

Recognition of Mannosylated Ligands and Influenza A Virus by Human Surfactant Protein D: Contributions of an Extended Site and Residue 343^{†,‡}

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ABSTRACT: Surfactant protein D (SP-D) plays important roles in antiviral host defense. Although SP-D shows a preference for glucose/maltose, the protein also recognizes D-mannose and a variety of mannose-rich microbial ligands. This latter preference prompted an examination of the mechanisms of mannose recognition, particularly as they relate to high-mannose viral glycans. Trimeric neck plus carbohydrate recognition domains from human SP-D (hNCRD) preferred α 1–2-linked dimannose (DM) over the branched trimannose (TM) core, α 1–3 or α 1–6 DM, or D-mannose. Previous studies have shown residues flanking the carbohydrate binding site can fine-tune ligand recognition. A mutant with valine at 343 (R343V) showed enhanced binding to mannan relative to wild type and R343A. No alteration in affinity was observed for D-mannose or for α 1–3- or α 1–6-linked DM; however, substantially increased affinity was observed for α 1–2 DM. Both proteins showed efficient recognition of linear and branched subdomains of high-mannose glycans on carbohydrate microarrays, and R343V showed increased binding to a subset of the oligosaccharides. Crystallographic analysis of an R343V complex with 1,2-DM showed a novel mode of binding. The disaccharide is bound to calcium by the reducing sugar ring, and a stabilizing H-bond is formed between the 2-OH of the nonreducing sugar ring and Arg349. Although hNCRDs show negligible binding to influenza A virus (IAV), R343V showed markedly enhanced viral neutralizing activity. Hydrophobic substitutions for Arg343 selectively blocked binding of a monoclonal antibody (Hyb 246-05) that inhibits IAV binding activity. Our findings demonstrate an extended ligand binding site for mannosylated ligands and the significant contribution of the 343 side chain to specific recognition of multivalent microbial ligands, including high-mannose viral glycans.

SP-D¹ is an effector of antimicrobial host defense in the lung and at extrapulmonary sites of expression (1–5). Animals deficient in SP-D are more susceptible to pulmonary challenge with *Pseudomonas aeruginosa* (6), and intranasal administration of recombinant SP-D can protect wild-type mice from lethal challenge with *Aspergillus fumigatus* (7). In addition, SP-D appears to play particularly important roles in the neutralization and clearance of viruses. Animals deficient in SP-D also show delayed clearance and heightened inflammatory responses to strains of influenza A virus (IAV)

and respiratory syncytial virus (RSV) (8, 9). Clearance is selectively impaired for IAV strains reactive with SP-D *in vitro* (8) and can be rescued with recombinant SP-D or by transgenic overexpression of trimeric subunits of SP-D (8, 10). Notably, specific polymorphisms in the human gene have been associated with enhanced inflammatory reactions and morbidity secondary to RSV (11).

SP-D is a member of a family of collagenous C-type lectins, and recognition of microorganisms by this collectin involves the carbohydrate recognition domain (CRD) (1, 2). Although SP-D is often characterized by its preference for glucose-containing sugars, such as maltose, human SP-D binds with only slightly lower affinity to D-mannose, readily binds to yeast mannan, and can be purified on mannose–Sephacrose (12)(unpublished data). In addition, SP-D preferentially binds to specific serotypes of *Klebsiella pneumoniae* and isolated lipopolysaccharides expressing mannose-rich O-antigens (13).

Interactions of SP-D with viruses are also predominantly mediated by interactions with asparagine-linked glycans expressed on viral envelope proteins (14–16). In particular, SP-D shows calcium-dependent and sugar-sensitive interactions with IAV, RSV (9, 17), human immunodeficiency virus (HIV) (15), rotaviruses (18), and the SARS coronavirus (16).

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[‡] Atomic coordinates for the crystal structures of the wild-type and mutant protein are available in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB codes 3G81, 3G83, and 3G84).

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¹ Abbreviations: BSA, bovine serum albumin; CRD, carbohydrate recognition domain; DM, dimannose; GlcNAc, N-acetylglucosamine; hNCRD, human SP-D NCRD; LPS, lipopolysaccharide; mAb, monoclonal antibody; ManNAc, N-acetylmannosamine; NCRD, trimeric neck plus CRD; SP-D, surfactant protein D; TM, trimannose.

