Ligand Specificity of Human Surfactant Protein D

EXPRESSION OF A MUTANT TRIMERIC COLLECTIN THAT SHOWS ENHANCED INTERACTIONS WITH INFLUENZA A VIRUS

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Surfactant protein D is a pattern recognition molecule that plays diverse roles in immune regulation and anti-microbial host defense. Its interactions with known ligands are calcium-dependent and involve binding to the trimeric, C-type carbohydrate recognition domain. Surfactant protein D preferentially binds to glucose and related sugars. However, CL-43, a bovine serum lectin, which evolved through duplication of the surfactant protein D gene in ruminants, prefers mannose and mannose-rich polysaccharides. Surfactant protein D is characterized by two relatively conserved motifs at the binding face, along the edges of the shallow carbohydrate-binding groove. For CL-43, sequence alignments demonstrate a basic insertion, Arg-Ala-Lys (RAK), immediately N-terminal to the first motif. We hypothesized that this insertion contributes to the differences in saccharide selectivity and host defense function and compared the activities of recombinant trimeric neck + carbohydrate recognition domains of human surfactant protein D (NCRD) with CL-43 (RCL-43-NCRD) and selected NCRD mutants. Insertion of the CL-43 RAK sequence or a control Ala-Ala-Ala sequence (AAA) into the corresponding position in NCRD increased the efficiency of binding to mannan and changed the inhibitory potencies of competing saccharides to more closely resemble those of CL-43. In addition, RAK resembled CL-43 in its greater capacity to inhibit the infectivity of influenza A virus and to increase uptake of influenza by neutrophils.

Surfactant protein D (SP-D)§ plays important roles in the innate defense against microorganisms and contributes to the pulmonary response to antigenic challenge (1–4). Like many other effectors of innate immunity, SP-D is a pattern recognition molecule with a variety of potential ligands (5, 6). These include cell wall glycoconjugates expressed by bacteria and fungi, viral coat proteins, and various organic antigens. In addition, SP-D, or SP-D-ligand complexes, can interact with specific binding sites on phagocytes and lymphoid cells. The consequences of these interactions include agglutination, opsonization with enhanced internalization, inhibition of T-lymphotoocyte proliferation, clearance of apoptotic cells, modulation of phagocyte cytokine production, and enhanced antigen presentation. Significantly, SP-D null animals show defective host responses to respiratory viruses, including influenza A virus (7, 8), which can be rescued with exogenous SP-D or by the targeted overexpression of SP-D in the alveolar epithelium (7, 9, 10).

SP-D is a member of a family of collagenous, C-type lectins, or collectins (1, 11). In humans, this family includes surfactant protein A (SP-A) and serum mannose-binding lectin (MBL). In cows, there are at least three other secreted collectins. Serum conglutinin (BC), serum CL-43, and CL-46 all appear to have evolved from SP-D in ruminants (12). Nearly all secreted collectins, including SP-D, are assembled as multimers of trimeric subunits associated at their amino termini and stabilized by interchain disulfide bonds (13). Each trimeric subunit consists of four major domains: an amino-terminal cross-linking domain, a collagen domain, a trimeric coiled-coil neck domain (N), and the C-terminal, C-type lectin CRD. Notably, bovine CL-43 is secreted exclusively as trimers (14, 15).

The carbohydrate binding activity of SP-D and MBL, and probably other collectins, is directly mediated by a coordinated calcium ion in their CRD. However, there are differences in saccharide selectivity among members of this family, even for collectins closely related to SP-D (11). For example, rat SP-D preferentially interacts with maltose and myo-inositol, with lower affinity for mannose (16). By contrast, CL-43 shows preferential binding to mannose with comparatively low affinity for glucose (17–19). Whereas CL-43 binds efficiently to fungal cell wall mannann (14, 15), SP-D has been reported to show relatively poor binding to this ligand (20). For all collectins, high affinity binding to saccharide ligands appears to require trimerization of the CRDs, which is mediated by the contiguous neck domain (21).

By analogy with studies for rat MBL (22, 23), it has been shown that site-directed substitution of two residues known to coordinate with calcium at the carbohydrate binding site of SP-D can alter ligand affinity and specificity (24). The so-called QPD substitution (Gln for Glu for Asn) of rat SP-D) reversed the relative inhibitory potencies of galactose compared with mannose, maltose, or glucose in phosphatidylinositol binding assays and increased the amount of calcium.
required for optimal phosphatidylinositol binding. Thus, alterations in amino acids that directly coordinate with calcium can alter ligand preferences.

Studies of the crystal structure of the trimeric recombinant human SP-D neck + CRD domains demonstrated the presence of bulky charged residues at two edges of the shallow carbohydrate binding groove, the so-called “SP-D groove” (25). Comparisons of the primary sequence of five different species of SP-D show marked conservation of this feature, which consists of two relatively conserved motifs immediately C-terminal to the site of the QPD mutation and near the ends of the short Cys331–Cys345 loop: (D/N)GG(S/A) and (R/K)(A/V)CEGXR (Fig. 1A). CL-43 is distinguished from SP-Ds by an insertion of three amino acids (RAK) immediately N-terminal to, and contiguous with, the first motif (Fig. 1A) (11, 25). This insertion is predicted to flank the carbohydrate binding site (Fig. 1, B and C). Given the above, we used site-directed mutagenesis to test the hypothesis that this unique insertion near the primary carbohydrate binding site contributes to the different ligand binding properties of SP-D and CL-43 and that this region adjoining the SP-D groove contributes to the unique ligand selectivity of SP-D.

