The Carbohydrate Recognition Domain of Surfactant Protein A Mediates Binding to the Major Surface Glycoprotein of *Pneumocystis carinii*†

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ABSTRACT: *Pneumocystis carinii* is a common cause of life-threatening pneumonia in immunodeficient patients. Pulmonary surfactant protein A (SP-A), an alveolar glycoprotein containing collagen-like and carbohydrate recognition domains (CRD), binds *P. carinii* and enhances adherence to alveolar macrophages. In this study, we examined the structural basis of the interaction between SP-A and the major surface glycoprotein of *P. carinii* (MSG). Rat SP-A bound to purified rat *P. carinii*-derived MSG in a saturable and calcium-dependent manner, which was partially reversible by coincubation with excess monosaccharides, or pretreatment of MSG with *N*-glycanase. Mutant recombinant SP-As with neutral amino acid substitutions for the predicted calcium- and carbohydrate-coordinating residues of the CRD were synthesized in insect cells using baculovirus vectors and tested for binding to MSG. Substitutions of negatively charged (Glu^{195}, Glu^{202}, and Asp^{215}) and polar residues (Asn^{214}) of the CRD with alanine but not substitution of the Arg^{197} with glycine reduced the binding of SP-A to mannose−Sepharose beads and to MSG. Deletion of the N-linked oligosaccharides from SP-A by mutagenesis of the consensus sequences for glycosylation had no effect on binding. We conclude that the CRD mediates the binding of SP-A to oligosaccharides attached to MSG.

Inhaled pathogens which reach the distal pulmonary airspaces encounter the host immune system in the milieu of the alveolar lining fluid. In addition to the immunoglobulins and inflammatory cells of classical immune defense, this compartment contains surfactant phospholipids and proteins which have been shown to have immunomodulatory properties (Pison et al., 1994). Recent attention has focused on the role of the most abundant surfactant protein, surfactant protein A (SP-A),[1] in the clearance of microorganisms from the alveolar space. Structural homologies identify SP-A as a member of the "collectin" protein family (Sastry & Ezekowitz, 1993), which also includes mannose-binding protein (Drickamer et al., 1986), bovine conglutinin (Lee et al., 1991), CL-43 (Lim et al., 1994), and surfactant protein D (Kuan et al., 1992). The collectins are thought to function as antibody-independent opsonins by pattern recognition-dependent binding to mannose-rich oligosaccharides on the surface of pathogens. SP-A binds to the surface of bacterial (McNeely & Coonrod, 1994; Tino & Wright, 1996; van Iwaarden et al., 1994), viral (Benne et al., 1995; van Iwaarden et al., 1991, 1992), and fungal (Schelenz et al., 1995) organisms and enhances microbial adherence and phagocytosis by macrophages (Gaynor et al., 1994; Pikaar et al., 1995).

Pneumocystis carinii is a ubiquitous, opportunistic pathogen which is most closely related to the fungi. Patients with the acquired immunodeficiency syndrome (AIDS) who are infected with *P. carinii* have 2−3-fold elevations of SP-A in bronchoalveolar lavage (Phelps & Rose, 1991; Sternberg et al., 1995). Human SP-A enhances the attachment of *P. carinii* to rat alveolar macrophages, suggesting an opsonic role for the protein (Williams et al., 1996). Immunosuppressed rats with *P. carinii* pneumonia also have elevated SP-A in lavage (Guo & Kaneshiro, 1995), and rat SP-A has been shown to bind to the organism (Zimmerman et al., 1992). The binding of SP-A to the *P. carinii* cell wall is mediated by an abundant protein species with an electrophoretic mobility consistent with the major surface glycoproteins (MSGs) (Zimmerman et al., 1992). The MSGs are a closely related family of polymorphic proteins, encoded by as many as 100 homologous but distinct genes, which are thought to function in host evasion (Kovacs et al., 1993; Lundgren et al., 1991). MSGs are modified with up to 9−10% N-linked oligosaccharides, on the basis of the increase in migration on SDS−PAGE after treatment with endoglycosidase F (Lundgren et al., 1991). Mannosylated albumin and α-methyl mannoside have been shown to block calcium-dependent binding of SP-A to whole *P. carinii* (Zimmerman et al., 1992), but a direct role for the oligosaccharides of MSG as ligands for SP-A has not been rigorously confirmed.

