Species Differences in the Carbohydrate Binding Preferences of Surfactant Protein D

Erika C. Crouch, Kelly Smith, Barbara McDonald, David Briner, Bruce Linders, Joseph McDonald, Uffe Holmskov, James Head, and Kevan Hartshorn

Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri; Medical Biology Center, University of Southern Denmark, Odense, Denmark; and Department of Physiology and Department of Medicine, Boston University School of Medicine, Boston, Massachusetts

Interactions of surfactant protein D (SP-D) with micro-organisms and organic antigens involve binding to the trimeric neck plus carbohydrate recognition domain (neck+CRD). In these studies, we compared the ligand binding of homologous human, rat, and mouse trimeric neck+CRD fusion proteins, each with identical N-terminal tags remote from the ligand-binding surface. Although rat and mouse showed similar affinities for saccharide competitors, both differed markedly from the human protein. The human neck+CRD preferentially recognized N-acetyl-mannosamine, whereas the rat and mouse proteins showed greater affinity for myoinositol, maltose, and glucose. Although human neck+CRDs bound to maltosyl-agarose and fungal mannan, only rat and mouse neck+CRDs showed significant binding to maltosyl-Toyopearl beads, solid-phase maltosyl-albumin neo-glycoprotein, or the Phil82 strain of influenza A virus. Likewise, human SP-D dodecamers and trimeric subunits of full-length rat, but not full-length human SP-D trimers, bound to maltosyl-Toyopearl. Site-directed mutagenesis of the human neck+CRD demonstrated an important role of Asp324-Asp325 in the recognition of N-acetyl-mannosamine, and substitution of the corresponding murine sequence (Asn324-Asn325) conferred a capacity to interact with immobilized maltose. Thus, ligand recognition by human SP-D involves a complex interplay between saccharide presentation, the valency of trimeric subunits, and species-specific residues that flank the primary carbohydrate binding site.

Keywords: carbohydrate recognition domain; collectin; lectin; SP-D

Surfactant protein D (SP-D) plays important roles in the innate defense against micro-organisms and contributes to the lung's response to antigenic challenge (1, 2). Like many other effectors of innate immunity, SP-D is a pattern recognition molecule with a variety of potential ligands (3, 4). These include glycoconjugates expressed by micro-organisms and complex oligosaccharides associated with viral envelope proteins, fungal cell walls, and particulate organic antigens. In addition, at least some interactions involve specific interactions with host cells. The consequences of these interactions include microbial aggregation and enhanced cellular uptake, inhibition of bacterial and fungal growth through effects on membrane permeability, modulation of cytokine pro-

duction by phagocytic cells in response to micro-organisms, enhanced clearance of apoptotic cells, and modulation of acquired immunity.

SP-D is a member of a family of collagenous, C-type lectins, designated collectins (5). In most mammals, this family also includes pulmonary surfactant protein A (SP-A) and serum mannose binding lectin (MBL). SP-D is preferentially assembled as multimers of homotrimeric subunits. Although rat lung SP-D is predominantly assembled as dodecamers, humans and certain other species can show varying proportions of "four-arm" dodecamers, multimers of dodecamers, and/or "single-arm" trimeric subunits. As for other collectins, each subunit of SP-D includes an amino-terminal, disulfide crosslinking domain; a collagen domain; a trimeric coiled-coil neck domain (N); and a C-terminal trimeric array of carbohydrate recognition domains (CRDs). Cooperative interactions among the three CRDs of a trimeric subunit are essential for high-affinity, functional binding to a variety of microbial ligands (5).

The carbohydrate-binding activity of SP-D, SP-A, and MBL is calcium dependent and involves direct interactions of the sugar ligand with a coordinated calcium ion within the C-type lectin CRD (6–8). Although the CRDs of all known secreted collectins are homologous to rat MBL-A (i.e., of the so-called "mannose-type"), our initial characterization of rat SP-D indicated a preference for maltose and glucose over mannose (9). Subsequent studies, using a variety of indirect immunologic assays and solid-phase ligands, suggested similar saccharide selectivities for human SP-D (10) and for SP-D molecules isolated from other species (11, 12). Although all known SP-Ds can be purified by maltosyl-agarose affinity chromatography and maltose can inhibit many binding activities, there have been no direct interspecies comparisons of SP-Ds using comparably oligomerized molecules. As a result, rat and human proteins have been used almost interchangeably for a variety of *in vitro* and *in vivo* studies.

In the present studies, the functional activities of trimeric human, rat, and mouse neck+CRD fusion proteins were directly compared. We observed species differences in preferences for saccharide ligands and in interactions with carbohydrate ligands presented on solid supports, a situation that models physiologic interactions with micro-organisms and other multivalent, particulate ligands. The unique presence of Asp, rather than Asn, at positions 324 and 325 in the human protein was found to contribute to the distinctive ligand-binding properties of human SP-D. Higher-order multimerization of human, but not rat or mouse, trimeric neck+CRDs was required for interactions with certain maltose-substituted affinity supports, maltosyl-BSA, and influenza A virus. Our findings further demonstrate the importance of subunit multimerization for ligand recognition by human SP-D and have important implications for the interpretation of animal models of SP-D function and deficiency.

MATERIALS AND METHODS

The full-length human and rat SP-D cDNAs were described previously (13, 14). Recombinant human SP-D dodecamers and trimers, recombinant

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Joseph McDonald is presently at Pfizer Inc., St. Louis, MO, joseph.mcdonald@pfizer.com

Correspondence and requests for reprints should be addressed to Erika C. Crouch, M.D., Ph.D., Dept. of Pathology and Immunology, Campus Box 8118, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110. E-mail: crouch@path.wustl.edu

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rat SP-D, and a single-arm trimeric mutant of rat SP-D (RrSP-Dser15,20) were prepared and characterized as previously described (13–15). RrSP-Dser15,20 has the same domain structure as rat or human SP-D but consists exclusively as trimeric subunits because of its inability to form interchain disulfide bonds. The full-length mouse SP-D cDNA was obtained as a kind gift of Dr. Sam Hawgood (University of California, San Francisco). The pET-30a(+) vector, S-protein horseradish peroxidase (S-protein HRP), and RosettaBlue competent cells were from Novagen (Madison, WI). Enterokinase, light chain (P8070L) was from New England Biolabs (Beverly, MA). BSA (Fraction V; A4503), BSA-fatty acid free (Fraction V, low endotoxin; A8806), yeast mannan, and maltosyl-BSA (A5283 or A8460) were from Sigma-Aldrich (St. Louis, MO). All mono- and disaccharides were the D-anomers and of the highest purity available from Sigma. Pustulan (54051) was from Calbiochem (San Diego, CA). Purified synthetic oligomers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA).

Generation of Fusion Constructs

We used a bacterial expression system to generate a panel of N-terminally tagged, trimeric neck+CRD (NCRD) fusion proteins containing the 153-amino-acid, neck+CRD domains of human, rat, or mouse SP-D (Figure 1). Previous studies have shown that the human neck+CRD domain contains all the information necessary to direct normal CRD folding, intrachain disulfide bond formation, coordination of calcium ions, and ligand binding (6, 16). All three of the expressed neck+CRD sequences lack consensus sequences for glycosylation or other known post-translational modifications.