Our studies demonstrate that sequences near the primary carbohydrate binding site contribute to SP-D’s recognition of saccharide ligands and strongly suggest that these sequences also contribute to certain unique antiviral properties of CL-43. The RAK insertion increased the capacity of SP-D to inhibit the infectivity of influenza A and to enhance viral uptake by neutrophils, effects probably mediated by enhanced interactions with mannose-rich oligosaccharides associated with the viral hemagglutinin. Because wild-type trimeric NCRDs have demonstrated protective effects in various animal models of microbial and antigenic challenge, our studies also demonstrate feasibility of “fine-tuning” host defense functions through site-directed modifications of the CRD.

MATERIALS AND METHODS

The pET-30a (+) vector, S-protein HRP conjugate, anti-His tag antibodies, and competent cells were from Novagen (Madison, WI). BamHI and HindIII restriction enzymes were from Promega (Madison, WI). BSA (Fraction V; A4503), BSA (Fraction V, fatty acid-free, low endotoxin; A8806), and yeast mannan were from Sigma. All saccharides were the D anomers and of the highest purity available from Sigma. Purified synthetic oligomers were obtained from the oligonucleotide synthesis center at Washington University School of Medicine.

**Generation of Fusion Constructs**

For these studies, we generated a panel of bacterial expression constructs encoding homologous, N-terminally tagged, trimeric neck + CRD (NCRD) fusion proteins. Bacterial expression is feasible, because the neck domains can efficiently trimerize and mediate the formation of trimeric CRDs (26, 27) and because the selected sequences lack known posttranslational modifications. Previous studies have shown that the human neck + CRD domain contains all information necessary to direct normal CRD folding, intrachain disulfide bond formation, coordination of calcium ions, and ligand binding (25, 28).

The full-length human SP-D and bovine CL-43 cDNAs were described previously (14, 29). SP-D and CL-43 neck + CRD domains were generated by thermal amplification of these cDNAs, using forward and reverse primers that incorporated restriction sites for BamHI and HindIII, ligated, and transformed into RosettaBlue competent cells (Novagen, Madison, WI). Sequence was verified by automated sequencing. This encoded a fusion protein with a His tag, S tag, and enterokinase (EK) cleavage site N-terminal to the neck + CRD domain (Fig. 1A). All amplification products were purified using the QiAquick gel extraction kit (Qiagen, Valencia CA). DNA sequencing was performed by the Protein and Nucleic Acid Chemistry Laboratory at Washington University School of Medicine.

**Site-directed Mutagenesis**

Site-directed mutagenesis of the hSP-D-NCRD cDNA to generate RAK and AAA mutants was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and/or overlap extension using the full-length hSP-D cDNA in pGEM-3Z (Promega) as the template. The mutated neck + CRD was then amplified by PCR using forward and reverse primers for the hSP-D-NCRD, which contained restriction sites for BamHI and HindIII. DNA sequences were verified by automated sequencing of the entire coding sequence of the fusion protein.

**Expression of Trimeric Neck + CRD Domains in Bacteria**

Transformed competent cells were inoculated into 100 ml of Luria-Bertani medium (Fisher) containing 50 μg/ml kanamycin and grown for 16 h at 37 °C with shaking. The cells were then diluted into 1 liter of medium and grown for an additional 4 h to obtain an A500 of 0.6–0.8. Following induction with 1 mM isopropyl-β-D-thiogalactopyranoside, cells were incubated for 4 h at 30 or 37 °C, harvested by centrifugation at 2500 × g for 15 min at 4 °C, and temporarily stored at –80 °C.

Bacteria transformed with the NCRD construct showed a major isoform of a 180-kDa-thiogalactopyranoside-inducible protein of the predicted size in Coomassie Blue-stained gels of whole cell lysates (data not shown). The reduced protein migrated slightly more slowly than soybean trypsin inhibitor (21.1 kDa), approximating the predicted mass of 21 kDa for the fusion protein (data not shown). The identity of the NCRD and human mutant fusion proteins was confirmed by immunoblotting using antibodies to human SP-D and/or by blotting with S-protein-HRP conjugate as described below (data not shown).

**Isolation of Recombinant Proteins**

Initially, recombinant proteins were isolated from soluble extracts of BL21 or NovaBlue cells. However, maximum yields of trimeric protein were in the range of several hundred μg/liter. In subsequent experiments, the recombinant proteins were isolated in much larger amounts from inclusion bodies (IBs) produced in RosettaBlue competent cells (Novagen, Madison, WI).

**Isolation of Soluble Protein from Periplasm**—For some control experiments using soluble fusion proteins, the bacterial pellet was resuspended in Tris-buffered saline buffer with lysozyme (0.1 mg/ml) in the absence of added calcium, with or without 0.1% (v/v) Triton X-100. After incubating for 15 min at 30 °C, the cells were lysed by sonication on ice with three 20-s bursts (30% of maximum power using a CE-3240 sonicator and microprobe; Heat Systems, Farmingdale, NY) until the suspension was no longer viscous. The homogenate was centrifuged at 40,000 × g at 4 °C for 25 min.