The domains of SP-A that mediate binding to MSG have not been directly examined. SP-A is an oligomer of nearly

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[1] Abbreviations: SP-A, surfactant protein A; MSG, major surface glycoprotein; CRD, carbohydrate recognition domain; MBP, mannose-binding protein; Sf-9, *Spodoptera frugiperda*; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunoadsorbent assay; Con A, Concanavalin A.

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identical subunits which contain four distinct structural domains (White et al., 1985): (1) a short NH_2-terminal globular segment, (2) a collagen-like domain of proline- and hydroxyproline-rich Gly-X-Y repeats, (3) a hydrophobic "neck" region, and (4) a COOH-terminal Ca^{2+}-dependent carbohydrate recognition domain (CRD) which bears extensive sequence homology to several mammalian lectins (Drickamer et al., 1986). Subunits of SP-A fold into trimers by the formation of collagen-like triple helices (King et al., 1989; Voss et al., 1988). The fully assembled molecule is a hexagonal array of trimers which are laterally associated through the first half of the collagen-like region and stabilized by inter- and intratrimeric bonds near the NH_2 terminus (Voss et al., 1988).

The purpose of this study was to explore the structural basis of the binding of SP-A to purified MSG. A panel of mutant recombinant SP-A proteins were developed by site-directed mutagenesis and tested for binding activity in a solid phase assay. We found that the CRD of SP-A binds to oligosaccharide domains attached to MSG.

EXPERIMENTAL PROCEDURES

DNA Constructs. The isolation and sequencing of the 1.6 kb cDNA for rat SP-A was previously reported (Sano et al., 1987). This insert was ligated into the unique *Eco*R1 site of the PVL 1392 vector (Invitrogen) (Luckow & Summers, 1988), and the proper orientation was confirmed by restriction mapping with *Kpn*I. Directed mutations were created in the cDNA for SP-A by the overlap extension technique (Horton et al., 1989), using mutagenic oligonucleotides and the polymerase chain reaction, as described (McCormack et al., 1994a). All mutant cDNAs were confirmed to contain the designed mutations and to be free of spurious mutations by DNA sequencing (Sanger et al., 1977).

Cell Culture. Sf-9 cells used for plaque assays and viral amplifications were maintained in 150 mL spinner cultures at 25−28 °C in an air atmosphere in media composed of IPL-41 insect culture media, tryptose phosphate broth, 0.1% pluronic (shear-reducing surfactant compound) (Sigma), antibiotics, and 10% fetal calf serum (McCormack et al., 1994a,b). *Trichoplusia ni* (*T. ni*) cells passaged in adherent cultures were used for the production of recombinant proteins.

Expression of the cDNAs for SP-A in T. ni Cells. The expression of recombinant proteins in the baculovirus system was performed as described (McCormack et al., 1994a,b; Sanger et al., 1977). Briefly, recombinant baculoviruses containing mutant cDNAs for SP-A were produced by homologous recombination in Sf-9 cells following contransfection with linear viral DNA and the recombinant PVL 1392−SP-A constructs (Baculogold, Pharmingen). Fresh monolayers of 10^7 *T. ni* cells were infected with plaque-purified recombinant viruses and incubated with serum-free media (IPL-41) supplemented with 0.4 mM ascorbic acid and antibiotics for 72 h. Recombinant SP-A was purified from the culture media by adsorption to mannose−Sepharose 6B columns in the presence of 1 mM calcium and elution with 2 mM EDTA (Fornstedt & Porath, 1975). The purified recombinant SP-A was dialyzed against 5 mM Tris (pH 7.4) and stored at −20 °C.

Purification/Modification of Native SP-A. Surfactant was isolated from Sprague-Dawley rats 4 weeks after the intratracheal instillation of 40 mg/(kg of silica) (Dethloff et al., 1986). SP-A was isolated from NaBr gradient-purified surfactant by delipidation, mannose−Sepharose affinity chromatography, and gel permeation chromatography with Biogel A-15m (Hawgood et al., 1985). The yield was approximately 500 μg of SP-A/(silica-treated rat), which is significantly enhanced over that from the untreated animal (approximately 20 μg of SP-A/rat).

