol and inositol 1-phosphate, as compared to the complex with a known carbohydrate ligand, *N*-acetylmannosamine. Panels A and B show the binding site for myoinositol together with electron density for the bound ligand. Panel A shows the ligand bound in one orientation; the other orientation is shown in panel B. Insets show the top view of the ligand, indicating that the electron density corresponds to the ligand primarily in these two orientations. Panel C shows the equivalent view of *N*-acetylmannosamine, a preferred carbohydrate ligand of human SP-D (*17*), bound to the same site via its C3− and C4−OH groups. Panel D shows the same view of inositol 1-phosphate. Panels A, B, and D show subunit B of the hNCRD, and panel C shows subunit A. Electron density maps are $F_o - F_c$ sa-omit maps of the ligands, contoured at $2\sigma$.

the nonconserved residue at position 325, which is positioned on the opposite side of the carbohydrate binding groove, contributes to differences in the recognition of *N*-acetylmannosamine and enhances the binding of human SP-D to certain solid-phase glycoconjugates (*17*). Notably, the substitution of asparagine for residue 324 and/or 325 in the human NCRD did not significantly alter binding to PI (data not shown).

The contributions of the lectin domain and its interactions with PI to surfactant homeostasis are uncertain. Interactions of SP-D with surfactant lipids could be critical for normal surfactant homeostasis, at least within the context of murine lung development (*15*, *16*). On the other hand, recent findings using murine models suggest potential direct contributions of the collagen domain to interactions with surfactant small aggregates, as well as effects on surfactant ultrastructure and surfactant reuptake by type II cells (*38*, *39*). Although our original work suggested that the majority of rat and human lavage SP-D is soluble, it was recently shown that most SP-D in the high-speed supernatant of murine lavage co-isolates with the small aggregate fraction of surfactant lipid (*16*). Thus, interactions with PI, and compartmentalization of SP-D between soluble and surfactant lipid-associated phases, could have important consequences for the host defense and innate immune function, in a manner independent of any possible contributions to surfactant homeostasis. PI could also be liberated at sites of active cellular injury, and similar interactions might contribute to the recognition of certain pathogens, such as mycobacteria (*40*).

Full-length human trimers exhibited weaker binding to PI liposomes than dodecamers, as detected in the liposome precipitation assay. However, human dodecamers, like rat dodecamers and rat and mouse NCRDs, were efficiently coprecipitated in the absence of competing inositol. Together, our data indicate that the human NCRDs and trimeric subunits bind with a comparatively low affinity for PI.

However, higher-order multimers of human SP-D exhibited efficient binding. The data suggest, but do not prove, that this is a consequence of cooperative binding among trimeric NCRDs. Other effects of the N-terminal truncation on function of the human protein cannot be entirely excluded. Although rat and human proteins show similar binding to mannan and certain forms of LPS (ref *17* and unpublished data), human SP-D appears to be much more highly dependent on higher-order multimerization for interactions with maltose-substituted supports and specific strains of influenza virus (*17*, *30*). Because trimers are not found in appreciable amounts in rodent lung, it is possible that the human protein has co-evolved modifications in quaternary structure and lectin activity that favor interactions with specific exogenous ligands. Future studies will examine interactions of these molecules with purified surfactant and surfactant-like phospholipid mixtures.

Because there are polymorphic variations that appear to alter the extent of assembly and the proportion of trimers (*30*), our data raise the possibility of genotype-dependent differences in association of SP-D with surfactant. This might contribute to individual variation in the risk of developing perturbations of surfactant homeostasis following lung injury. Interestingly, lavage from patients with alveolar lipoproteinosis often contains a trimeric variant of SP-D (*41*). Polymorphic variations in assembly might also influence the availability for interactions with inhaled pathogens, in part through differential interactions with surfactant.

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