Cloning and mutagenesis. A DNA fragment encoding the NCRD was subcloned into pET30a(+) as previously described (17). Site-directed mutagenesis was performed using a QuikChange II XL Site-Directed Mutagenesis Kit (200521; Stratagene, LA Jolla, CA) and the hSP-D neck+CRD DNA in pET-30a(+) as the template. All DNA

sequences were verified by automated sequencing of the entire coding sequence of the fusion protein.

Expression, Purification, and Biochemical Characterization of Trimeric Neck+CRD Domains

Bacterial expression. RosettaBlue competent cells were transformed with the desired plasmid. After induction, expressed proteins were isolated from inclusion bodies (IBs) and refolded and oligomerized as previously described (17). We observed no evidence of proteolysis of proteins partitioned to IBs. However, for some control experiments using soluble proteins, the extraction buffer was supplemented with a bacterial protease inhibitor cocktail (P8849; Sigma-Aldrich) and 1 mM o-phenanthroline (Sigma-Aldrich).

Chelation affinity chromatography. Refolded proteins extracted from IBs were dialyzed at 4°C against 0.5M NaCl, 0.05M Tris-HCl (pH 7.5), containing 10 mM imidazole (binding buffer). Insoluble material was removed by centrifugation. The soluble His-tagged fusion proteins were purified by sequential nickel-affinity chromatography, dialysis to remove imidazole or any eluted nickel, and gel filtration chromatography, as previously described (17). Yields of the mouse protein (5–10 mg/liter) were ~ 4-fold lower than for the human and rat proteins under identical conditions of IB isolation, reflecting differences in compartmentalization between the periplasm and inclusion bodies. Aliquots of protein were stored at –80°C. After thawing and mixing, the purified proteins eluted exclusively as trimers (data not shown).

SDS-PAGE and blotting. SDS-PAGE was performed using 12% separating or 10–20% gradient minigels. Recombinant proteins were routinely visualized by rapid staining with Bio-Safe Coomassie Blue (Bio-Rad), and molecular weights were estimated using Mark 12 standards (Invitrogen, Carlsbad, CA). Blotting and detection with S-protein HRP or specific antibodies to SP-D was performed as previously described (17).

Figure 1. Homologous NCRD fusion proteins. (A) Alignment of corresponding C-terminal, CRD sequences of human, rat, and mouse SP-D. The three proteins are identical in length, and all terminate at residue 355 of the mature protein. A consensus sequence is shown. *Upper case letters* identify residues conserved in all three proteins; *lower case letters* indicate identity for rat and mouse. Critical residues involved in the coordination of calcium ion 1 and saccharide binding are identified with an *asterisk*. Characteristic sequences that surround the carbohydrate binding site and define the so-called "SP-D groove" are indicated. The *small box* in the human sequence identifies sites of site directed substitutions. (B) Sequence of human NCRD fusion protein showing critical structural elements. The *underline* identifies the neck domain. The site of enterokinase (EK) cleavage is identified by the *top arrow*. The *small box* and *arrow* near the end of the sequence identify sites of site-directed substitutions. (C) Fusion proteins were isolated from inclusion bodies, refolded, oligomerized, dialyzed, resolved by gel filtration chromatography, and examined by SDS-PAGE. The approximate molecular mass (kD) is indicated at left. *Lane 1*, Mark 12 protein standard. *Lane 2*, rat NCRD, unreduced. *Lane 3*, human NCRD, unreduced. *Lane 4*, mouse NCRD, unreduced. *Lane 5*, rat NCRD, reduced. The figure was prepared using identical exposures of the same gel; some lanes were deleted for clarity.

Enterokinase cleavage and crosslinking assays. For some control experiments, fusion tags were cleaved from the recombinant proteins at the enterokinase cleavage site using a protocol provided with the enzyme. The enzyme was adsorbed with trypsin inhibitor agarose beads before isolating the neck+CRD by gel filtration chromatography. The extent of oligomerization of proteins was further confirmed by chemical crosslinking (17).

Endotoxin precautions. All chromatography and binding assay buffers were prepared using freshly purified, endotoxin-free water, and columns were depyrogenated as previously described (17). Endotoxin was routinely quantified using an end-point chromogenic assay (QCL-1000; Cambrex Corp., E. Rutherford, NJ). All recombinant protein stocks isolated from IBs had very low endotoxin values, with maximum concentrations for the current preparations ranging from 3.2 to 7.9 pg/ μ g purified protein.

Saccharide Affinity Chromatography

Maltosyl-agarose. Maltosyl-agarose was prepared using divinyl sulfone-mediated covalent crosslinking of maltose to Sepharose 4B. For these separations, we used the low pressure chromatographic system originally described (9). After dialysis versus chromatography buffer, purified proteins (0.5–1 mg total in 3 ml) were applied to a 20-ml column of maltosyl-agarose equilibrated with 150 mM NaCl, Tris-HCl (pH 7.5) (tris-buffered saline) containing 5 mM calcium. After washing with five column volumes, bound proteins were eluted with calcium-free column buffer containing 10 mM EDTA. Volume-adjusted aliquots of the starting material, wash, and eluted proteins were resolved by SDS-PAGE, detected by protein staining or immunoblotting, and quantified by densitometry.

Maltosyl-Toyopearl. Maltose was also coupled to Toyopearl TSK HW/75(F) beads (Tosoh, Tokyo, Japan) using divinyl sulfone (18). Briefly, the beads were suspended in 10% (vol/vol) divinyl sulfone in 0.5 M Na₂CO₃, incubated for 70 min, washed with 0.5M Na₂CO₃, incubated in 10% (wt/vol) maltose in 0.5 M Na₂CO₃ (pH 11) for 16 h at room temperature, washed, and blocked by thorough washing with tris-buffered saline. For affinity chromatography, we used an HR10/10 column (Amersham Pharmacia, GE Healthcare) with a 7.5-ml bed volume. Proteins were applied and eluted at room temperature at a flow rate of 1 ml/min using the AKTA system. Elution profiles were monitored by A₂₈₀. Pre-equilibration, sample, and elution buffers were identical to those described for maltosyl-agarose.

Binding and Competition Assays

The binding of trimeric fusion proteins to surface-adsorbed mannan or other ligands was assessed using 96-well plates and an S-protein–HRP detection system as previously described (17). Immediately before each assay, thawed aliquots of the protein stock solutions were briefly centrifuged in a microfuge, and the concentration of protein in the supernatant was reconfirmed using a BCA protein assay.

Binding assays. The binding of trimeric neck+CRD fusion proteins to yeast mannan- or neoglycoprotein-coated plates was performed using limited modifications of published methods (11) as previously described (17). For most experiments, the wells of 96-well plates were coated with 50 μ g/ml mannan. Previous studies of hNCRDs showed that binding increases linearly as the mannan concentration is increased from 1 to 50–100 μ g/ml, reaching a plateau at slightly higher concentrations (17).

Coated wells were washed and incubated with blocking/binding buffer containing low endotoxin, fatty-acid free, BSA. The fusion proteins were diluted in blocking/binding buffer containing 5 mM calcium. Mannan-coated plates were incubated with fusion protein for 1 h at room temperature, washed, and incubated with an empirically optimized dilution of S-protein–HRP conjugate (Novagen). After washing, color was developed using peroxidase substrate, and the absorbance was measured at 405 nm. Background binding in the absence of fusion protein was subtracted to give total binding. Where indicated, specific binding (defined as binding in the presence of calcium minus binding in the absence of calcium) was determined. Control experiments confirmed equivalent dose-dependent detection of the plastic-adsorbed fusion proteins, indicating the presence of comparably accessible N-terminal, S-protein binding sites within the range of protein concentrations used in the mannan binding studies (17).