**Isolation from Inclusion Bodies**—Bacteria from 1 liter of cells were collected by centrifugation and lysed using 10 ml of CellLytic B reagent (Sigma) per gram of bacterial pellet. After a 15-min incubation at room temperature, the IBs were collected and washed, dissolved in 10 or 20 ml of CellLytic IB in the presence of 5 mM dithiothreitol, incubated for 30 min at room temperature, dialyzed into 6 mM ube, and then refolded and oligomerized by dialysis against decreasing concentrations of urea and increasing concentrations of NaCl at 4 °C according to the manufacturer’s instructions. For the AAA mutant, it was necessary to include a bacterial protease inhibitor mixture (P8849; Sigma) prior to and immediately following refolding and oligomerization to prevent degradation during chelation chromatography.

**Endotoxin Precautions**

Given that endotoxin is a ligand for SP-D, particular precautions were undertaken to minimize endotoxin contamination during protein purification and characterization. Endotoxin was quantified using an endotoxin chromogenic assay (GCT-1000; Cambrex Corp., Northford, NJ). Water was purified using a MQ Biocol A10 water purification system with TOC and Pyroguard 5000UF cartridge (Millipore Corp.). All chromatography and binding assay buffers were prepared using freshly purified, endotoxin-free water and stored in deyprotogenated or pyrogen-free glass and plasticware. Saccharide affinity columns were routinely precleaned with washing by PyroCLEAN (AleCherk, Portland, ME). Truncin gel slurry was then washed with washing with 0.5 mM sodium hydroxide and stored in ethanol.

**Purification of Neck + CRD Fusion Proteins**

**Chelation Affinity Chromatography**—Refridged proteins isolated from IBs or soluble proteins from cell lysates were dialyzed against 0.5 mM NaCl, 0.05 mM Tris-HCl, pH 7.5, containing 10 mM imidazole (binding buffer). After centrifugation for 30 min at 20,000 × g, the soluble
His-tagged fusion proteins were purified by nickel affinity chromatography using an AKTA Purifier chromatography system (Amershams Biosciences) and a fresh charged, 5-mL Hitrap Chelating HP column (Amershams Biosciences). Samples were bound to the column in the binding buffer (see above) at a flow rate of 1.5 mL/min. The column was then washed with 5 column volumes of binding buffer prior to elution with a two-step imidazol gradient with 5 column volumes per step (step 1, 10–50 mM imidazole; step 2, 50–500 mM). Elution was monitored at A280 with subtraction of buffer absorbance.

All of the fusion proteins eluted near the same position from the metal columns. Yields of the human proteins were in the range of 40 mg of total protein were dialyzed versus Tris-buffered saline (pH 7.4) containing 0.1% (w/v) fatty acid-free BSA.

**SDS-PAGE**

SDS-PAGE sample buffer and boiling for 3 min prior to analysis by SDS-PAGE.

**Gel Filtration Chromatography**—Aliquots (up to 0.5 mL) of pooled affinity-purified protein containing as much 10 mg of total protein were dialyzed versus 500 mM NaCl, 25 mM Hepes, pH 7.5, and concentrated as needed by ultrafiltration (Centricon-20; Millipore Corp.). Following centrifugation at 20,000 g for 30 min at 4 °C, samples were applied to a Tricorn 10/300 GL Superose 12 column on the AKTA purifier. The column was precalibrated with commercial globular protein standards (151-1901; Bio-Rad). Proteins were eluted at a flow rate of 0.3 mL/min at room temperature. All recombinant protein stocks isolated from IBs had very low endotoxin values, with maximum concentrations ranging from 1.9 to 3.1 pg/mL purged protein. Aliquots of protein were stored at −80 °C.

Samples dialyzed into 150 mM NaCl prior to gel filtration showed an increased proportion of larger aggregates on the chromatogram. Careful pooling of the trimer fractions and storage at higher ionic strength was implemented to minimize aggregation of concentrated stocks and ensure reproducible results in these assays. Fusion protein purified by gel filtration in the presence of 0.5 mM NaCl showed negligible aggregation at concentrations up to several hundred μg/mL, even following freezing and thawing (data not shown).

**Saccharide Affinity Chromatography**—For some preliminary experiments, whole or refolded IB proteins were dialyzed versus Tris-buffered saline at 4 °C. Following the addition of calcium to 5 mM, samples were centrifuged at 2500 × g for 15 min, and the supernatant was applied at 30 mL/h to a deprotonated column of maltosyl-agarose or mannosyl-agarose equilibrated with Tris-buffered saline containing 5 mM calcium (30). After extensive washing with several column volumes, bound proteins were eluted with calcium-free column buffer containing 10 mM EDTA.

**SIPS-PAGE and Blotting**—SIPS-PAGE was performed using pre-poured 10 or 12% separating or 10–20% gradient gels. Recombinant proteins were routinely visualized by rapid staining with BioSafe Coomassie Blue (Bio-Rad). Molecular weights were estimated using Mark 12 standards (Invitrogen). The identity of the proteins was confirmed by blotting using S-protein-HRP (1:5000; Novagen) or by indirect immunoassay. For blotting, the proteins were transferred to nitrocellulose membranes.