Protein Assays. The SP-A content of tissue culture media containing recombinant SP-A was determined using a rabbit polyclonal IgG against rat SP-A in a sandwich ELISA (McCormack et al., 1990). The lower limit of sensitivity of the assay was 0.20 ng/mL, and the linear range extended from 0.16 to 10.0 ng/mL. Routine protein concentrations were determined with the bicinchoninic protein assay kit (BCA) (Pierce) using bovine serum albumin as a standard.

Analysis of SP-A and MSG. Protein species were separated by 8−16% SDS−PAGE and either stained with Coomassie blue or transferred to nitrocellulose membranes. For immunoblotting, the membranes were incubated either with horseradish peroxidase (HRP)-conjugated polyclonal anti-rat SP-A (McCormack et al., 1990) or with the 4E7 monoclonal anti-rat MSG antibody followed by HRP-conjugated anti-mouse IgG (Linke et al., 1994). For lectin blotting experiments, the membranes were incubated with biotinylated Concanavalin A (Con A), followed by incubation with avidin−HRP (Theus et al., 1993). Blots were developed by the HRP-dependent oxidation of *o*-phenylenediamine.

Isolation of Rat P. carinii and Purification of MSG. P. carinii organisms were isolated from the lungs of immunosuppressed rats after 8 weeks of corticosteroid treatment as described previously (Theus et al., 1993). Briefly, infected lungs were removed, minced in phosphate-buffered saline (PBS), and ground through a 60-mesh screen. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C, and the resulting pellet was treated with 0.85% ammonium chloride to lyse erythrocytes. The pellet was washed twice and resuspended in PBS. Aliquots of the homogenates were examined by Diff-quik stain (American Scientific Products) for quantitation of organisms (routinely over 10^9 *P. carnii*/rat). Preparations with evidence of bacterial or fungal contamination on Mueller-Hinton and Sabouraud dextrose plates were discarded.

MSG was purified from the *P. carinii* organisms by zymolyase digestion, differential centrifugation, and Con A−Sepharose 4B affinity column chromatography, as described previously (Linke & Walzer, 1991). MSG was deglycosylated with *N*-glycanase (Genzyme, Cambridge, MA) according to the manufacturer's instructions. Briefly, MSG was electroeluted from SDS−PAGE gels and boiled in 0.5% SDS and 50 mM 2-mercaptoethanol for 5 min. The MSG was incubated overnight in the presence or absence (sham) of *N*-glycanase and 1.5% NP-40 and purified by Sephadex G-50 column chromatography and dialysis.

Binding of Mutant SP-A to Carbohydrate-Linked Beads. The carbohydrate binding activities of recombinant proteins with mutations in the CRD were examined by competition binding for mannose−Sepharose beads. Nonspecific binding sites were blocked by preincubation of the beads with binding buffer containing 50 mM Tris, 2 mM Ca^{2+}, 100 mM NaCl, and 1% albumin. Variant SP-As (200 ng) were incubated with 100 μL of mannose−Sepharose beads (50 μL bed volume) in the presence of 0−2 M mannose at 4 °C for 1 h in binding buffer. The mixture was centrifuged at 1000*g*,

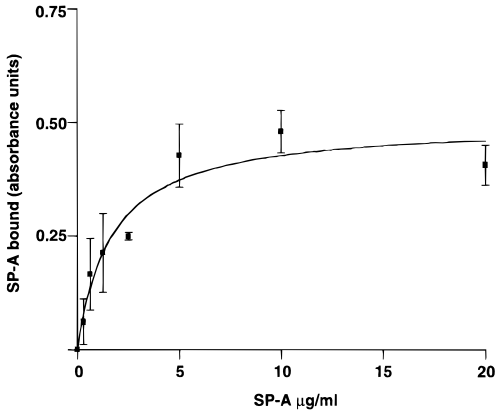

FIGURE 1: Specific binding of rat SP-A to whole *P. carinii* organisms adsorbed to microtiter plates. Rat-derived *P. carinii* organisms were adsorbed to microtiter plates (10^5 per well) as outlined in Experimental Procedures. Varying concentrations of rat SP-A were added, and binding was quantified with an HRP-conjugated polyclonal anti-SP-A antibody and peroxidase-dependent oxidation of *o*-phenylenediamine. Nonspecific binding was determined by substitution of EDTA for Ca^{2+} in the binding buffer. Data are the mean ± SE of 3 separate experiments.