Competition assays. Proteins were added to wells in the presence of various concentrations of competing inhibitors. Unless otherwise stated, all values are given as the mean $\pm$ SD of at least triplicate determinations. Most binding data were plotted and analyzed using Sigmaplot 8.0 (SPSS Inc., Chicago, IL).

Influenza Virus Assays

The Phil82 strain of Influenza A virus (IAV) was grown and isolated as previously described (19, 20). Final viral stocks contained $\sim 5 \times 10^8$ plaque-forming units/ml. Hemagglutination assays were also performed as previously described. Viral binding assays were performed essentially as for the mannan binding assays, except for modifications to the coating procedure. Briefly, wells were incubated overnight at 4°C with a 1:250 dilution of the Phil82 stock in coating buffer. After a brief centrifugation, plates were washed with PBS containing calcium chloride before blocking and incubation with the fusion proteins.

Computer Modeling and Docking

The crystal structure coordinates of human SP-D neck+CRD complexed with maltose (PDB accession 1 pwb) (6) were used for modeling studies. Docking simulations of mannose, glucose, and ManNAc were performed using GLIDE and GLIDE XP (Schrodinger, Inc.). For each saccharide, 15 poses were examined, and ranked based on the calculated energy potentials. Ligands were minimized using the OPLS2001 force field before docking. Side chain conformers for residue 325 were chosen for docking based on a conformational analysis of Asp and the Asn mutant side chains in the context of the SP-D crystal coordinates. These simulations were performed using the torsional sampling function within Maestro (Schrodinger, Inc.). The minimum energy conformer was used for docking calculations.

RESULTS

Expression of Recombinant Trimeric Neck+CRDs

For these studies, we used fusion proteins containing the homologous wild-type human, rat, or mouse neck+CRD domain (hNCRD, rNCRD, and mNCRD, respectively) linked to identical N-terminal tags (17). This design permits interactions of the binding surface of trimeric CRDs with solid-phase ligands while leaving the tags accessible to S-protein conjugates added to the aqueous phase. It also circumvents potential confounding effects of differing levels of oligomerization and potential effects of antibody binding or covalent labeling procedures on ligand interactions. It also permits the reproducible detection of the lectin domains from different species, despite incomplete immunologic cross-reactivity.

Expressed recombinant rat and mouse fusion proteins were extracted from inclusion bodies, as previously described for the human fusion protein (17). After refolding, oligomerization, and metal affinity chromatography, the fusion proteins were further purified by gel filtration to ensure the isolation of trimeric oligomers. Essentially identical chromatographic profiles were obtained for the human (17) and the rat and mouse proteins (data not shown). All showed a major peak eluting in the positions of trimers and minor higher molecular weight species. Fractions in the major peak were pooled, and SDS-PAGE analysis of equivalent amounts of the purified fusion proteins is shown in Figure 1C.

Chemical crosslinking assays and gel filtration chromatography of the enterokinase-cleaved proteins were used to further confirm trimerization of the fusion proteins (17). The major EK-cleavage fragments of all three wild-type NCRDs eluted from the gel filtration column with an estimated mass of ~ 54 kD, consistent with the predicted mass of trimeric neck+CRD domains, and SDS-PAGE of the purified cleavage products demonstrated a single band that migrated with an estimated mass of ~ 18 kD (reduced) (data not shown). As previously shown for the human NCRD (17), the cleaved and uncleaved rat and mouse

NCRDs migrated more slowly after reduction with dithiothreitol, indicating the presence of intact, intra-chain disulfide bonds (Figure 1C).

Murine and Human NCRDs Are Differentially Retained on Maltose-Substituted Supports

In our initial experiments, carbohydrate-binding activity of the fusion proteins was confirmed by maltosyl-agarose affinity chromatography using conditions routinely used for the isolation of natural and full-length recombinant rat SP-D and natural and recombinant human SP-D multimers, dodecamers, and trimers (9, 14). Consistent with previous studies, all NCRD fusion proteins bound to the column in the presence of calcium. For example, immunoblots using a polyclonal antibody to human SP-D CRD (P13) demonstrated essentially complete binding of the purified human NCRD fusion protein to maltosyl-agarose, with subsequent elution by EDTA (Figure 2A). By contrast, a control, mutant trimeric human NCRD with the substitution of Q₃₂₁PD₃₂₃ for E₃₂₁PN₃₂₃ at the carbohydrate binding site (17) did not bind (data not shown), confirming specific, CRD-dependent binding to the substituted-agarose beads.

While these experiments were in progress, we implemented a more efficient, semi-automated procedure for affinity chromatography using the AKTA system. Maltose-substituted, cross-linked supports were prepared using divinyl sulfone activation of Toyopearl beads. Although nickel-affinity-purified rat and

mouse NCRDs were efficiently bound and specifically eluted under these conditions (Figures 2B and 2C), the hNCRD was poorly retained (Figure 2C and data not shown). Based on integrated peak areas of bound and specifically eluted fractions, ~5-fold less hNCRD than rNCRD bound to the column (Figure 2C). Total recoveries from the column were ~80%, and blotting confirmed that the majority of the hNCRD was unbound and eluted as a broad peak during the wash (data not shown). Consistent with these findings, a single-arm form of rat SP-D (RrSP-Dser15,20) bound efficiently to maltosyl-Toyopearl (Figure 2D, rat single arm). Although full-length, human SP-D trimers showed low binding (Figure 2D, human trimer), wild-type human SP-D dodecamers bound to the column (Figure 2D, human dodecamers).

Human NCRDs Bind to Mannan but Interact Poorly with Maltose-Containing Neoglycoproteins in Solid-Phase Binding Assays

Given these unexpected results and the importance of interactions with multivalent, particulate ligands for SP-D function, we compared the saccharide activities of the three fusion proteins in solid-phase binding assays using the S-protein detection system. All three fusion proteins showed similar dose-dependent binding to mannan-coated wells (Figure 3A). Comparable results were obtained using at least two different preparations of each protein. There was negligible binding in the absence of added

Figure 2. Saccharide affinity chromatography. Maltosyl-agarose and maltosyl-Toyopearl columns were prepared, and chelation chromatography-purified NCRD fusion proteins were examined by affinity chromatography as described in MATERIALS AND METHODS. (A) Maltosyl-agarose chromatography of human NCRD. After sample loading and washing of the column, bound proteins were eluted with EDTA. Volume-corrected aliquots from pooled fractions were examined by SDS-PAGE. In this experiment, proteins were visualized by blotting with S-protein-HRP. Lane 1, starting material. Lane 2, wash, 0–20 ml. Lane 3, wash, 20–60 ml. Lane 4, EDTA eluate. The hNCRD efficiently bound to the column. (B) Maltosyl-Toyopearl chromatography of rat NCRD. After sample loading and washing of the column, bound proteins were eluted with EDTA. The absorbance profile is shown. Aliquots from pooled fractions were examined by SDS-PAGE. *Inset*, volume-adjusted aliquots of fractions were resolved by SDS-PAGE, and proteins were visualized by blotting with S-protein-HRP. Lane 1, starting material. Lanes 2 and 3, sequential pools of wash. Lanes 4 and 5, leading and trailing fractions of EDTA-eluted NCRD. All but a small fraction of the protein was bound and subsequently eluted from the column. (C) Maltosyl-Toyopearl chromatography of NCRDs. Equivalent amounts of rat, mouse, and human NCRD (250 μ g) were applied to the column and eluted as shown