**Enterokinase Cleavage**—For selected control experiments, fusion tags were cleaved from the recombinant proteins at the enterokinase cleavage site using a protocol provided with the enzyme (P89070L; New England BioLabs, Beverly, MA). The proteins were incubated with 0.005% (w/w) enzyme for 16 h at room temperature in Tris-buffered saline containing 0.1% CaCl2. The reaction was terminated by the addition of a 2-fold volume excess of trypsin inhibitor-agarose (T-0637, Sigma). The suspension was incubated for 30 min at 4 °C, and the beads were removed by centrifugation. The EK-cleaved trimeric neck + CRD domain was isolated by gel filtration as described above.

**Cross-linking Assays**—The extent of oligomerization of proteins recovered by gel filtration was confirmed by chemical cross-linking using the covalent homobifunctional cross-linker bis(sulfo succinimidyl) suberate (BS3; Pierce), as previously described (33). BS3 was added to the purified neck + CRD fusion protein (1 μg/100 μL) in HEPES-buffered saline, pH 7.4, at the indicated concentrations. The samples were incubated at 25 °C for 30 min. Reactions were terminated by the addition of SDS-PAGE sample buffer and boiling for 3 min prior to analysis by SDS-PAGE.

**Function of Recombinant Proteins**

The binding of trimeric fusion proteins to surface-adsorbed mannan or other ligands was assessed using 96-well plates and a S-protein-HRP detection system. Buffers for binding assays were prepared as follows: coating buffer, 15 mM Na2CO3, 55 mM NaHCO3, 0.05% (w/v) NaN3, pH 9.6; wash buffer, 20 mM Tris-HCl, 140 mM NaCl, 5 mM CaCl2, 0.05% Tween 20, pH 7.4; blocking/binding buffer, 20 mM Tris-HCl, 140 mM NaCl, 5 mM CaCl2, pH 7.4, and containing 0.1% (w/v) fatty acid-free BSA. For some experiments, calcium was deleted from buffers used for the initial wash and blocking/binding steps. Immediately prior to each assay, thawed aliquots of the protein stock solutions were briefly centrifuged in a microcentrifuge, and the concentration of protein in the supernatant was confirmed using a BCA protein assay.

**Mannan Binding Assays**—The binding of trimeric neck + CRD to yeast mannan- or LPS-coated plates was performed using limited modifications of the method of Lim et al. (27). Briefly, the wells of 96-well plates (catalog no. 3590; Corning) were incubated for at least 1 h at room temperature with 100 μL of 50 μg/mL mannan dissolved in coating buffer. After washing, the wells were incubated for at least 1 h with coating/binding buffer. Neck + CRDs were diluted as required in the calcium-containing blocking/binding buffer and added to wells to give a final volume of 100 μL/well. The plates were incubated with protein for 1 h at room temperature, washed, and then incubated for 1 h at room temperature with S-protein-HRP conjugate (Novagen) at a verified optimal dilution of 1:5000. For some control experiments, bound recombinant SP-Ds were detected by an indirect immunoperoxidase using the P13 antibody and goat anti-rabbit HRP IgG (1:2000; Bio-Rad). After washing, the wells were incubated with ABTS chromogenic substrate (KPL) (0.1% ABTS, 0.02% H2O2) for 20 min at 80 °C. 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.

The fusion proteins showed essentially identical signals on blotting of equivalent amounts of protein with S-protein-conjugate. However, to confirm comparable S-protein-binding activities of the nonadenanured trimeric fusion proteins, equivalent dilutions of proteins were adsorbed to untreated 96-well plates. After blocking and washing, bound fusion proteins were detected with S-protein-HRP conjugate as described above.

**Competition assays** were performed as for direct mannan binding except that the proteins were added to wells in the presence of various concentrations of competing inhibitors. For critical comparisons of IC50 values (μM) of RhSP-D-NCRD and RAK, replicates were derived from dilutions of three independent stocks of saccharide. Unless otherwise stated, all values are given as the mean ± S.D. of triplicate determinations, and the data are representative of two to three independent experiments using independent dilutions of protein and/or competitors. Binding data were plotted and analyzed using SigmaPlot 8.0 (SPSS Inc., Chicago, IL).

**Influenza Virus Assays**

**Preparation of Influenza A virus (IAV) and Neutrophil Isolation**—IAV,Phil82 strain, was grown in the chorioallantoic fluid of 10-day-old chicken eggs and purified on a discontinuous sucrose gradient. The virus was dialyzed against phosphate-buffered saline to remove sucrose, and aliquots were stored at −80 °C. After thawing, the viral stocks contained ∼5 × 106 plaque-forming units/mL. Neutrophils from healthy volunteers were isolated to >95% purity by using dextran precipitation, followed by Ficoll-Paque gradient separation for the removal of mononuclear cells, and hypotonic lysis to eliminate any erythrocytes. Cell viability was determined to be >98% by trypan blue exclusion. The cells were resuspended in phosphate-buffered saline containing divalent cations and used within 2 h.