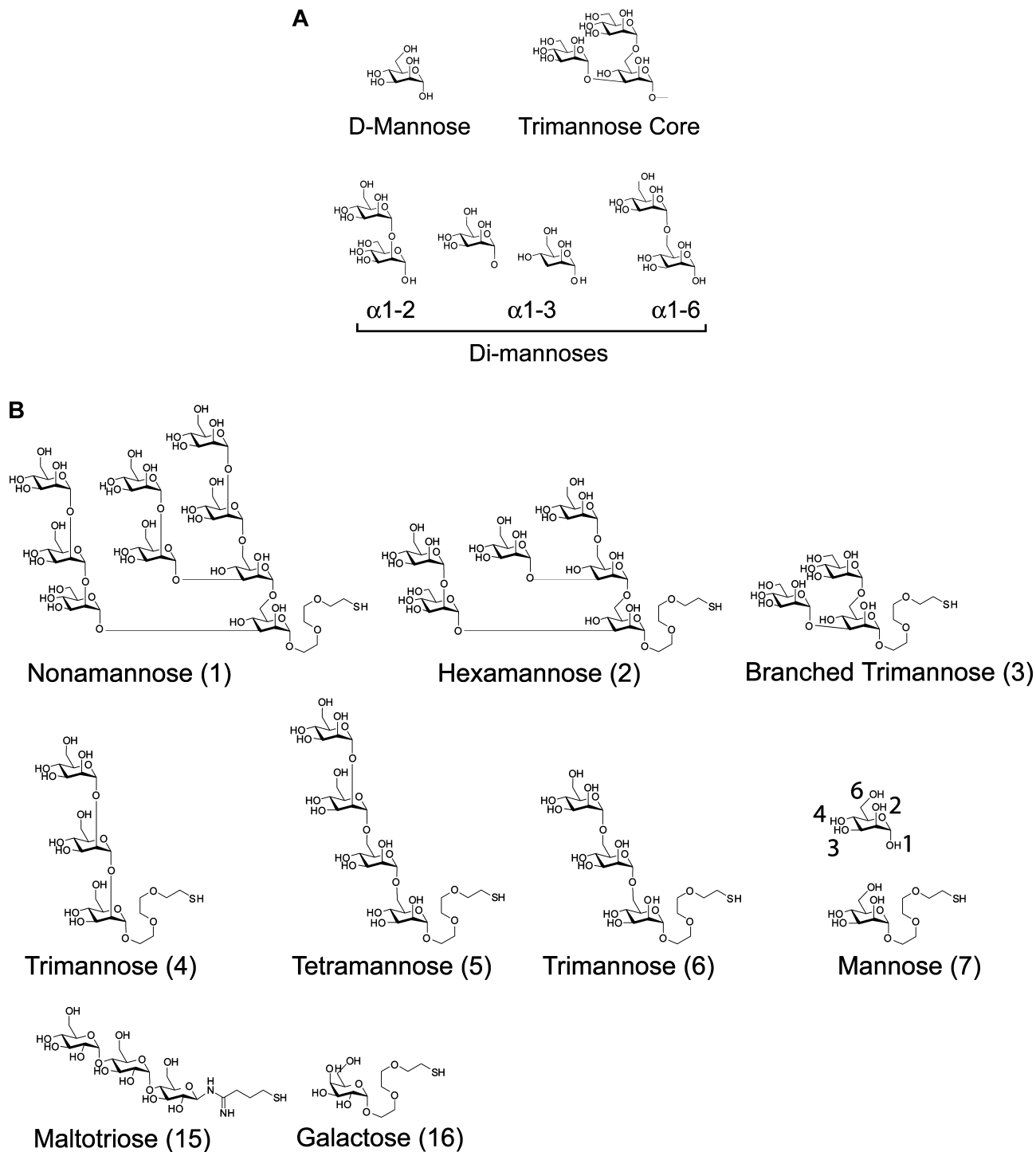


FIGURE 1: Mannose ligands. (A) Carbohydrates used as solution-phase competitors in competition assays. (B) Carbohydrate ligands used for array experiments.

In the case of IAV, binding was localized to specific high-mannose glycans associated with the hemagglutinin (HA) (14), while binding to HIV was localized to glycans on gp120 (15). Subsequent studies have correlated SP-D reactivity with IAV *in vitro*, clearance *in vivo*, and delayed clearance in SP-D deficient mice with the presence or absence of specific glycans associated with the head of the HA (8, 19–22). For example, an IAV strain that is reactive with SP-D *in vitro* shows decreased clearance in SP-D deficient mice, while a strain that binds poorly to SP-D showed no alteration in clearance (8, 19, 20). Although SP-D can also bind to glycans associated with the neuraminidase (14), inhibitory effects on

neuraminidase activity appear to primarily depend on steric interference as a consequence of binding to the HA (23).

Bovine serum collectins, probable evolutionary descendants of SP-D in ruminants, differ from SP-D in their affinities for various microbial ligands, including mannan and IAV, and CL-43 shows particularly high affinity for both of these ligands (24, 25). Notably, chimeras consisting of the N-terminal peptide and collagen domains of SP-D combined with the neck and CRD of bovine conglutinin show enhanced interactions with IAV, in part attributed to the greater IAV binding activities of the lectin domains (26, 27). The ligand binding surfaces of the serum conglutinin

and CL-43 diverge from SP-D at several locations, but are most notably characterized by insertions near the ligand binding site and by the substitution of valine or isoleucine for arginine at position 343. We have previously shown modest increases in antiviral activity with the insertions of RAK (or AAA) to resemble CL-43 (24). Our recent studies have shown that residue 343 plays a critical role in mediating interactions of SP-D with phosphatidylinositol (PI), its major surfactant-associated ligand (28), and with the core oligosaccharide domain of rough bacterial lipopolysaccharides (29). In addition, earlier studies by Allen and co-workers demonstrated that a full-length human SP-D with valine at position 343 shows altered saccharide preferences with enhanced affinity for GlcNAc and certain other monosaccharide ligands (30).

In the present studies, we employed a combination of site-directed mutagenesis, carbohydrate microarray studies, and crystallographic analysis to further study the contributions of the 343 side chain to ligand recognition by human SP-D. Our experiments demonstrate greatly enhanced antiviral activity of R343V associated with utilization of a modified carbohydrate binding site for α 1–2-linked mannoses, which are commonly displayed at the termini of asparagine-linked viral glycans. The findings indicate that the unique presence of arginine at position 343 in human SP-D greatly influences its ligand binding preferences.

EXPERIMENTAL PROCEDURES

The pET-30a(+) vector, S-protein horseradish peroxidase (S-protein-HRP), and RosettaBlue competent cells were from Novagen (Madison, WI). Yeast mannan was from Sigma-Aldrich (St. Louis, MO). Trimannose (methyl 3,6-di-*O*-(α -D-mannopyranosyl)- α -D-mannopyranoside, M302500), corresponding to the conserved trimannose core oligosaccharide of high-mannose viral glycans (Figure 1A), was from Toronto Research Chemicals, Inc., (Toronto, Canada). All other mono- and disaccharides were the D-anomers and of the highest purity available from Sigma. Fatty acid free BSA (BAH66-0050) was from Equitech-Bio, Inc. (Kerrville, TX). Purified synthetic oligomers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA).

Expression and Characterization of Trimeric Neck plus CRD Fusion Proteins. The expression and characterization of the N-terminally tagged, trimeric human neck plus CRD (NCRD) fusion proteins have been previously described (12, 24, 28, 31). The use of bacterially expressed proteins is feasible because there are no known posttranslational modifications within the human SP-D CRD or neck domains, which self-assemble *in vitro* as stable trimers with lectin activity (24, 32).

Site-directed mutagenesis was performed using a QuikChange II XL site-directed mutagenesis kit (200521; Stratagene, La Jolla, CA) and the hSP-D neck plus CRD DNA as template. In particular, we performed site-directed substitutions for arginine 343 within the CRD, as defined by numbering of the full-length protein: valine (R343V), alanine (R343A), and lysine (R343K) (28). Tagless NCRDs were generated by reengineering the expression vector to delete the entirety of the fusion tag. Sequences were verified by automated sequencing of the entire coding sequence of the fusion protein. Glycerol stocks of transformed bacteria were stored in single-use aliquots at -80°C .

RosettaBlue competent cells were transformed with the wild-type or mutant construct in pET-30a(+) vector, and expressed proteins were isolated from inclusion bodies (IBs) (28, 31). After refolding and oligomerization, the fusion proteins were purified by nickel affinity chromatography. Trimers were isolated by gel filtration chromatography on an AKTA system (24). All proteins were isolated in similar yields with similarly low levels of endotoxin. The fusion proteins and tagless NCRDs showed the expected mobility on SDS-PAGE in the absence and presence of sulfhydryl reduction, as previously demonstrated for the wild-type protein (24). Purified proteins were stored in small aliquots at -80°C .