in B. Profiles of the bound and eluted protein were compared, and recoveries were estimated by integration of the peak area after baseline correction. As compared with rat, recoveries of mouse and human NCRD were 89% and 21%, respectively. The small amount of bound human NCRD probably represents aggregates that are normally removed by gel filtration. There was no significant binding of the gel filtration purified trimeric NCRDs. (D) Maltosyl-Toyopearl chromatography of a rat single arm, trimeric mutant (RrSP-Dser15,20), and recombinant full-length human trimers and human dodecamers. Chromatography was performed as for C, except that 84 μ g of each protein was loaded. *Insets* show volume-corrected aliquots as visualized by immunoblotting using P13 rabbit anti-human SP-D or P9 rabbit anti-rat SP-D, as required. For each panel: Lane 1, starting material. Lane 2, wash. Lane 3, proteins eluted with EDTA. Although the maximal absorbance of the human dodecamer peak was lower than for the NCRD peak, integration of peak area indicated that the recovery of RrSP-Dser15,20 was ~80% of the recovery for human dodecamers. By contrast, the recovery of human trimers was < 13%. Thus, rat trimers are efficiently bound, whereas human trimers predominantly elute with the wash.

Figure 3. Binding of NCRDs to mannan, maltosyl-BSA neoglycoprotein, and viral particles. (A) Binding of NCRDs to mannan. Microtiter wells were coated with 50 μg/ml mannan, and bound fusion proteins were quantified using the S-protein-HRP detection system. Each point corresponds to the mean of three independent assays for human (closed circles), rat (open circles), and mouse (closed triangle) NCRD fusion proteins. The fusion proteins show negligible binding to mannan in the absence of added calcium (data not shown), and binding is efficiently competed by saccharide inhibitors (see below). (B) Mannan dose-response assay. The assay was performed as for A, except that wells were coated with increasing concentrations of mannan. Representative curves are shown for human (closed circles) and rat (open circles) NCRD fusion protein (10 μg/ml). The single points at right show absorbance in the absence of calcium. (C) Maltosyl-BSA dose-response assay. This assay was performed as in A, except that wells were coated with increasing concentrations of

maltosyl-BSA. After blocking and washing, the coated plates were incubated with human (closed circles) or rat (open circles) NCRD fusion protein (10 μg/ml) in the presence of 5 mM calcium. (D) Wells were coated with Phil82 strain of IAV as described in MATERIALS AND METHODS, and binding assays were performed essentially as for other solid-phase ligands. Left: Binding of rat (open squares) compared with the human (closed squares) NCRD. Right: Separate experiment showing binding of mouse (open squares) compared with the human (closed squares) NCRD. Note the different scales on the vertical axes. The data represent the mean and SE for at least three independent assays.

calcium (Figure 3B and data not shown), and binding was largely ablated with inhibitory concentrations of various competing sugars (see below). In addition, the human QPD mutant described previously showed no detectable specific binding (17).

Consistent with the chromatographic results, the rat and mouse proteins showed nearly identical specific and dose-dependent binding to wells coated with maltosyl-BSA (10 μg/ml), whereas the human NCRD showed little binding at protein concentrations as high as 80 μg/ml (data not shown). Inefficient binding of the human protein was not attributable to a general deficiency in carbohydrate binding activity or differential effects of ligand coating density. The human and rat NCRDs showed similar increases in binding as a function of mannan concentration (Figure 3B), and there was minimal binding of the human protein to maltosyl-BSA across a wide range of coating concentrations (Figure 3C).

Rat and Mouse NCRDs Bind More Efficiently to IAV

There is considerable evidence that SP-D plays important roles in the host response to IAV infection and that SP-D dodecamers efficiently neutralize virus *in vitro* and *in vivo*. These interactions are mediated by binding of the SP-D lectin domains to specific, high-mannose oligosaccharides on the viral hemagglutinin (sialic acid receptor) and/or the neuraminidase (21). Nevertheless, recent studies have shown that natural human trimeric subunits isolated from amniotic fluid show minimal binding to the Phil82 strain of IAV under conditions in which the binding of human SP-D dodecamers and multimers is readily demonstrated (20).

Consistent with these findings, we observed minimal binding of the hNCRD fusion protein to this strain of virus when adsorbed to the well (Figure 3D). However, rat and mouse trimeric NCRDs showed significant dose-dependent binding to the solid-phase virions. Binding of the rat NCRD was reproducibly greater than for the mouse protein ($P < 0.05$). The rat NCRD, but not the mouse or human proteins, also showed significant hemagglutination inhibition activity (24 ± 1 μg/ml), albeit at considerably higher concentrations than previously obtained for wild-type recombinant rat or human SP-D dodecamers.

Human NCRDs Show a Distinctive Saccharide Inhibition Profile

Previous crystallographic studies have shown that maltose interacts with a single carbohydrate binding site involving calcium ion 1 of the human SP-D CRD. Accordingly, we used inhibition assays to assess the relative preferences of CRDs for soluble mono- and disaccharides. Representative inhibition curves are shown in Figure 4, and binding data from several experiments using independent dilutions of protein and competitor are summarized in Table 1. Rat and mouse NCRDs showed similar profiles that resembled those previously described for natural rat SP-D (Table 2) with an approximate rank order of myo-inositol < maltose < glucose < mannose, galactose < N-acetyl-mannosamine < N-acetyl-glucosamine. By comparison, the hNCRD showed a different profile: N-acetyl-mannosamine, maltose < glucose < mannose, myo-inositol < galactose, N-acetyl-glucosamine (Table 1). Most notable are the preference

Figure 4. Competition assays. Fusion proteins were incubated with mannan-coated plates in the presence of selected mono- and disaccharides. Data are presented as percentage of control binding in the absence of competitor. Representative inhibition curves are shown for rat (closed circle, solid line), mouse (open circle, dashed line), and human (closed triangle, dashed line) NCRD fusion proteins. (A) Mannose. (B) Maltose. (C) Glucose. (D) N-acetyl-mannosamine. (E) Myo-inositol. (F) N-acetyl-glucosamine. The I_{50} (mM) data for these and other experiments and competitors are compiled in Table 1. The ability of solution phase mannan (G) or pustulan (H) to compete for binding of the fusion proteins to solid-phase mannan was compared. Inhibition curves are shown for rat (closed circle), mouse (open circle), and human (closed triangle) NCRD fusion proteins. Mannan showed similar inhibitory activity for rat, mouse, and human NCRDs. By contrast, pustulan preferentially inhibited the binding of rat and mouse NCRDs to mannan. Because mannan and pustulan are polydisperse with different average molecular weights, the inhibitory concentrations cannot be directly compared.

of the human protein for ManNAc, comparable to maltose, and the higher apparent affinity of the rat and mouse proteins for myo-inositol, maltose, and glucose. These differences were highly significant and reproducible among several different preparations of each protein. When the rat and human proteins were compared, there were significant differences ($P < 0.01$) for all competitors, except mannose and GlcNAc. When the mouse

and human proteins were compared, there were significant differences for ManNAc and maltose; however, myo-inositol, glucose, and galactose also approached significance. None of the small differences between rat and mouse reached statistical significance.