**Neutrophil Uptake of IAV**—Uptake assays were performed as recently described for CL-43 (14). Briefly, fluorescent isothiocyanate-labeled IAV were preincubated with control buffer or various concentrations of NCRDs for 30 min at 37 °C, followed by incubation of aliquots of these samples with neutrophils for 30 min at 37 °C. Trypan blue (0.2 mg/mL) was added to quench extracellular fluorescence. Uptake was determined by phagocyte-buffered saline containing divalent cations and used within 2 h.

**Neutrophil Uptake of IAV**—uptake assays were performed as recently described for CL-43 (14). Briefly, fluorescent isothiocyanate-labeled IAV were preincubated with control buffer or various concentrations of NCRDs for 30 min at 37 °C, followed by incubation of aliquots of these samples with neutrophils for 30 min at 37 °C. Trypan blue (0.2 mg/mL) was added to quench extracellular fluorescence. Uptake was determined by phagocyte-buffered saline containing divalent cations and used within 2 h.

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Fig. 1. The SP-D groove and RAK insertion. A, top, alignment of corresponding subsequences of SP-D and CL-43 CRDs showing conserved sequences of the SP-D groove and sites of insertions. Middle, schematic diagram of NCRD fusion proteins showing His tag (6× His), S-tag, EK, and the neck + CRD domains. Bottom, primary sequence of human SP-D NCRD fusion protein. The His tag, S-protein binding sequence, and enterokinase cleavage motif are boxed. The EK cleavage site is depicted by a downward arrow. The neck domain is underlined, and the sites of the RAK or AAA insertion and QPD substitution are indicated. The EK cleavage site is indicated (E) in D. B, ribbon diagram of a portion of a single hSP-D CRD (25). Residues contributing to the C-terminal and N-terminal motifs of the SP-D groove are identified (ball and stick, yellow). C, ribbon diagram of trimeric CRD derived from crystal structure of human SP-D neck + CRD trimer (25). The two aspartate residues (Asp<sup>324</sup> and Asp<sup>325</sup>) that flank the site of the RAK or AAA insertion are shown (ball and stick, yellow). The ligand binding face is at the bottom and perpendicular to the axis of the neck. The red ball identifies calcium ion 1 at the primary carbohydrate binding site. D, schematic diagram of fusion protein depicting the predicted association of the binding face of a trimeric CRD with a ligand-coated well. E indicates the location of the EK cleavage sites.
confluence. The layers were infected with diluted IAV for 30 min at 37 °C, followed by washing three times in serum-free Dulbecco’s modified Eagle’s medium containing 1% penicillin and streptomycin. The monolayers were incubated for 7 h at 37 °C in Dulbecco’s modified Eagle’s medium, washed, and fixed with 80% (v/v) acetone at 4 °C. Monolayers were then labeled by incubating with monoclonal antibody directed against the IAV viral nucleoprotein (monoclonal antibody A-3; gracious gift of Nancy Cox, Centers for Disease Control and Prevention, Influenza Branch, Atlanta, GA) for 30 min at 4 °C, washed three times, and incubated with fluorescein isothiocyanate-labeled goat anti-mouse IgG. The fluorescent foci were counted by fluorescence microscopy, and areas labeled by fluorescein were measured with ImagePro Plus software.

RESULTS

Design of Recombinant Trimeric Neck + CRDs—We expressed four trimeric neck + CRD fusion proteins in bacteria (25, 27, 34, 35). These included a wild-type protein containing the NCRD and a mutated human NCRD containing an RAK insertion at the position of the natural insertion in CL-43 (RhSP-D-NCRD-RAK, or RAK) (Fig. 1A). We also expressed two control fusion proteins containing 1) a mutated NCRD with alanine in all three positions of the insertion (RhSP-D-NCRD-AAA, or AAA) and 2) the neck + CRD domain of wild-type bovine CL-43 (RCL-43-NCRD).

All four constructs encoded a contiguous S-protein binding site and an EK with the neck + CRD splice junction at the N-terminal exon boundary of the neck domain (Fig. 1A). This design is well suited to solid-phase binding assays, because interactions of the binding surface of trimeric CRDs with solid-phase ligands leaves the tag accessible to S-protein conjugates added to the aqueous phase (Fig. 1D).

Wild-type and Mutant NCRD Domains Are Expressed as Trimeric Fusion Proteins—Recombinant proteins were extracted from inclusion bodies, refolded, and oligomerized and purified by sequential metal affinity and gel filtration chromatography as described under “Materials and Methods.” The N-terminal exon boundary of the neck domain (Fig. 1A) was ligated into the N-terminus of the S-protein for binding of the fusion proteins to the S-protein ligand and a His-tag was added to the C-terminus of the NCRD domain for metal affinity purification. The His-tagged fusion proteins were bound to a HiTrap Chelating HP column (5 ml) and eluted with a gradient of imidazole. Profiles of total absorbance at 280 nm (solid line) and absorbance corrected for the contribution of buffer (dashed line) are shown. The major peak eluted at ~280 mM imidazole (70–80% B). RCL-43-NCRD, RAK, and AAA showed virtually identical elution profiles. A, the affinity-purified fusion protein was further purified by gel filtration, as described under “Materials and Methods.” The protein eluted as a major peak (solid line) between immunoglobulin (I) and ovalbumin (Ov) standards (dashed line), near the position of bovine serum albumin (68 kDa).
Thus, the purified proteins consist almost exclusively of trimeric assemblies.