Direct Binding and Competition Assays. Binding of trimeric NCRD fusion proteins to yeast mannan was measured as previously described using the S-protein-HRP conjugate (24). To determine the level of nonspecific binding, 50 mM maltose was included as a competitor during the binding step. For competition assays, proteins were added to wells in the presence of various concentrations of competing inhibitors. The concentration of competitor (millimolar) required for 50% inhibition of binding (I_{50}) was calculated.

Binding and competition data were analyzed using the ligand binding module in Sigmaplot 9.0 (SPSS Inc., Chicago, IL). Apparent dissociation constants for mannan were calculated by nonlinear regression of the saturation binding curves assuming a molecular mass of 66 kDa per trimeric fusion protein. Although it is not possible to calculate true dissociation constants or binding capacities with this type of assay, the analysis permits quantitative comparison of the binding properties of the homologous NCRD fusion proteins using unmodified ligands. All values are given as the mean \pm standard error of the mean (SEM) of at least three independent assays using at least two independent preparations of recombinant protein.

Fabrication of High-Mannose Microarrays. GAPS II slides (Corning) were submerged in DMF containing 2% diisopropylethylamine and 0.5 mg/mL maleimido-*N*-hydroxysuccinimide hexanoic acid for 14 h at room temperature. Functionalized slides were washed three times with methanol, dried, and stored under an argon atmosphere. The high-mannose-containing oligosaccharides have been synthesized as described before (33, 34) (Figure 1B). Compounds were dissolved in PBS with 1 molar equiv of TCEP to reduce disulfides. One nanoliter of the solution was printed onto each spot of the maleimide functionalized slides using an automated printing robot. Slides were incubated in a humid chamber to complete reaction for 24 h and stored in a desiccator until usage.

Binding Experiments Using High-Mannose Microarrays. Slides were washed with water and methanol and quenched in 0.1% (v/v) β -mercaptoethanol in PBS for 1 h at room temperature. Slides were blocked with 2.5% (w/v) BSA in 20 mM Hepes, pH 7.5, with 100 mM NaCl and 20 mM CaCl_2 for 1 h at room temperature, washed twice with buffer, and centrifuged for 5 min at 50g at room temperature. Blocked slides were incubated with NCRD solutions (20 μg /mL NCRD in binding buffer: 20 mM Hepes, pH 7.5, with 100 mM NaCl, 20 mM CaCl_2 , 1% (w/v) BSA, and 0.5% (v/v) Tween-20) or concanavalin A (10 μg /mL in binding buffer) for 1 h at room temperature. For inhibition, 50 μg /

mL yeast mannan (Sigma Aldrich), 125 mM maltose, and 20 mM EDTA were added to binding buffer lacking calcium chloride. Incubated slides were washed twice with buffer, centrifuged, and overlaid with 2 μ g/mL penta-His Alexa Fluor 555 antibody conjugate (Quiagen) in binding buffer in a similar fashion. After two washes and centrifugation, slides were scanned with a fluorescent microarray scanner (Tecan). Binding intensities were analyzed using microarray evaluation software (Genespotter, MicroDiscovery).

Crystallographic Analysis of Ligand Complexes. For crystallographic analysis, the proteins were expressed as enterokinase-cleaved fusion proteins (31) or as NCRDs lacking the fusion tags (28, 31). The latter begin with methionine, followed by the first residue of the natural neck domain. Tagless proteins were isolated by maltosyl-agarose affinity chromatography (12) prior to purification of trimers by gel filtration on Superose 12 in 150 mM NaCl and 10 mM Hepes, pH 7.5, containing 10 mM calcium chloride (HBSC). The trimer fractions were pooled, and the protein was concentrated by ultrafiltration to approximately 15 mg/mL prior to freezing at -80°C . Protein concentrations were determined using the bicinchoninic acid assay (BCA; Pierce, Rockford, IL) with BSA as standard. Purity was verified by SDS-PAGE, and homogeneity was confirmed by dynamic light scattering using a 1 mg/mL solution in the above buffer.

Crystals were prepared as previously described (28, 31). D-Mannose or α 1,2 dimannose was soaked into crystals by dissolving the ligand in mother liquor and transferring a crystal into the soaking solution for a period of 1–2 h. After soaking, the crystal was flash frozen in a 100 K stream of nitrogen gas at the beamline. Data were collected at beamline X8C at the National Synchrotron Light Source, Brookhaven National Laboratory, and processed using DENZO and SCALEPACK (35). Data collection statistics are shown in the Results. Model building and structure refinement were performed using O (36), PHENIX (37), CNS (38), and REFMAC (39). R343V crystals belong to the same space group with a similar unit cell as the wild-type protein. However, these crystals were found to exhibit pseudomero-hedral twinning. The twinning operator ($l, -k, h$) and twin fraction (0.482) were determined using xtriage (PHENIX), and the structure was refined in PHENIX.

Viral Binding and Neutralization Assay. The Phil82 strain of IAV was grown and isolated as previously described. Binding of NCRD fusion proteins to solid-phase IAV, hemagglutination inhibition assays, and fluorescent focus assays of viral neutralization were performed as described in recent publications (24).

Monoclonal Antibody Binding Assays. Monoclonal antibodies were originally prepared by Holmskov and co-workers using purified human SP-D as antigen (40). Binding of antibody to non-NCRDs and NCRD mutants was characterized by an ELISA-type binding assay (41). Proteins were adsorbed to wells without prior denaturation.

RESULTS

Substitutions for the Arg343 Side Chain Alter Binding to Mannan. In conjunction with earlier experiments examining interactions of SP-D with PI, we observed that substitutions at the 343 position could substantially and differentially alter the apparent affinity for fungal mannan. For example,

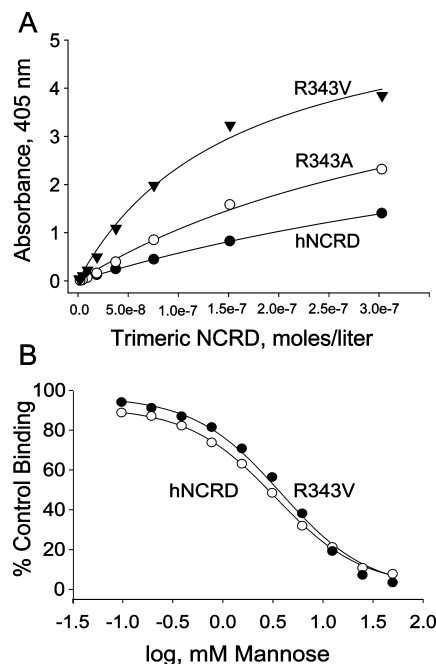


FIGURE 2: Binding of NCRD fusion protein to mannan and mannose. (A) Binding to mannan. Trimeric hNCRD fusion proteins were incubated with mannan-coated plates as described in the Experimental Procedures. Bound protein was detected using an S-protein peroxidase conjugate as previously described. Specific binding of the wild-type hNCRD is compared to mutant hNCRDs with substitutions of valine (R343V) or alanine (R343A) for Arg343. Specific binding was calculated by subtracting binding in the presence of maltose from total binding. Nonspecific binding was $<10\%$ for all proteins. The data are representative of five experiments. (B) Mannose competition assay. The ability of saccharides to compete for binding of NCRD fusion proteins to mannan was performed as described in the Experimental Procedures. The concentration required to inhibit binding by 50% is shown.