The apparent relative preference of the hNCRD for galactose and GlcNAc, two weak competitors, are different than previously

TABLE 1. SACCHARIDE COMPETITION OF BINDING TO MANNAN, I_{50} (mM)

	hNCRD			rNCRD			<i>P</i> value*	mNCRD			<i>P</i> value**
	Mean	SEM	<i>n</i>	Mean	SEM	<i>n</i>		Mean	SEM	<i>n</i>	
Glc	4.1	0.5	6	1.3	0.1	7	<0.0001	1.1	0.1	3	0.011
Man	5.3	0.9	9	3.5	0.3	8	NS	3.2	0.6	4	NS
Mal	2.7	0.4	11	0.55	0.08	11	<0.001	0.43	0.04	4	<0.01
Gal	10	2	8	3.5	0.4	8	<0.01	2.6	0.3	4	0.012
GlcNAc	14	2	7	15	2	7	NS	15	6	2	NS
ManNAc	2.1	0.4	11	10	1	11	<0.00001	9.3	1.9	6	<0.001
In	5.7	1.1	10	0.44	0.05	11	<0.001	0.29	0.08	4	0.011

Definition of abbreviations: Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; hNCRD, human neck plus carbohydrate recognition domain; In, myo-inositol; Mal, maltose; Man, mannose; ManNAc, N-acetyl-mannosamine; mNCRD, mouse neck plus carbohydrate recognition domain; NS, not significant; rNCRD, rat neck plus carbohydrate recognition domain.

Representative inhibition curves are illustrated in Figure 4. The data are expressed as the mean and SEM for the indicated number of independent experiments (*n*), using at least two different preparations of NCRD for each species. Differences between means for rat versus human NCRDs (*) and mouse versus human NCRDs (**) were compared using a two-tailed, unpaired *t* test. Values of *P* < 0.01 were considered significant. Selected values that closely approach the cutoff for significance are shown. Apparent differences between rat and mouse for inositol and galactose were not statistically significant.

reported (17). Although most assays included in Table 1 were performed by a different individual using different stocks of competitors, this difference is primarily attributed to variability among different lots of GlcNAc.

Pustulan Is a Comparatively Weak Competitor of Human NCRD Binding to Mannan

Previous studies have shown that pustulan, a linear β -1,6-glucose homopolymer, is a specific, high-affinity ligand for recombinant human SP-D that can efficiently inhibit the binding of SP-D to certain fungal organisms (22). Given differences in the inhibitory activities of glucose and closely related mono- and disaccharides, in a single experiment we compared the inhibitory activities of solution-phase mannan and pustulan for fusion protein binding to adsorbed mannan. Consistent with the direct binding data, mannan efficiently competed the binding of all three fusion proteins, with nearly identical I_{50} s (Figure 4G). Although pustulan showed dose-dependent inhibition of binding of all three fusion proteins, as predicted, it was considerably less efficient as a competitor for human NCRDs (Figure 4H).

Preferential Binding of hSP-D to ManNAc Is Dependent on Asp325

Given the observed species differences in ligand preferences, we examined the sequence divergence of residues known to be exposed at the ligand binding surface of the human SP-D CRD.

Previous studies have identified relatively conserved motifs immediately adjoining the primary carbohydrate binding site that define the so-called “SP-D groove” (Figure 1) (16). However, all known SP-Ds, except human and bovine SP-D, have an asparagine residue at position 325 of the mature protein, and rat and mouse SP-D have asparagine residues at positions 324 and 325 (Figure 1A and not shown). Although Asp325 does not coordinate with calcium at the carbohydrate binding site (16) or obviously hydrogen bond with maltose (6), modeling studies suggest that Asp325 is in close proximity to the carbohydrates that coordinate with calcium ion 1 (23). Accordingly, we created a site-directed human NCRD mutant, with a combined substitution of asparagine for aspartic acid at positions 324 and 325 (hNCRD_{N324,N325}). Thus, the mutant DNA encoded a human protein that resembles rat and mouse SP-D along the N-terminal lip of the “SP-D groove” (Figure 1A). Because bovine SP-D contains the sequence Asn324-Asp325, we also created a mutant with the single site substitution of asparagine for aspartate at position 324 (hNCRD_{N324}). Both mutants were efficiently expressed, isolated from IBs, refolded and oligomerized, and purified as described for the wild-type fusion proteins. The purified proteins eluted as trimers by gel filtration (data not shown). Both showed specific, dose-dependent binding to mannan (data not shown).

The double mutant (hNCRD_{N324,N325}) showed a marked and selective decrease in apparent binding affinity for ManNAc (Figure 5A), with a mean I_{50} approaching that of the rat and mouse proteins (I_{50} = 7.6 $\pm$ 1.2 mM [mean $\pm$ SEM]; *n* = 6 independent experiments). There was no significant effect of the substitutions on the inhibitory effectiveness of inositol, maltose, or mannose (Figures 5B–5D). The single-site mutant (hNCRD_{N324}), which preserved aspartate at position 325, functionally resembled the wild-type human protein, with no significant alteration in the apparent affinity for ManNAc (Figure 5A) (I_{50} = 1.7 $\pm$ 0.6 mM; *n* = 3). There were also no significant differences in the inhibitory potency of inositol, maltose, glucose, mannose, or GlcNAc (data not shown).

Asp325 Limits Binding of Human NCRDs to Immobilized Maltose

A significant proportion of hNCRD_{N324,N325} bound to maltosyl-Toyopearl and eluted with EDTA under conditions where the wild-type protein and hNCRD_{N324} showed negligible binding (Figure 6A and data not shown, respectively). Based on the absorbance profile, the recovery of bound double mutant was ~ 50% of that obtained for wild-type rat NCRD in a sequential run. Consistent with this finding, the double mutant also showed increased dose- and calcium-dependent binding to maltosyl-BSA (Figure 6B). On the other hand, the asparagine substitutions did not confer the capacity to inhibit hemagglutination of the Phil82 strain of IAV (data not shown).

TABLE 2. RELATIVE SACCHARIDE SELECTIVITIES OF NATURAL AND FULL-LENGTH RECOMBINANT WILD-TYPE PROTEINS REPORTED IN THE LITERATURE

Protein	Structure	Assay	Relative Preferences	Reference
rSP-D	Dodecamer	Maltosyl-BSA	<i>In</i> < Mal < Glc < Man < Gal < <GlcNAc	9
RrSP-D	Dodecamer	PI-vesicles	<i>In</i> < Mal < Glc < Man < Gal	28
hSP-D	Multimer	Maltosyl-BSA	Mal < Man,Glc < Gal < GlcNAc	10
RhSP-D	Dodecamer	Mannan-BSA	ManNAc < Man < <i>In</i> ,Mal,Glc < Gal < GlcNAc	12
pSP-D	Dodecamer?	Mannan-BSA	<i>In</i> < Mal < Glc < Man < Gal,ManNAc < GlcNAc	12

Definition of abbreviations: hSP-D, human surfactant protein D; PI, phosphatidylinositol; pSP-D, porcine surfactant protein D; RhSP-D, recombinant human surfactant protein D; RrSP-D, recombinant rat surfactant protein D; rSD-D, rat surfactant protein D.