Enterokinase cleavage of NCRD, followed by removal of the enzyme, showed a single major cleavage product that migrated on SDS-PAGE with an estimated size of 18 kDa (Fig. 4B), consistent with the predicted size of the cleaved protein. The cleavage product migrated more slowly after reduction with dithiothreitol, indicating the presence of intrachain disulfide bonds. RAK, AAA, and RCL-43-NCRD showed similar cleavage products (data not shown). The EK-cleaved proteins eluted from the gel filtration column with an estimated mass of 54 kDa, consistent with trimeric neck/H11001/CRD domains (Fig. 4C).

Saccharide Affinity Chromatography—All the fusion proteins bound to mannosyl- or maltosyl-agarose in the presence of calcium and were eluted with EDTA (data not shown). In preliminary experiments using a different vector, we expressed a His-tagged mutant human NCRD with the substitution of Gln321-Pro-Asp323 for Glu321-Pro-Asn323, analogous to the rat QPD mutant described in the Introduction. Consistent with previous studies using full-length rat QPD mutants, the trimeric human QPD mutant did not bind to either affinity support. This confirmed specific, CRD-dependent binding of the fusion proteins to the affinity columns (data not shown).

RAK and AAA Show Enhanced Interactions with Solid-phase Mannan—Previous studies have shown that CL-43 preferen...
tially interacts with mannose and mannose-rich glycoconjugates, including fungal cell wall mannan (14). In order to characterize the effect of the insertions on binding to mannose-rich polysaccharides, we developed a modified mannan binding assay using the S-protein detection system. Control experiments demonstrated essentially equivalent dose-dependent detection of all four trimeric fusion proteins using the S-protein conjugate (Fig. 5A). This indicates the presence of comparably accessible, N-terminal, S-protein binding sites. Similar results were obtained with three independent assays using at least two different preparations of each protein and with preparations of soluble protein isolated from bacterial lysates (data not shown).

Consistent with previous studies of full-length, mammalian cell-expressed, recombinant CL-43 and natural bovine serum CL-43 (14), RCL-43-NCRD showed dose-dependent binding to solid-phase mannan (Fig. 5B). This binding was not observed in the absence of added calcium, consistent with specific, calcium-dependent binding mediated by the CRD (Fig. 5B).

We subsequently compared the binding activities of purified NCRD, RAK, and AAA trimers using the same assay system.

As illustrated in Fig. 6A, RAK showed more efficient binding to mannan than the wild-type NCRD, with ∼2-fold greater binding at equivalent protein concentrations. Comparable results were obtained in at least six independent experiments using aliquots of purified protein derived from different preparations of recombinant protein. Although there was greater variability for AAA, binding of AAA was always greater than NCRD and less than or equivalent to RAK. Similar to RCL-43-NCRD, binding of all constructs required added calcium (Fig. 6B). The comparatively low efficiency of binding of NCRD to mannan is consistent with previous studies of natural human SP-D, which showed low binding to mannan under conditions where the protein bound efficiently to maltosyl-albumin (20). There was no significant binding of the trimeric human QPD mutant (data not shown).

In order to further characterize binding specificity and assess possible effects of ligand density, we compared the binding of identical amounts of NCRD, RAK, and AAA as a function of the coating concentration of mannan (Fig. 7A). Although the proteins showed little binding at mannan concentrations of less than ∼0.5 μg/ml, binding of all three constructs increased as the mannan concentration increased from 1 to 50–100 μg/ml.

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Notably, RAK and AAA showed greater binding at all concentrations examined, and maximum binding for RAK was ~2-fold greater than for equivalent concentrations of NCRD. As compared with NCRD, RAK showed dose-dependent enhancement of binding to solid-phase LPS isolated from *Klebsiella pneumoniae* serotypes with mannose-rich O-antigens (36) but reduced binding to maltosyl-BSA or to pustulan, a glucose homopolymer isolated from yeast cell walls (data not shown).

Given the proximity of the insertions to three of four known calcium binding sites, we compared the calcium requirements for NCRD, RAK, and AAA binding to mannan. All three constructs showed a similar minimum calcium concentration for detectable binding to mannan-coated plates in the presence of 5 mM calcium and the presence or absence of selected mono- or disaccharides, as described under “Materials and Methods.” Data are presented as percentage of control binding in the absence of competitor. For this figure, the percentage of total binding is plotted to more accurately illustrate the specificity of binding to the mannan-coated well.

**FIG. 8. Mannan competition assays.** Fusion proteins were incubated with mannan-coated plates in the presence of 5 mM calcium and the presence or absence of selected mono- or disaccharides, as described under “Materials and Methods.” Data are presented as percentage of control binding in the absence of competitor. For this figure, the percentage of total binding is plotted to more accurately illustrate the specificity of binding to the mannan-coated well. A, RCL-43-NCRD (left to right), mannose (open circles), N-acetylmannosamine (closed diamonds), N-acetylgalactosamine (open squares), glucose (closed circles), maltose (closed triangles), myo-inositol (open diamonds), galactose (open triangles), and lactose (closed squares). B–E, representative competition assays comparing NCRD (closed circles) with RAK (open circles). B, myo-inositol; C, maltose; D, GlcNAc; E, mannose. F shows competition curves for RAK using three separately prepared stock solutions of mannose (three curves at left), and three stocks of glucose (three curves at right). Inhibition data for these and other experiments and competitors are compiled in Table I.
maltose over mannose (20). The profiles for RAK and AAA resembled published profiles of mannose competition of binding to mannan.