substitution of lysine for Arg343 (R343K) greatly increased binding to PI and *Escherichia coli* J5 or Rd-LPS, while largely abrogating binding to mannan (28, 29). By contrast, the substitution of valine for Arg343 (R343V) showed little effect on binding to PI or LPS. However, the apparent affinity for R343V for mannan was increased by 2–3-fold (Figure 2A). These effects were highly dependent on the specific hydrophobic side chain. Notably, the substitution of alanine (or isoleucine) resulted in a more modest enhancement in mannan binding (Figure 2A, data not shown).

Despite these effects, R343V showed no significant increase in affinity for D-mannose (Figure 2B). This observation is consistent with the fact that residues corresponding to 343 do not coordinate calcium in SP-D or other C-type lectins (42–44). Further, Arg343 of human SP-D does not participate in H-bonding interactions with inositol or the preferred monosaccharide ligand, *N*-acetylmannosamine (28).

Human SP-D NCRDs Bind to the α 1–2-Linked Dimannose and Trimannose Core with Higher Affinity than Mannan. Our recent studies have shown that SP-D can interact with specific larger oligosaccharides via an extended carbohydrate binding site, i.e., a binding site that provides secondary interactions with the side chains of amino acids that do not provide calcium ligands (31). We examined binding of hNCRDs with mannose disaccharides (dimannose, DM) and a 1-methyl derivative (Man α 1–6(Man α 1–3)Man- α -OMe-) of the trimannose (TM) core of high-mannose glycans. The hNCRD showed a reproducible, approximately

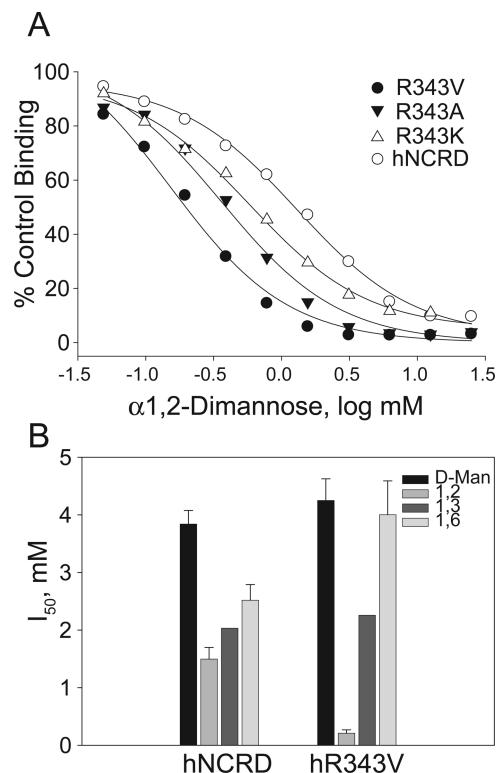


FIGURE 3: Competition assays with dimannoses. (A) Representative competition assay. Mannan competition assays were performed using hNCRD, R343V, R343A, and R343K as described in the Experimental Procedures. (B) Histogram comparing apparent affinities of hNCRD and R343V fusion proteins. The concentration of competing sugar (mM) required to inhibit binding to mannan by 50% (I_{50}) is shown. The data represent the mean and standard error of the mean from three to ten independent determinations except for α 1–3 DM, which corresponds to a single assay.

2-fold higher affinity for α 1–2-linked DM and the TM core than for mannose (Figure 3). Both differences were highly significant ($p < 0.001$). The affinities for α 1–3 and α 1–6 DM were also increased relative to mannose, but to a significantly lesser degree. The affinity for α 1–2-linked DM was also significantly greater than for α 1–6 DM ($p < 0.02$). Although the affinity of the wild-type hNCRD for the TM core (1.6 ± 0.15 mM, $n = 3$) was increased relative to D-mannose (3.8 mM), the affinity was less than observed for maltotriose, a linear glucose trisaccharide ($I_{50} = 0.94 \pm 0.08$ mM, $n = 11$) (31).

The Side Chain of Residue 343 Influences Interactions with Dimannoses and the Trimannose Core. As shown in Figure 3, mutations at the 343 position altered the apparent affinity for specific mannose-containing carbohydrates. In particular, R343V showed a 7-fold increase in affinity for α 1–2 DM ($I_{50} = 210 \pm 60$ μ M, $n = 5$), as compared to wild type. In one experiment, R343A showed a similar but less marked increase in affinity ($I_{50} = 362$ μ M). R343K also showed a detectable increase in affinity for α 1–2 DM ($I_{50} = 552 \pm 35$ μ M, average of three determinations); however, this finding must be interpreted with some caution given the markedly lower levels of total binding of R343K to mannan.

Human NCRD Fusion Proteins Bind to High-Mannose Oligosaccharides on Carbohydrate Arrays. Ligand binding preferences of wild-type hNCRD and R343V were examined in array experiments using a small panel of mannoses, mannose-rich oligosaccharides, and selected other simple

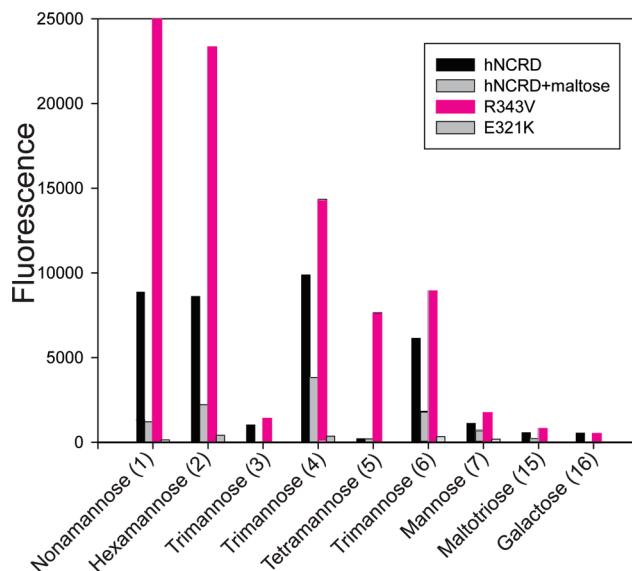


FIGURE 4: hNCRD binding to carbohydrate microarrays. Histogram comparing binding of hNCRD fusion proteins to the high-mannose microarray. Binding of hNCRD fusion proteins was assessed using penta-His Alexa Fluor 555 antibody conjugate as described in the Experimental Procedures. Solid-phase carbohydrates are illustrated in Figure 1B. A selected experiment is shown.

sugars covalently linked to the slide (Figures 1B and 4). A selected assay for several independent experiments is shown. Uniformity of ligand printing was verified using ConA binding, as described in the Experimental Procedures. Equivalent retention of the N-terminal His tags was reconfirmed in control immunoblotting experiments (data not shown).