Figure 5. Competition assays using human NCRD mutants with substitutions of asparagine for Asp324 and/or Asp325. Fusion proteins were incubated with mannan-coated plates in the presence of selected mono- and disaccharides. Data are presented as percentage of control binding in the absence of competitor. The double mutation shifts the inhibition curve for ManNAc but does not significantly alter the apparent affinity of the human NCRD for maltose, myo-inositol, or mannose. (A) N-acetyl-mannosamine (ManNAc) as competitor. Curves left to right: human (open circle), N324 mutant (open triangle), rat (closed circle), and N324N325 double mutant (closed triangle). The binding curve of the double mutant resembles the rat, whereas the single mutant resembles the wild-type human protein. (B) Maltose as competitor. Curves left to right: mouse (closed square), rat (closed circle), human (open circle), and double mutant (closed triangle). (C) Myo-inositol as competitor. Curves left to right: mouse (closed square), rat (closed circle), human (open circle), and double mutant (closed triangle). (D) Mannose as competitor. Curves left to right: mouse (closed square), rat (closed circle), human (open circle), and double mutant (closed triangle).

Because the affinity of hNCRD_{N324,N325} for maltose or glucose was not increased, we hypothesized that the nature of residue 325, which is exposed at the binding interface, might influence interactions of the nearby carbohydrate binding site with immobilized maltose residues. Accordingly, we expressed an additional trimeric mutant with a single site substitution of asparagine for aspartate at position 325 (hNCRD_{N325}). The mean I_{50} for ManNAc was ~ 3 -fold higher than for the wild-type protein, intermediate between the wild-type human fusion protein and the rat protein or hNCRD_{N324,N325}, whereas affinities for maltose and other sugars were unchanged (data not shown). hNCRD_{N324,N325} and hNCRD_{N325} showed dose- and calcium-dependent binding to maltosyl-BSA under conditions where the wild-type protein and hNCRD_{N324} were poorly retained (Figure 6B and data not shown). The binding of the wild-type and mutant proteins (20 μ g/ml) was largely abrogated with 50 mM maltose (Figure 6B).

ManNAc Recognition Could Involve Hydrogen Bonding of the N-Acetyl Group to the Side Chain of Asp325

To better understand the mechanism of preferred recognition of ManNAc and the contributions of Asp325 to ManNAc binding, docking experiments were performed with ManNAc and other selected saccharide ligands. Preliminary dockings of glucose to the hSP-D/maltose complex confirmed that 3-OH and 4-OH groups coordinate with calcium and participate in hydrogen bonds with Glu329 and Glu321 in a manner identical to that previously observed for maltose (6). Although visual inspection suggested that ManNAc might bind in an orientation similar to glucose, with the amide -NH- moiety hydrogen bonded to Asp325, the crystal structure side chain conformation of Asp325 did not allow this interaction.

Because the precise coordinates for Asp325 differ in the published studies and vary in the absence and presence of maltose (6), alternative conformations of the side chain were explored via energy minimization and conformational sampling using available hSP-D or hSP-D/maltose coordinates. For both sets of coordinates, the C β -C γ torsional angle was predicted to be ~ 140 degrees, as compared with 94 degrees for the crystal structures (data not shown). Using the calculated torsional angle, ManNAc consistently docked in orientations that produced a hydrogen bond between the -NH- moiety of ManNAc and Asp325 while preserving the hydrogen bonds of the 3-OH and 4-OH groups of Glc1 of maltose with Glu329 and Glu321 (Figure 7). Torsional angles outside this range produced “atypical poses” such that the 5-OH and 6-OH groups provided the key interactions with Glu329 and Glu321.

When Asp325 was “mutated” to Asn and docking was performed using the previous parameters, the Asp325-like conformation was not favored due to steric hindrance with the backbone of Gly326. As a result, there was no predicted hydrogen bonding between the mutant asparagine side chain and the -NAC substituents (Figure 7).

DISCUSSION

In this study, we document significant species differences in the ligand preferences of SP-D using assays that control for potential differences in CRD valency and that do not rely on antibodies to the carbohydrate binding domain for detection. Rat and mouse trimeric neck+CRDs showed relative saccharide selectivities comparable to those previously demonstrated for natural rat and full-length recombinant rat SP-D dodecamers, with a preference

Figure 6. Effects of mutations on binding to solid-phase, maltose-containing ligands. (A) Maltosyl-Toyopearl chromatography. Maltosyl-Toyopearl affinity chromatography was performed as shown in Figures 2B and 2C, using 250 μ g of gel-filtration purified trimeric NCRD fusion protein. Volume-corrected aliquots of the pooled wash (W) and pooled EDTA eluate containing the bound proteins (B) were examined by SDS-PAGE and blotting with S-protein-HRP. Lanes 1 and 2, rat NCRD, Lanes 3 and 4, human NCRD. Lanes 5 and 6, N324N325 double mutant. Approximately half of the mutant protein bound was bound to the affinity matrix. (B) Maltosyl-BSA binding. Wells were coated with 10 μ g/ml maltosyl-BSA. After blocking and washing, the coated wells were incubated with the indicated concentration of rat (open triangles), mouse (open circles), human (closed circles), double mutant (closed triangle), or N325 single mutant (open diamonds) in the presence of 5 mM calcium. The low signals obtained in the absence of calcium are indicated for wells containing 20 μ g/ml of fusion protein. Binding of the double mutant was significantly increased relative to wild-type human ($P < 0.03$ in five independent experiments).

for myo-inositol, maltose, and glucose (see Tables 1 and 2). The high potency of myo-inositol is also consistent with earlier studies of natural rat SP-D (24, 25). By contrast, inositol, maltose, and glucose were much less efficient competitors of human NCRD binding to mannan, and N-acetyl-mannosamine (ManNAc) was at least as potent as the “prototypical” SP-D competitor (maltose). The difference for inositol was particularly striking, with the murine proteins showing ~ 10 - to 20-fold higher affinity. ManNAc is uncommonly included in panels of competitors for SP-D; however, our findings are consistent with the recent observation that ManNAc can potently inhibit the binding of recombinant human SP-D dodecamers to mannan-neoglycoproteins (12) (see Table 2).

Although the three species of NCRD showed similar calcium-dependent binding to solid-phase fungal mannan, there were conspicuous differences in the interactions of these proteins with

maltose-containing ligands presented on solid supports and with influenza virus particles. All three fusion proteins bound efficiently to our preparations of maltosyl-agarose. However, rat and mouse NCRDs were preferentially retained on the cross-linked maltose-substituted Toyopearl gels, maltosyl-BSA coated wells, and adsorbed Phil82 IAV. In addition, there are marked differences in interactions with phosphatidylinositol and specific bacterial lipopolysaccharides (manuscripts in preparation). Because full-length human SP-D trimers were not efficiently retained on the maltosyl-Toyopearl matrix under conditions where trimeric subunits of rat SP-D bound, we infer that the different binding properties of the NCRDs contribute to the different biological properties of full-length molecules.

The similar saccharide preferences of rat and mouse NCRDs seem to be consistent with the marked evolutionary conservation of their primary sequences (see Figure 1A). There are no differences in residues that coordinate with calcium ligand 1 at the carbohydrate binding site, or of surface exposed regions of the “SP-D groove” (see Figure 1A). Observed differences between mouse and rat NCRDs in binding to solid-phase ligands, including IAV, are more difficult to explain. Given substitutions in core structural regions of the CRD, it is possible that the observed differences result from somewhat different spatial presentations of the conserved ligand binding sequences within a trimeric CRD, or slight differences in the spatial distribution of CRDs within a trimer.