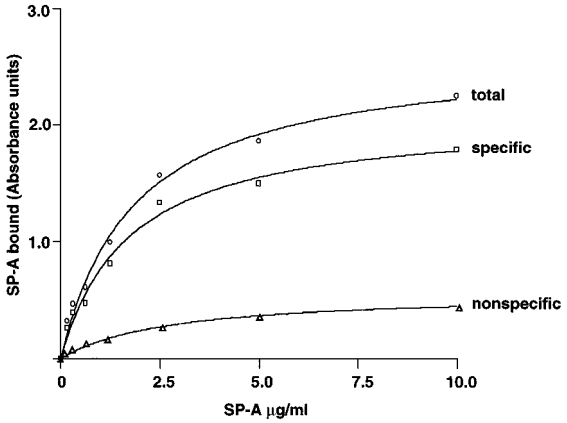

FIGURE 2: Direct binding of rat SP-A to purified MSG on microtiter plates. MSG purified from rat *P. carinii* (10 μg/mL) was adsorbed to microtiter plates. Varying concentrations of SP-A were incubated with the well overnight. After washing, binding was quantified with an HRP-conjugated anti-SP-A antibody and peroxidase-dependent oxidation of *o*-phenylenediamine. Nonspecific binding was determined by substitution of EDTA for Ca^{2+} in the binding buffer. Data are from one representative experiment of three separate experiments.

the supernatant harvested, and the pellet washed twice with ice-cold binding buffer. The pellet was transferred to a new tube and eluted with 5 mM EDTA. SP-A in the (supernatant plus wash) fractions and the pellet were separately quantified by ELISA. For determination of nonspecific binding, EDTA was substituted for Ca^{2+} in the binding buffer.

ELISA for Binding of SP-A to Rat P. carinii-Derived MSG or Whole Rat P. carinii Organisms. Purified MSG (10 μg/mL) or EDTA-washed *P. carinii* (10^5/well) were adsorbed to Immulon 1 microtiter plates (Dynatech) overnight in the presence of 0.1 M $NaHCO_3$ (pH 8.1). The plates were washed three times with 10 mM Tris containing 2 mM $CaCl_2$, 50 mM NaCl, 3% bovine serum albumin, and 0.1% Triton X-100 (buffer A), and nonspecific sites were blocked for 30 min with the same buffer. SP-A at various concentrations in buffer A or buffer B (same as buffer A except 2 mM EDTA was substituted for $CaCl_2$) was added to the wells and incubated overnight at 4 °C. In some experiments, 0−2 M mannose or galactose was added as a competitor. The plates were washed three times with the appropriate buffer and incubated for 2 h with 15 μg/mL HRP-linked polyclonal anti-rat SP-A antibody. After washing, *o*-phenylenediamine (1 mg/mL) was added in 0.1 M citrate buffer containing 0.03% H_2O_2 and color development was read at 490 nm in a spectrophotometer. Specific binding was determined by subtracting the binding which occurred in the presence of EDTA from total binding.

Data Analysis. Binding curves were computer fitted using a hyperbolic equation for one-site binding ($Y = B_{max}X/K_d + X$) (Prism, GraphPad Inc.). Differences in the extent of binding between the wild type recombinant and mutant proteins were compared using the one-tailed *t* test. Statistical significance was accepted for $p < 0.01$.