Both fusion proteins bound to known saccharide competitors, maltotriose (ligand 14) and D-mannose (7). However, there was no reproducible binding to D-galactose (16), which is a poor competitor of SP-D binding to mannan and other ligands. Binding of hNCRDs to the arrays was inhibited by maltose, mannan, or mannan plus EDTA. Notably, a human loss-of-function mutant (E321K) with a point mutation at Glu321 and abnormal coordination of calcium at the sugar binding site consistently showed negligible binding to all ligands (Figure 4).

The proteins showed preferential recognition of long linear and branched mannose oligosaccharides as compared to D-mannose (ligand 7). The best ligand for the wild-type protein was the α 1–2-linked mannose trisaccharide (ligand 4, Man α 1–2Man α 1–2Man-). However, binding was increased to the high-mannose nonasaccharide (1), hexamannose (2), and the α 1–6-linked TM (6, Man α 1–6Man α 1–6Man-). Interestingly, binding to the trimannose core (3) was not appreciably increased as compared to mannose (7), and binding to all of the oligomannoses was greater than maltotriose (14), a classical SP-D ligand.

The binding profile of R343V was generally similar to the wild-type protein. There was preferential binding to the α 1–2-linked mannose trisaccharide (ligand 4), high-mannose nonsaccharide (1), and hexamannose (2) (Figure 4). Relative binding to hexamannose (2) and a tetramannose arm of the nonamannose (5), Man α 1–2Man α 1–6Man α 1–6Man-, was greater for the mutant (Figure 4). Although variability in absolute fluorescence intensities

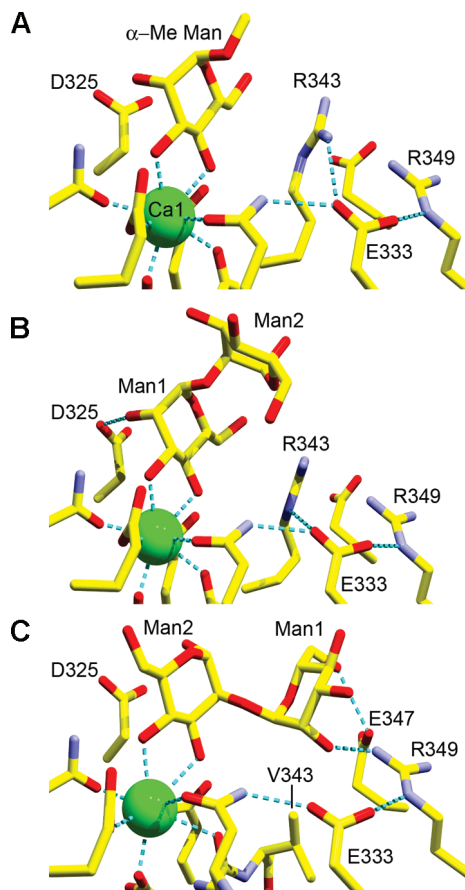


FIGURE 5: Crystallographic studies. (A) Crystal structure of trimeric hNCRD wild type with α -methyl-mannoside (α -Me-Man). (B) Crystal structure of wild-type hNCRD with α 1–2 dimannose. (C) Crystal structure of R343V with α 1–2 dimannose. Calcium ion 1 at the primary sugar binding site (green) and key flanking amino acid residues are shown.

precluded direct, quantitative comparisons among individual binding experiments, the differences in the rank ordering of binding to specific ligands was reproducible.

Crystallographic Complexes Show Differential Interactions of α 1–2 DM with Wild-Type or R343V hNCRDs. Given the binding data, we examined complexes of tagless wild-type and mutant hNCRDs with α 1–2 linked dimannose (DM) using X-ray crystallography (Figure 5, Table 1). Crystal structures of the trimeric NCRD complexes were obtained at 1.8 and 2.3 Å resolution for wild-type or R343V proteins, respectively.

The ligand was bound to the lectin calcium ion of wild-type hNCRD via the 3-OH and 4-OH groups of the nonreducing, terminal sugar in the same manner as α -methyl-D-mannose (Figure 5A,B). The dimannose showed the same orientation with respect to the plane of the ring of Man1. While strong electron density is observed for Man1 in all subunits, density for Man 2 is weaker in subunits B and C of the NCRD trimer. Notably, α 1–2 DM showed additional weak interactions of Man1 O2 with Asp325, and an interaction of Man2 with Arg343 in subunit B, consistent with differences in affinity in competition assays.

R343V showed very different interactions with the dimannose (Figure 5C). Notably, the binding orientation of the α 1–2 DM was reversed, with the reducing sugar bound to Ca1. This orientation was stabilized by a strong H-bond between the 2-OH of the nonreducing Man1 and the guanidyl

group of R349. In subunit A of the trimer (shown in Figure 5C) an additional H-bond was formed between the 6-OH of Man1 and the carboxylate of E347. In subunit B a similar interaction was mediated by a water molecule. Similar orientations were observed in all three subunits. Superimposition of α 1–2 DM from the mutant and wild-type complexes showed very little difference in torsional bond angles (Figure 6).