Contributions of Asp324-Asp325 to Ligand Binding by Human SP-D

Our mutagenesis studies establish that Asp325, a nonconserved residue displayed along the N-terminal ridge of the “SP-D groove,” contributes to the distinctive saccharide preferences of human SP-D. Substitution of Asn for Asp at position 324 showed no significant effect on saccharide preferences in competition assays; however, the double mutant with Asn at positions 324 and 325 and a single-site mutant with a substitution of Asn at position 325 resembled the rat and mouse proteins with respect to their preference for mannose over ManNAc. Bovine SP-D, which is unique among known SP-Ds in containing the sequence Asn-Asp at the corresponding position, reportedly also shows a greater affinity for ManNAc than mannose (11). There was no significant effect of mutations at positions 324 and/or 325 on other tested saccharide competitors, including myo-inositol, maltose, and glucose.

Modeling suggests that the 2-NAc substituent of ManNAc can hydrogen bond to the side chain of Asp325, contributing to the greater affinity of the human protein for ManNAc, as compared with mannose (Figure 7). Furthermore, observed pose variations of ManNAc as a function of Asp325 side chain conformation suggest a role for the side chain in helping to guide the docked binding mode for the carbohydrate ligand. This interpretation is consistent with a preliminary refinement of the crystal structure of the EK-cleaved, human trimeric NCRD complexed with ManNAc, which supports the current computational model in showing the side chain carboxylate of Asp325 to be within hydrogen-bonding distance of the ManNAc N-acetyl nitrogen in all subunits (Dr. James F. Head, Boston University, manuscript in preparation). The modeling also provides a potential mechanism for the reduced affinity of the double mutant and hNCRD_{N325} for ManNAc and for observed species differences in ManNAc recognition. With asparagine at position 325, ManNAc is expected to show reduced affinity because the predicted conformation would not allow hydrogen bonding between the carboxamide side chain of asparagine and the N-acetyl group of ManNAc.

Figure 7. Docking of ManNAc to the carbohydrate binding site. The conformations of Asp325 and Asn325 in the human CRD were calculated by minimization and conformational analysis using crystallographic coordinates of human SP-D complexed with maltose as described in MATERIALS AND METHODS. The model illustrates the carbohydrate binding subregion of a single CRD of human SP-D, superimposed on the Asn 325 “mutant,” and docked with N-acetylmannosamine (green). Asp/Asn325 and Asp324 are identified and highlighted with gray backbones. ManNAc is complexed to calcium ion 1 (orange atom at center) via the indicated 3- and 4-hydroxyl groups of the mannose ring. The carboxyl group of Asp325 forms a hydrogen bond with the nitrogen of the NAc substituent of ManNAc (yellow line). By contrast, the energetically favored orientation of the asparagine β amide carbonyl group of Asn325 (tinted yellow) does not permit hydrogen bonding with ManNAc. There is no predicted interaction of either side chain with docked mannose or glucose (not shown). Asp324 (right) is oriented away from the carbohydrate binding site; it coordinates with calcium ion 2 (not shown). Asn323 and Glu329 residues (blue side chains), which are seen immediately above and below the Asp/Asn325 side chain in the

figure, participate in the coordination of calcium ion 1. Arg343 (lower left), which resides on the opposite edge of the SP-D groove from Asp325, is thought to sterically interfere with one possible binding orientation of N-acetyl-glucosamine, thereby contributing to its relative ineffectiveness as a saccharide competitor (26).

The calculated, energetically favored C β -C γ torsional angle for Asp325 differs from that shown in the crystal structure (*see RESULTS*). When considered in the context of the mutagenesis data, this implies some conformational flexibility of the Asp325 side chain. As indicated above, Shrive and coworkers observed that the positions of Arg343, Asp325, and the length of a potential Asp325-Gly326 hydrogen bond are altered in the presence of bound maltose (6).

Our mutagenesis (and modeling) studies suggest that Asp325 can directly participate in ManNAc recognition by human SP-D, providing the first experimental evidence for a direct interaction of a bound carbohydrate with a residue that does not coordinate with calcium ion 1. This is distinct from the proposed contributions of Arg343, which seems to influence discrimination between glucose and GlcNAc by sterically limiting the available binding orientations of GlcNAc (26). The rat and mouse proteins show substantially less affinity for ManNAc than mannose. Although Arg343 is not conserved in the rat and mouse, we speculate that similar steric constraints involving Lys343 and the 2-Nac substituent of ManNAc influence the discrimination between mannose and ManNAc in these species.

The physiologic significance of enhanced ManNAc recognition by hSP-D is not known. This sugar is found in potential microbial ligands, including specific bacterial lipopolysaccharides, lipoteichoic acids, capsular polysaccharides, and glycolipids. It has also been identified as a preferred competitor for other innate immune molecules (27), including SP-A (16). On the other hand, enhanced affinity for ManNAc could be an indirect consequence of structural adaptations required for the recognition of other ligands.

Contributions of Asp325 and Asp324 to Interactions with Solid-Phase Ligands

Given our findings, it is unlikely that species differences in the recognition of solid-phase ligands and viral particles are solely determined by differences in the intrinsic saccharide-binding preferences of the CRD. For example, the double mutant and hNCRD₃₂₅ showed increased binding to maltosyl-BSA and

maltosyl-Toyopearl beads, even though the affinity for glucose or maltose was not appreciably enhanced.

Because Asp325 does not directly interact with maltose (6), we hypothesize that the side chains of bulky or charged residues that extend into the ligand-binding interface influence the capacity of ligands presented on surfaces to approach and/or occupy the carbohydrate site within the SP-D groove. The mechanism could be steric, electrostatic, or indirect (e.g., involving differences in the local ordering of bound water). It could also depend on the composition, spatial complexity, and/or environment of the solid-phase ligand. In any case, our findings have important implications for the recognition of natural ligands, including glycoconjugates or polysaccharides, presented on the surfaces of microorganisms or organic antigens.

hNCRD_{N324,N325} more fully reproduced the rat and mouse phenotypes than hNCRD_{N325}, at least with respect to ManNAc recognition. Because Asp324 coordinates with calcium ion 2 and is not displayed at the binding interface (6), it is possible that the substitution of Asn influences the spatial distribution of Asp325 or other residues residing on the same loop. We recently observed that a three-amino acid insertion between residues 324 and 325, which was predicted by modeling to deflect Asp325 away from the carbohydrate binding site, greatly alters the saccharide preferences, mannan binding, and antiviral activity of human NCRDs (17). Crystallographic analysis of the rat or mouse NCRD is needed to further define the specific contributions of residues 324 and 325 to ligand binding.

Subunit Multimerization Is an Important Determinant of Ligand Recognition by Human SP-D

The trimeric human NCRDs and human SP-D trimeric subunits differed from the corresponding rat and mouse proteins in their limited capacity to interact with maltose-substituted supports and viral particles that interact with human SP-D dodecamers. Thus, human SP-D seems to be more highly dependent on cooperative binding among trimeric CRDs to recognize certain ligands. This is consistent with recent studies that showed that natural human multimers, but not natural trimers, bind to

influenza virus and various bacteria (20). Different proportions of dodecamers, multimers of dodecamers, and trimeric subunits are found in various preparations of natural human SP-D, and recent studies indicate that certain common polymorphic variations in the SP-D gene are associated with differences in assembly. In particular, the substitution of threonine for methionine at position 11 is associated with the preferential secretion of trimers (20). Thus, our functional data further support the hypothesis that the proportions of trimers and higher-order multimers could profoundly influence the host defense and innate immune functions of human SP-D.