**Table I.** Saccharide competition of binding to mannan

| Table II | Relative saccharide selectivities |

The table shows the approximate rank order of I<sub>50</sub> values for recombinant, trimeric neck + CRD fusion proteins, based on I<sub>50</sub> values (mM) for binding to mannan. This is largely derived from data presented in Table I. RAK shows the inhibition profile for RAK isolated from bacterial lysates. RAK (sol) shows the profile for soluble protein isolated from inclusion bodies. AAA (sol) shows the profile for soluble protein isolated from bacterial lysates. AAA is in boldface type, and manno-inositol (In) is italicized to facilitate comparisons between proteins. The last row shows the published sugar inhibition profile for natural bovine serum CL-43 using mannan as ligand and an anti-CL-43 detection system (14); manno-inositol was not examined in that study. Mal, maltose.

The RAK Sequence Modifies Collectin-dependent Interactions of IAV with Neutrophils—As indicated in the Introduction, there is now considerable evidence that SP-D plays important roles in the host response to influenza infection and that it can efficiently neutralize virus both in vitro and in vivo. These interactions are mediated by binding of the SP-D CRD to specific high mannose-containing oligosaccharides on the viral hemagglutinin (sialic acid receptor) and/or the neuraminidase (37). In recent studies, we compared the activities of natural and full-length recombinant CL-43 trimers with respect to their interactions with influenza virus (14). Similar to SP-D, conglutinin, and MBL, bovine CL-43 showed specific binding to various strains of IAV and exhibited potent hemagglutination inhibition activity. However, unlike wild-type SP-D dodecamers, the trimeric CL-43 did not grossly agglutinate virus and only weakly stimulated the oxidant response of neutrophils to virus. Nevertheless, natural and full-length recombinant CL-43 showed potent stimulation of viral uptake by neutrophils and inhibition of viral infectivity in vitro. Because a single arm trimeric mutant of rat SP-D (RrSP-Dser15,20) and trimeric human neck + CRDs provided by other investigators show little capacity to stimulate uptake or inhibit infectivity, we inferred that the CRD of CL-43 binds with higher affinity to the viral envelope glycoproteins.

In preliminary experiments, we observed that the human fusion proteins show differential binding to IAV. As compared with wild-type NCRD, the RAK and AAA insertions caused a significant, >2-fold increase in specific binding to adsorbed virions using the microplate assay (data not shown). Given these findings, we performed selected functional assays known to involve binding of collectins to viral glycoconjugates. As predicted by our earlier studies, the trimeric NCRD showed no significant stimulation of IAV uptake by isolated neutrophils (Fig. 9A). By contrast, RAK significantly stimulated viral uptake (Fig. 9A). In separate control experiments, we also examined the effects of RCL-43-NCRD and AAA. As predicted, RCL-43-NCRD significantly increased uptake, albeit at a lower concentration than for RAK (Fig. 9B) and with lower potency than full-length recombinant CL-43 (data not shown). Surprisingly, AAA showed no significant stimulation (Fig. 9B).

As illustrated in Fig. 9C, RAK, AAA, and RCL-43-NCRD significantly inhibited viral infection of Madin-Darby canine kidney cells (Fig. 9C). However, consistent with our previous findings, the NCRD showed no detectable inhibitory activity.

**DISCUSSION**

In this study, we demonstrate that a small subregion of the carbohydrate recognition domain contiguous with the N-terminal

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**DISCUSSION**

In this study, we demonstrate that a small subregion of the carbohydrate recognition domain contiguous with the N-terminal
motif of the conserved SP-D groove contributes to the ligand preferences of SP-D. Insertion of RAK or AAA at a position corresponding to the natural RAK insertion in CL-43 substantially increased the efficiency of binding of trimeric human NCRDs to mannan. It also slightly increased the apparent affinity for mannose while simultaneously decreasing the relative affinity for maltose and myoinositol (Fig. 8, Table I). To our knowledge, this is the first functional evidence for participation of this subregion of the CRD in ligand recognition by SP-D. The effects of the RAK insertion are quite distinct from the rat QPD substitution, which alters the coordination of calcium at the primary carbohydrate binding site, increases the affinity for galactose, and decreases affinity for mannone sufficient to preclude purification by saccharide affinity chromatography (24). Strikingly, the RAK insertion also conferred specific functional attributes of CL-43 on the trimeric, human SP-D NCRD, including the capacity of a trimeric molecule to efficiently stimulate IAV uptake by neutrophils and inhibit viral infectivity. The effects of collectins on viral uptake and infectivity involve binding to viral glycoconjugates. For example, binding of SP-D to IAV involves the recognition of oligomannose sugars on the viral hemagglutinin, which plays critical roles in the attachment of IAV to host cells (37). Binding of CL-43 to IAV is similarly dependent on the presence of a specific oligosaccharide on the viral hemagglutinin, and these interactions are most efficiently inhibited by mannone (14). Given the above, the enhanced antiviral properties of RAK appear consistent with its enhanced affinity for mannan, a complex mannone-containing polysaccharide. Together, our findings strongly suggest that the RAK insertion confers increased binding affinity for mannone-containing viral glycoconjugates.