R343V and R343A Show Increased Antiviral Activity. Consistent with previous studies, wild-type hNCRD showed no measurable binding to Phil82 IAV as assessed by solid-phase binding assay (Figure 7) (15), despite efficient binding to mannan. The wild-type trimeric hNCRD also lacked hemagglutination inhibition (Table 2) or neutralizing activity (Figure 8, right panel). Significantly, R343V, and to a lesser extent R343A, bound to virus (Figure 7). The valine mutant also showed greatly increased HA inhibitory (Table 2) and neutralizing activity (Figure 8, left panel). Strikingly, neutralizing activity was in a range previously associated with native collectins and much greater than the previously characterized RAK mutant. Although neutralizing activity was still less than native SP-D dodecamers (Figure 8, left panel), it was greater than native human mannose binding protein (data not shown). Despite marked increases in binding of R343V to Phil82 IAV, there was no alteration in binding or neutralization of PR8 (data not shown), an SP-D resistant strain that lacks high-mannose glycans on the head of the HA. R343A showed a much smaller increase in hemagglutination inhibition (Table 2). Although R343A showed no neutralizing activity at the same concentrations as used for R343V, dose-related neutralizing activity was observed at considerably higher protein concentrations (Figure 8, right panel). The R343K mutant showed no significant binding (data not shown) and no detectable hemagglutination inhibition (Table 2) or neutralizing activity (Figure 8)), despite its greatly enhanced interactions with PI and inositol (28), as well as rough LPS (29).

Residue 343 Contributes to an Epitope Required for Binding to IAV. To further characterize the effects of the mutations on CRD structure, we examined the binding of the 343 mutants to selected monoclonal antibodies (246-02, 246-05, and 246-6) known to react with the SP-D CRD (40). All three monoclonals bound to wild-type SP-D dodecamers (not shown) and to hNCRDs (Figure 9, panel A). However, there were differential interactions of the antibodies with the 343 mutants. Although 246-02 bound to all three mutants and 246-06 showed some decrease in binding, binding of 246-05 was largely abrogated by the substitution of valine or alanine for Arg343. The more conservative R343K mutation caused a less pronounced reduction of binding to 246-05. As shown in panels B and C, the 246-02 and 246-05 mAbs blocked HA inhibitory activity of SP-D, while 246-06 did not. Although 246-02 blocked the HA inhibitory activity of R343V, 246-05 was ineffective, consistent with poor binding of the antibody to this mutant.

DISCUSSION

Mannose Sugars Are Preferred Ligands for Human SP-D. SP-D is often identified as a D-glucose or maltose binding protein. However, our recent studies identified *N*-acetylmannosamine as a preferred monosaccharide competitor for

Table 1: Crystallographic Data^a

	WT hNCRD α -methyl-mannoside	WT hNCRD 1,2 dimannose	R343V 1,2 dimannose
Data Set			
wavelength (\AA)	1.1	1.1	1.542
resolution range (\AA)	30–1.8	30–1.9	50–2.3
measured reflections	689057	643901	399674
unique reflections	67243	51917	29151
completeness (%) overall (final shell)	99.8 (99.8)	92.7 (72.6)	99.7 (99.9)
$\langle I/\sigma I \rangle$ (final shell)	18.3 (10.2)	46.2 (14.2)	15.9 (5.0)
R_{merge} (%) (final shell)	0.04 (0.17)	0.04 (0.06)	0.06 (0.30)
Refinement			
R_{free}	0.20	0.22	0.19
R_{cryst}	0.18	0.19	0.15
atoms/AU			
protein	3457	3457	3450
water	614	554	129
ions	9	9	9
ligand	39	69	69
total	4119	4089	3657
average B (\AA^2)			
main chain	21.5	24.1	32.9
side chain	24.7	26.9	33.3
ligand (as occupancy = 1)	27.3	38.5	35.7
all atoms	24.3	25.7	33.5
rms deviation			
bonds (\AA)	0.008	0.006	0.005
angles (deg)	1.156	0.932	0.869

^a Ramachandran plots of the structures described showed all residues falling within the most favorable or allowed regions.

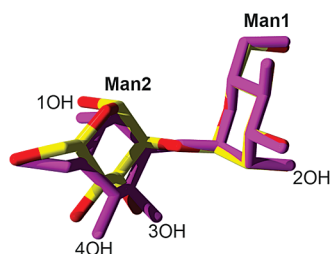


FIGURE 6: Superimposition of α 1–2 DM from wild type and R343V. The α 1–2 dimannoses from the corresponding hNCRD and R343V crystallographic complexes were superimposed. Note that the conformations are very similar.

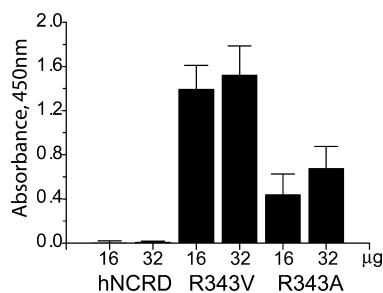


FIGURE 7: Binding of wild-type and mutant hNCRDs to IAV. Trimeric hNCRD fusion proteins were incubated with Phil82 IAV-coated plates, and bound protein was detected as described in the Experimental Procedures. Note the greatly increased binding of R343V and the more modest increase for R343A.

the human protein (12), and interactions with the core oligosaccharide of rough LPS are largely mediated by interactions with heptoses, D-mannose sugars with a side chain at the 5-position. The current studies demonstrate a significant preference of SP-D for mannose-rich sugars, including high-mannose oligosaccharides as associated with viral glycans. When presented on a solid phase, as might be encountered on a microbial surface, all of the oligomannoses

Table 2: Hemagglutination Inhibition by NCRDs vs SP-D

collectin	HA inhibitory concn ($\mu\text{g/mL}$)
SP-D dodecamers	0.040 ± 0.005
hNCRD wild type	>50
R343V	0.38 ± 0.05^a
R343A	2.66 ± 0.06
R343K	>50

^a $n = 5$. Significantly greater than SP-D dodecamers but significantly less than other NCRDs ($p < 0.02$).

were preferred over maltotriose, an α 1–4-linked trisaccharide of D-glucose.

Dimannoses Utilize an Extended Carbohydrate Binding Site. We have previously shown that Phe335, Thr336, Asn337, and Arg343 can participate in an extended sugar binding site, in which the side chains of residues that do not coordinate calcium provide secondary stabilizing interactions with nonterminal sugars (31). As indicated above, small peptide insertions (e.g., Ala-Ala-Ala) adjoining Asp325, a residue found on the opposite ridge of the shallow carbohydrate groove, can significantly enhance interactions with mannan and IAV (17) but have little effect on binding to rough LPS or PI (28, 29).