RESULTS

Direct Binding of SP-A to P. carinii. Direct binding of SP-A to rat *P. carinii* was assessed in a solid phase assay as outlined in Experimental Procedures, and the results are shown in Figure 1. The binding of rat SP-A to whole *P. carinii* organisms was calcium-dependent and saturable. Binding was half-maximal (K_d) at an SP-A concentration of 1.68 ± 0.53 μg/mL (2.58 ± 0.82 nM, based on a rat SP-A mass of 650 kDa) and reached a plateau at approximately 10 μg/mL SP-A. Nonspecific binding, defined as binding which occurred in the presence of EDTA, was less than 15% of the total binding throughout the range of concentrations tested. These results demonstrate that rat SP-A binds specifically to the surface of rat *P. carinii* adsorbed to microtiter plates.

Direct binding of SP-A to purified MSG was also determined, and the results are shown in Figure 2. MSG was isolated from rat *P. carinii* by zymolyase digestion and lectin affinity chromatography and adsorbed to microtiter plates. The binding of rat SP-A to purified MSG was calcium-dependent and saturable. Binding was half-maximal at an SP-A concentration of 1.70 ± 0.26 μg/mL (2.62 ± 0.40 nM), and nonspecific binding was less than 15% of the total binding at each concentration tested. Mannose or galactose has previously been shown to compete for binding of SP-A to polyvalent carbohydrate ligands (Haagsman et al., 1987; Haurum et al., 1993). As shown in Figure 3, the binding of SP-A (1.0 μg/mL) to solid phase MSG was inhibited in a dose-dependent manner by excess free monosaccharides. The EC_{50} was 244 mM for mannose (95% CI = 71.9−826.6, $r^2 = 0.954$) and 544 mM for galactose (95% CI = 113−2623, $r^2 = 0.948$). Collectively, these results demonstrate that SP-A binds to MSG in a specific manner which is reversible with excess carbohydrates.

To further investigate the role of the carbohydrate domains of MSG as ligands for SP-A, we performed binding analyses after partial removal of N-linked carbohydrates of MSG with *N*-glycanase. First, the efficiency of the enzymatic deglycosylation of MSG was assessed using SDS−PAGE and Con A lectin blotting (Figure 4). Under reducing conditions, sham-treated MSG migrates as a broad band with a molecular mass of 116 kDa and a minor band at 80 kDa, as described (Figure 4A, lane 1) (Lundgren et al., 1991). Treatment with *N*-glycanase resulted in an increase in the migration of the higher-molecular mass species (Figure 4A, lane 2), indicating liberation of N-linked oligosaccharides. Ligand blotting with

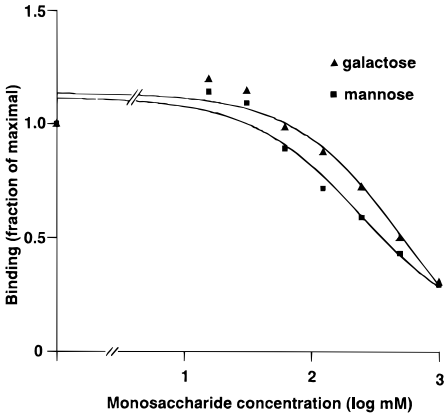

FIGURE 3: Soluble MSG and saccharide competition for binding to MSG on microtiter plates. MSG adsorbed to microtiter plates (10 μg/mL) was incubated with 1 μg/mL SP-A and 0−1000 mM galactose or mannose. Binding was quantified with an HRP-conjugated anti-rat SP-A antibody and peroxidase-dependent oxidation of *o*-phenylenediamine. Data are from one representative experiment of three separate experiments.

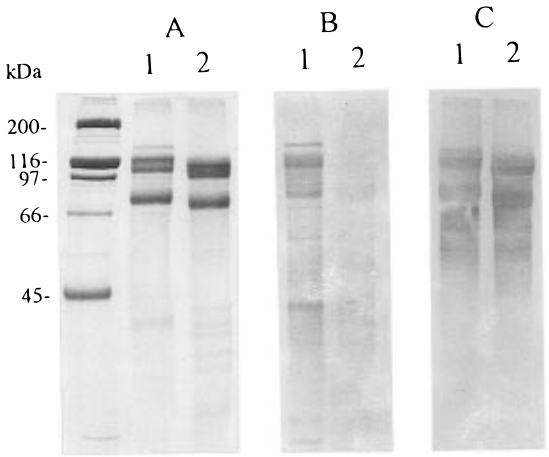

FIGURE 4: Deglycosylation of MSG with *N*-glycanase. MSG derived from rat *P. carinii* was incubated in the absence (lane 1) or presence of *N*-glycanase (lane 2) and then subjected to 8−16% SDS−PAGE (A). Proteins were then transferred to nitrocellulose and reacted with either Con A−biotin followed by avidin−HRP (B) or a monoclonal antibody to MSG followed by HRP-conjugated anti-mouse IgG (C). Blots were developed using 3,3′-diaminobenzidine as the substrate.