The AAA mutant resembled RAK (and CL-43) in its capacity to inhibit infection of mammalian cells by IAV. However, unlike RAK, it did not appreciably enhance viral uptake by neutrophils. Although this difference is unexplained, it might be attributable to the slightly greater apparent affinity of RAK for mannone-rich polysaccharides. Inhibition of infectivity requires binding of the CRD to the hemagglutinin, which sterically interferes with the ability of IAV to bind to sialic acid receptors on host cells (37). On the other hand, SP-D-dependent uptake is greatly enhanced by viral aggregation, which normally requires cooperative interactions among trimeric subunits (14). We speculate that the higher intrinsic affinity of RAK (and CL-43) for highly mannosylated viral proteins permits individual CRDs of a trimer to participate in stable interactions with virions, permitting bridging interactions, even in the absence of gross viral aggregation. Given that AAA was more susceptible to degradation in crude bacterial lysates, it is also possible that it is more susceptible to degradation in association with viral uptake by neutrophils. Studies are planned to investigate these possibilities and to characterize the effects of RAK within the context of more highly oligomerized molecules.

The mechanism of enhanced binding of RAK and CL-43 to solid-phase polysaccharides is not fully understood. Interaction of rat MBL with mannone involves the vicinal 3- and 4-OH groups of the sugar; these groups directly coordinate with calcium and hydrogen-bond with residues that serve as calcium ligands (38). Similar interactions have been demonstrated for human SP-D complexed with maltose (28). We initially hypothesized that the insertion contributes to an accessory or secondary binding site. Such sites have been shown to contribute to the interactions of other C-type lectins with specific ligands (38). For example, basic accessory sites for the anionic ligand, sialyl-LewisX were identified in studies of selectin/mannose binding lectin chimeras (39), and there is evidence for extended or secondary oligosaccharide binding sites in the dendritic cell receptors, DC-SIGN and DC-SIGNR (40). Furthermore, crystallographic comparisons of the binding orientations of various saccharides to rat MBL-A and MBL-C have demonstrated that interactions with sequences outside of the primary binding site can influence the orientation of ligands at the primary site, thereby altering interactions with multivalent ligands (41). Last, computer docking studies predict the participation of both Asp125 (the first residue of the N-terminal motif of SP-D)
and Arg\textsuperscript{343} (the first residue of the C-terminal motif) in interactions of hSP-D with the terminal glucosyl residues of a glucosyl-trisaccharide (42). The importance of Arg\textsuperscript{343} in glucose recognition and in sterically limiting interactions with GlcNac was confirmed in a recent mutagenesis study (43).

Nevertheless, it seems unlikely that the RAK sequence contributes to a secondary binding site. Both RAK and AAA show increased efficiency of binding to mannan and similar changes in relative saccharide preferences, with increased apparent affinity for mannos. Thus, the effects of RAK on carbohydrate recognition must largely result from conformational perturbations resulting from the three-peptide bond insertion rather than specific biochemical properties of the inserted amino acid sequence. Because RAK and AAA show greater maximal binding to mannan than NCRD, we infer that these mutants participate in additional modes of interaction with the adsorbed ligand. Given that mannan is a polysaccharide with three different patterns of glycosidic linkage (\(\alpha-1,6, \alpha-1,3, \) and \(\alpha-1,2\)), this might involve enhanced interactions with a distinct subpopulation of mannan chains and/or an increase in the number of potential binding orientations of the exposed polysaccharide chains. Such effects could involve the orientation of residues flanking the insertion. Although Asp\textsuperscript{324} coordinates with calcium ion 2, Asp\textsuperscript{325} closely approximates Glc1 in maltose (28), and preliminary model building suggests that the RAK insertion between Asp\textsuperscript{324} and Asp\textsuperscript{325} markedly alters the spatial distribution of Asp\textsuperscript{2}, deflecting it away from the primary carbohydrate binding site.\textsuperscript{2} It is problematic to accurately predict the conformational effects of insertions within loops, definitive assessment will probably require the characterisation of the crystal structure of RAK or CL-43 complexed with ligand.

The biological function and endogenous ligands of serum CL-43 are not known. However, based on solid-phase binding assays, it has been proposed that CL-43 contributes to host defense through interactions with high mannose sugars on complement proteins, particularly C3 (17). Although the CRDs of CL-43 and hSP-D show a number of conspicuous differences in primary sequence, our results suggest that the evolutionary acquisition of a three-residue insertion in trimeric CL-43 served to enhance interactions with surface arrays of mannose-containing glycoconjugates while limiting the aggregation mediated by multimers of trimeric subunits. In this regard, our experiments indicate that the RAK insertion contributes to one of the most intriguing host defense properties of CL-43, its unique capacity among tested natural collectins to increase the uptake of IAV by neutrophils in the absence of gross segregation of viral particles and without augmenting the release of potentially deleterious oxidants.

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