As shown here, dimannoses can make additional contacts with Asp325 and/or Arg 343, which are not seen in the mannose monomer, consistent with the greater affinity for these sugars over mannose in solution-phase competition assays. We propose that these secondary interactions contribute to the enhanced binding of the trimeric hNCRD to linear and branched mannose-rich oligosaccharides and high-mannose glycans, as compared to identically displayed mannose. Preferential binding of the wild-type protein to the linear α 1–2-linked trimannose (ligand 4), as compared to a tetramannose with the same terminal disaccharide (5), is consistent with secondary interactions and/or other significant effects of nonterminal sugars on binding affinity. Combined with previously reported observations (12, 28, 29), these

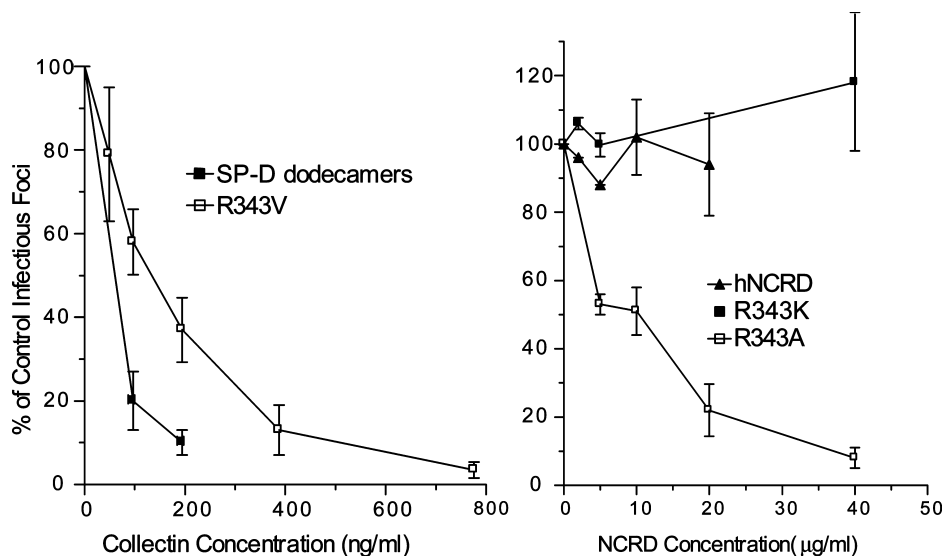


FIGURE 8: Viral neutralization assays. Wild-type and mutant hNCRDs were examined for viral neutralization using Phil82 IAV as described in the Experimental Procedures. The data in both graphs are expressed as the percent of control infectious foci. Note that the range of protein concentration is different for the two graphs; the plot at left is ng/mL, while the plot at right is in μg/mL. R343V is much more potent than the other examined hNCRDs.

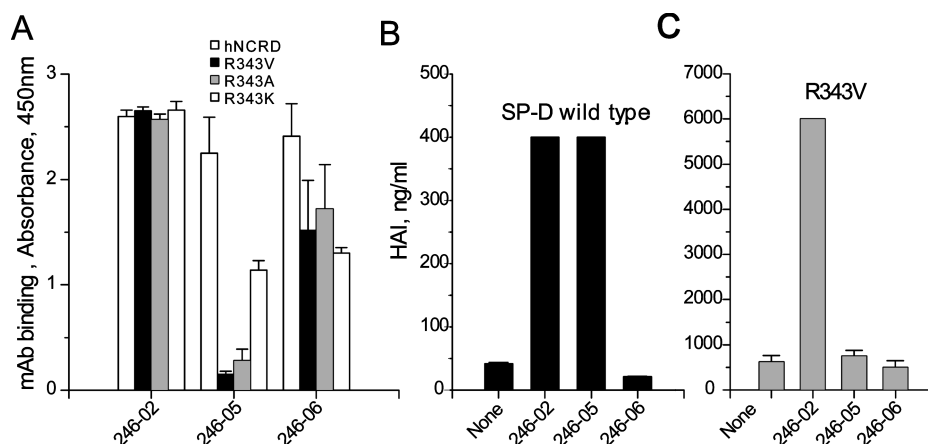


FIGURE 9: Effects of mutations on binding to anti-CRD mAbs. (A) Binding of monoclonals to solid-phase wild-type and mutant hNCRDs was examined by ELISA as described in the Experimental Procedures. (B) Effects of monoclonals on hemagglutination inhibition activity of native human SP-D. (C) Effects on hemagglutination inhibition activity of R343V. The R343V mutant is not susceptible to inhibition by the 246-05 monoclonal antibody.

studies indicate that different SP-D ligands can utilize distinct but partially overlapping binding sites. This seems consistent with the diversity of known microbial ligands.

We cannot entirely exclude differences between the NCRD and a full-length mutant protein. However, previous studies indicate the specificity of rat and human wild-type NCRDs is concordant with the specificity of the corresponding full-length proteins (12).

R343V Utilizes an Alternative Extended Binding Site for α 1–2 Dimannose. Substitution of Arg343 with valine greatly enhanced binding to α 1–2 DM in competition assays. This was quite selective for the α 1–2 linkage; there was no significant increase in affinity for α 1–3 and α 1–6 dimannoses or the trimannose core in competition assays. Comparison of crystallographic complexes of wild type and R343V with α 1–2 DM showed that the sugar ring conformations and geometry of the saccharide linkage were highly similar in the two complexes (Figure 6) and consistent with the preferred solution conformation. However, the orientation of the bound disaccharide was very different in the two complexes. Whereas the wild-type protein bound α 1–2 DM

in the more typical orientation, i.e., with the nonreducing terminal sugar bound to calcium, the binding orientation of the dimannose was flipped in R343V. In this alternate orientation, the reducing sugar is bound to calcium, while the terminal sugar participates in a strong H-bond with another conserved residue, Arg349. This flipped orientation has not been previously described for SP-D. However, we infer that loss of the Arg343 side chain increases steric access of oligosaccharides to the primary sugar binding site, while permitting additional H-bonding interactions with residues even more remote from this site. We cannot yet prove that this alternative binding orientation can be utilized by a solid-phase oligosaccharide. However, the alternate orientation, which is selected by R343V, should permit interactions with nonterminal mannoses of more complex glycans. For example, the alternate would position the reducing sugar of a linear trimannose or the corresponding internal residue of a high-mannose glycan in the region of Asp325. This region has been previously implicated in interactions with mannan and IAV (24). Thus, these observations offer a possible structural basis for the distinct binding preferences and

enhanced interactions with high-mannose oligosaccharides exhibited by R343V as compared with wild-type SP-D.