Implications for Future Investigations

Species differences in ligand binding introduce important complexities for *in vitro* studies examining the interactions of SP-D with micro-organisms, host cells, and organic antigens. Species differences should be considered in the design and interpretation of future *in vivo* studies using murine models of microbial host defense and pulmonary hypersensitivity. Although differences between human and murine proteins were most obvious, subtle differences between the rat and mouse proteins were also observed.

Our findings confirm that SP-D preferences for carbohydrate ligands can be engineered through relatively subtle modifications to sequences flanking the carbohydrate binding groove (17), raising the possibility of developing recombinant collectins with enhanced host defense or immunoregulatory properties. "Therapeutic interventions" in mice have been restricted to wild-type, trimeric human neck + CRDs.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

1. Crouch EC, Whitsett JA. Diverse roles of lung collectins in pulmonary innate immunity. In: Ezekowitz AR, Hoffman JA, editors. *Innate immunity*. Rotowa, NJ: Humana Press Inc.; 2002. pp. 205–229.
2. Wright JR. Immunoregulatory functions of surfactant proteins. *Nat Rev Immunol* 2005;5:58–68.
3. Crouch E, Wright JR. Surfactant proteins A and D and pulmonary host defense. *Annu Rev Physiol* 2001;63:521–554.
4. Palaniyar N, Nadesalingam J, Clark H, Shih MJ, Dodds AW, Reid KB. Nucleic acid is a novel ligand for innate, immune pattern recognition collectins surfactant proteins A and D and mannose-binding lectin. *J Biol Chem* 2004;279:32728–32736.
5. Holmskov UL. Collectins and collectin receptors in innate immunity. *APMIS Suppl* 2000;100:1–59.
6. Shrive AK, Tharia HA, Strong P, Kishore U, Burns I, Rizkallah PJ, Reid KB, Greenhough TJ. High-resolution structural insights into ligand binding and immune cell recognition by human lung surfactant protein D. *J Mol Biol* 2003;331:509–523.
7. Head JF, Mealy TR, McCormack FX, Seaton BA. Crystal structure of trimeric carbohydrate recognition and neck domains of surfactant protein A. *J Biol Chem* 2003;278:43254–43260.
8. Taylor ME, Drickamer K. Structure-function analysis of C-type animal lectins. *Methods Enzymol* 2003;363:3–16.
9. Persson A, Chang D, Crouch E. Surfactant protein D is a divalent cation-dependent carbohydrate-binding protein. *J Biol Chem* 1990;265:5755–5760.
10. Lu J, Willis AC, Reid KB. Purification, characterization and cDNA cloning of human lung surfactant protein D. *Biochem J* 1992;284:795–802.
11. Lim BL, Wang JY, Holmskov U, Hoppe HJ, Reid KB. Expression of the carbohydrate recognition domain of lung surfactant protein D and demonstration of its binding to lipopolysaccharides of gram-negative bacteria. *Biochem Biophys Res Commun* 1994;202:1674–1680.
12. van Eijk M, White MR, Crouch EC, Batenburg JJ, Vaandrager AB, van Golde LMG, Haagsman HP, Hartshorn KL. Porcine pulmonary collectins show distinct interactions with influenza A viruses: role of the N-linked oligosaccharides in the carbohydrate recognition domain. *J Immunol* 2003;171:1431–1440.
13. Crouch E, Persson A, Chang D, Heuser J. Molecular structure of pulmonary surfactant protein D (SP-D). *J Biol Chem* 1994;269:17311–17319.
14. Hartshorn K, Chang D, Rust K, Crouch EC. Interactions of recombinant human pulmonary surfactant protein D and SP-D multimers with influenza A. *Am J Physiol Lung Cell Mol Physiol* 1996;271:L753–L762.
15. Brown-Augsburger P, Hartshorn K, Chang D, Rust K, Fliszar C, Welgus HG, Crouch EC. Site-directed mutagenesis of Cys-15 and Cys-20 of pulmonary surfactant protein D. Expression of a trimeric protein with altered anti-viral properties. *J Biol Chem* 1996;271:13724–13730.
16. Hakansson K, Lim NK, Hoppe HJ, Reid KB. Crystal structure of the trimeric alpha-helical coiled-coil and the three lectin domains of human lung surfactant protein D. *Struct Fold Des* 1999;7:255–264.
17. Crouch E, Tu Y, Briner D, McDonald B, Smith K, Holmskov U, Hartshorn K. Ligand specificity of human surfactant protein D: expression of a mutant trimeric collectin that shows enhanced interactions with influenza A virus. *J Biol Chem* 2005;280:17046–17056.
18. Hansen S, Thiel S, Willis A, Holmskov U, Jensenius JC. Purification and characterization of two mannan-binding lectins from mouse serum. *J Immunol* 2000;164:2610–2618.
19. Hartshorn KL, Holmskov U, Hansen S, Zhang P, Meschi J, Mogues T, White MR, Crouch EC. Distinctive anti-influenza properties of recombinant collectin-43 (CL-43). *Biochem J* 2002;366:87–96.
20. Leth-Larsen R, Garred P, Jensenius H, Meschi J, Hartshorn K, Madsen J, Tornøe I, Madsen HO, Sørensen G, Crouch E, et al. A common polymorphism in the SFTPD gene influences assembly, function, and concentration of surfactant protein D. *J Immunol* 2005;174:1532–1538.
21. Hartshorn K, White MR, Voelker D, Coburn JP, Zaner KS, Crouch EC. Mechanism of binding of surfactant protein D to influenza A viruses: importance of binding to hemagglutinin to antiviral activity. *Biochem J* 2000;351:449–458.
22. Allen MJ, Voelker DR, Mason RJ. Interactions of surfactant proteins A and D with *Saccharomyces cerevisiae* and *Aspergillus fumigatus*. *Infect Immun* 2001;69:2037–2044.
23. Allen MJ, Laederach A, Reilly PJ, Mason RJ. Polysaccharide recognition by surfactant protein D: novel interactions of a C-type lectin with nonterminal glucosyl residues. *Biochemistry* 2001;40:7789–7798.
24. Ogasawara Y, Kuroki Y, Akino T. Pulmonary surfactant protein D specifically binds to phosphatidylinositol. *J Biol Chem* 1992;267:21244–21249.
25. Persson AV, Gibbons BJ, Shoemaker JD, Moxley MA, Longmore WJ. The major glycolipid recognized by SP-D in surfactant is phosphatidylinositol. *Biochemistry* 1992;31:12183–12189.
26. Allen MJ, Laederach A, Reilly PJ, Mason RJ, Voelker DR. Arg343 in human surfactant protein D governs discrimination between glucose and N-acetylglucosamine ligands. *Glycobiology* 2004;14:693–700.
27. Krist P, Herkommerova-Rajnochova E, Rauvolfova J, Semenuk T, Vavruskova P, Pavlicek J, Bezouska K, Petrus L, Kren V. Toward an optimal oligosaccharide ligand for rat natural killer cell activation receptor NKR-P1. *Biochem Biophys Res Commun* 1994;202:11–20.
28. Ogasawara Y, Voelker DR. Altered carbohydrate recognition specificity engineered into surfactant protein D reveals different binding mechanisms for phosphatidylinositol and glucosylceramide. *J Biol Chem* 1995;270:14725–14732.