HRP-linked Concanavalin A demonstrated reduced reactivity with the *N*-glycanase-treated MSG (Figure 4B, lane 2) relative to that with the sham-treated MSG (Figure 4B, lane 1), confirming the reduction in protein-associated carbohydrate. The protein was not degraded by the enzyme treatment, since the immunoreactivity of the MSG with the anti-MSG monoclonal antibody was not affected by the *N*-glycanase treatment (Figure 4C).

Direct binding of SP-A to *N*-glycanase-treated MSG was then compared to that to sham-treated MSG, and the results are shown in Figure 5. SP-A bound to deglycosylated MSG in a dose-dependent manner that was less than binding to sham-treated MSG throughout the range of SP-A concentrations tested. The K_d for SP-A binding to sham-treated MSG was 1.23 ± 0.14 μg/mL (1.89 ± 0.22 nM), and for deglycosylated MSG, the K_d was 0.51 ± 0.06 μg/mL (0.78 ± 0.09 nM). At 10 μg/mL added, the binding of SP-A to deglycosylated MSG (0.73 OD unit) was 66.8% of the binding to sham-treated MSG (1.134 OD units). The reduced SP-A binding was not attributable to less efficient adsorption

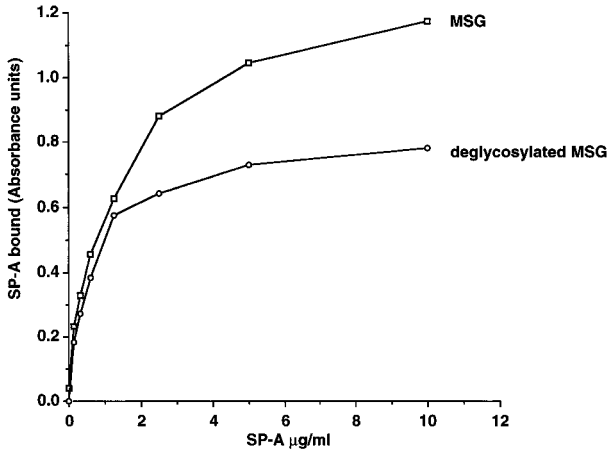

FIGURE 5: Binding of SP-A to *N*-glycanase-treated MSG. MSG and deglycosylated MSG (10 μg/mL each) were adsorbed to microtiter plates. Determination of bound SP-A was conducted as in Figure 2. Data are from one representative experiment of three separate experiments.

of the *N*-glycanase-treated MSG substrate to wells, since 1.6-fold more immunoreactive MSG bound to the plastic after glycosidase treatment (data not shown). These data indicate that N-linked carbohydrates of MSG participate in the calcium-dependent binding of SP-A to MSG. The residual SP-A binding observed after glycosidase treatment may be mediated by *N*-glycanase resistant carbohydrate moieties or noncarbohydrate domains of the protein.

Determination of MSG Binding Domains of SP-A Using Recombinant Proteins. Mutant recombinant forms of SP-A were generated by oligonucleotide-directed mutagenesis of the cDNA for SP-A and expression in insect cells using baculovirus vectors. We have reported the characterization of several SP-As synthesized using this system and have noted that hydroxylation of prolines within the collagen-like region is incomplete (denoted "hyp") (McCormack et al., 1994a,b). The SP-A^{hyp} ("wild type" recombinant SP-A) has measurable functional activities which are comparable to that of rat SP-A in assays of ligand binding, regulation of surfactant secretion, and uptake by alveolar type II cells (McCormack et al., 1994a). The average oligomeric mass of SP-A^{hyp} is greater than 500 kDa on the basis of migration on gel exclusion columns, consistent with the association of more than 10 subunits (McCormack et al., 1997).