R343V Shows Greatly Enhanced Interactions with SP-D Reactive Strains of IAV. The mutant showed increased binding to IAV as well as enhanced HA inhibition (HAI) and neutralization of infectivity using MDCK cells. As indicated previously, the wild-type protein shows no detectable IAV binding and negligible HAI or viral neutralization in these same cells. These findings are consistent with data from collectin chimeras which suggested greater affinity of the conglutinin and mannan binding lectin CRDs for collectin-sensitive strains of IAV (19, 20). Notably, both of these serum collectins have valine at the position corresponding to 343. Because the affinity for D-mannose was not significantly altered for R343V, we attribute enhanced binding to additional secondary interactions with the IAV oligomannoses. The available data strongly suggest that the valine side chain contributes to the enhanced recognition of viral glycans. In particular, the SP-D mutants R343A and R343I showed significantly lower viral binding, HAI, and neutralizing activity than R343V. R343K also showed no detectable binding to IAV. The reason for these differences is not yet apparent. Our inference is that the valine substitution eliminates the bulky Arg343 side chain, allowing the second sugar ring to make additional interactions with the Arg349 side chain. The longer isoleucine and lysine side chains could impose similar steric constraints as arginine, while the short alanine side chain might permit alteration of local backbone properties, leading to other functional consequences. Future crystallographic studies of these mutants should resolve these questions. Studies designed to examine the antiviral properties of R343V *in vivo* are in progress.

The related human collectin, surfactant protein A, has arginine at the position corresponding to arginine 343 in SP-D. It is not yet possible to generalize in relation to effects on interactions with mannose-containing oligosaccharides. Our previous studies have shown that SP-A shows little if any binding to virus; antiviral effects are mediated by binding of virus to glycans associated with SP-A.

Valine and Alanine Substitutions Interfere with mAb 246-05 Binding to IAV. Monoclonal antibody binding studies suggest that substitutions at 343 cause only localized perturbations in structure. This suggestion is consistent with the crystallographic analysis of R343V. Interestingly, the substitution of valine or alanine preferentially decreased binding of the 246-05 monoclonal, while the comparatively conservative lysine substitution caused a more modest decrease in antibody binding. Given that R343V shows enhanced viral binding and blocks the viral neutralizing activity of 246-05, our data effectively localize the 246-05 epitope and the primary site of viral interaction to the carbohydrate binding face of the trimer. We infer that binding of 246-05 sterically interferes with SP-D binding to glycans associated with the viral envelope.

Binding Determinants for SP-D on IAV. The wild-type hNCRD showed preferential binding to α 1–2-linked mannose, and most of the high-mannose oligosaccharide subdomains on the array have at least one terminal α 1–2-linked mannose. This feature is not required for efficient oligomannose binding, as indicated by recognition of the linear α 1–6 trimannose (ligand 6) on the array studies.

All available hNCRD mutants with enhanced affinity for

IAV show increased apparent affinity for mannan, and nearly all show increased affinity for α 1–2 DM. However, the extent of binding to mannan binding does not directly correlate with effectiveness in viral binding or neutralization. For example, a mutant that combines the RAK insertion with isoleucine at 343, resembling CL-43, shows a greater apparent affinity for mannan relative to R343V but is less active in viral neutralization (Hartshorn and co-workers, unpublished data). Yeast mannan consists of highly extended and branched polysaccharides with α 1–2- and α 1–3-linked mannose residues on an α 1–6-linked mannose backbone. Thus, there could be differential access of branched carbohydrates to the lectin calcium, which might lead to exclusion of specific types of sugar linkages or branched structures and differences in effective ligand density.

Although terminal α 1–2-linked mannose may be necessary for binding of the hNCRD to virus, it is unlikely to be sufficient. For example, the rat NCRD resembles R343V in its high affinity for α 1–2 DM but resembles the wild-type hNCRD with respect to viral binding and neutralization (Hartshorn and co-workers, data not shown).

Mechanism of Enhanced Antiviral Activity of R343V. Our data suggest that the enhanced antiviral activity of R343V results from its capacity to recognize nonterminal sugars of more complex glycans. Given the crystallographically observed orientation of bound α 1–2 DM, such interactions could involve the region of 325. Unpublished studies indicate that viral interactions and effects of 343 substitutions are greatly influenced by the nature of the 325 side chain and the conformation of the opposite side of the carbohydrate binding groove. Planned crystallographic studies with trimannoses and other dimannose ligands should contribute to understanding these synergistic effects.

Because R343V also shows enhanced recognition of α -linked GlcNAc (30), it is possible that contributions of more complex glycans also may influence binding. Thus, additional information of the specific linkage patterns on examined strains of IAV will also be needed to fully integrate the data.

Contributions of Arg343 to Ligand Recognition by Human SP-D. Human SP-D is uniquely characterized by arginine at position 343; all other SP-Ds have lysine, while evolutionary descendents of SP-D have valine or isoleucine. We have previously shown that the substitution of lysine for arginine can greatly enhance the apparent affinity of human SP-D lectin domains for PI, the major surfactant ligand, and for forms of rough bacterial LPS (28, 29). However, there is no associated enhancement of viral interactions (data not shown), and binding to yeast mannan is abrogated (28, 29). On the other hand, substitutions of shorter, noncharged amino acids (Ala, Ile, Val) for Arg343 increase binding to mannan and interactions with SP-D sensitive strains influenza but do not enhance binding to PI or rough LPS (28, 29). Thus, the presence of a long basic side chain at 343 in human SP-D plays a substantial role in determining relative ligand preferences, greatly decreasing affinity of the lectin domain for the major known endogenous airspace ligand, while preserving interactions with specific mannose- or heptose-rich ligands.

We speculate that the need for some specific antimicrobial defense or immunomodulatory function contributed to evolutionary divergence of human SP-D at the 343 position.

Although SP-D reactive strains of IAV have only recently emerged (45), other pathogens have evolved and disseminated with humans. For example, genomic analysis of the *M. tuberculosis* complex suggests that the worldwide dissemination of this organism closely parallels the spread of humans from Africa (46). SP-D interacts with mannose-rich glycoconjugates on certain mycobacteria (47, 48), and specific polymorphic forms of SP-D appear to be associated with an increased risk of tuberculosis (49). Mycobacteria are also significant pathogens for ruminants and could have similarly contributed to evolution of bovine serum collectins from SP-D.

Potential Intersubunit Interactions among CRDs. It is important to emphasize that some ligand interactions of SP-D can be substantially influenced by higher order oligomeric assembly. Like hNCRDs, native human SP-D trimers bind poorly to IAV; however, wild-type human SP-D multimers, like rat SP-D dodecamers, show efficient binding and neutralization (12, 50). Likewise, SP-D dodecamers can bind efficiently to PI liposomes, even though the hNCRDs and native trimeric subunits bind poorly (31). Given that all the examined glycans are too small to engage more than one CRD of a trimer, preferential binding to longer linear or branched oligosaccharides might result from cooperative interactions among the CRDs of a trimer, but with each CRD showing only incremental increases in binding affinity as a result of secondary interactions at the ligand binding surface. On the other hand, a more complex ligand could also provide more opportunities for energetically favorable binding at any given ligand density. Planned studies comparing the interactions of wild-type and mutant dodecamers should be informative.

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