Carbohydrate Recognition Domain of SP-A. The role of the CRD in the binding of SP-A to MSG was determined by substitution of the putative calcium- and carbohydrate-coordinating residues of the domain with alanine or glycine. The mutant proteins which were synthesized included SP-A^{hyp,E195A} (substitution of Glu^{195} by Ala), SP-A^{hyp,R197G} (substitution of Arg^{197} by Gly), SP-A^{hyp,E202A} (substitution of Glu^{202} by Ala), SP-A^{hyp,N214A} (substitution of Asn^{214} by Ala), and SP-A^{hyp,D215A} (substitution of Asp^{215} by Ala). Between 3 and 5 mg of each protein was purified from the culture media of recombinant baculovirus-infected cells by calcium-dependent adsorption to mannose−Sepharose affinity columns, indicating at least partial retention of the carbohydrate binding activity. The average yield per 100 mL of media harvested from cultures of 10^8 *T. ni* cells for SP-A^{hyp} = 1.45 ± 0.30 mg, SP-A^{hyp,E195A} = 1.68 ± 0.58 mg, SP-A^{hyp,R197G} = 1.43 ± 0.08 mg, SP-A^{hyp,E202A} = 0.88 ± 0.08 mg, SP-A^{hyp,N214A} = 1.36 ± 0.41 mg, and SP-A^{hyp,D215A} = 0.56 ± 0.05 mg. The electrophoretic analyses

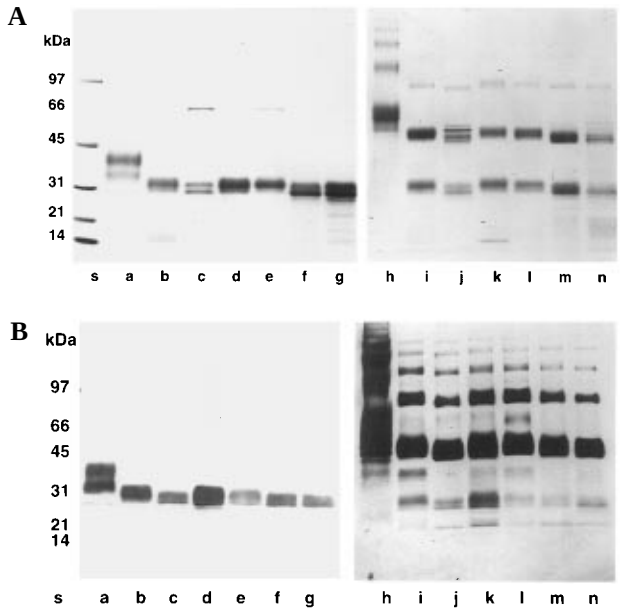

FIGURE 6: Electrophoretic and immunoblot analyses of recombinant SP-As. Proteins were separated on 8−16% SDS−PAGE gels under reducing (a−g) and nonreducing (h−n) conditions (panel A). Following transfer to nitrocellulose, the membrane was reacted with HRP-linked polyclonal anti-rat SP-A IgG and developed by HRP-dependent oxidation of *o*-phenylenediamine (panel B). Rat SP-A (a and h), SP-A^{hyp} (b and i), SP-$A^{hyp,E195A}$ (c and j), SP-$A^{hyp,R197G}$ (d and k), SP-$A^{hyp,E202A}$ (e and l), SP-$A^{hyp,N214A}$ (f and m), SP-$A^{hyp,D215a}$ (g and n), and standards (s) are shown.

of the native rat and recombinant SP-As are shown in Figure 6A. Under reducing conditions, rat SP-A migrated as a triplet at 26, 32, and 38 kDa, as described (McCormack et al., 1994a). The heterogeneity in molecular mass is due to differential glycosylation at the Asn^{1} and Asn^{187} consensus sites for oligosaccharide attachment (McCormack et al., 1994a). The simple pattern of glycosylation characteristic of invertebrate cells resulted in slightly faster migration of SP-A^{hyp}, which appeared as a broad band between 27 and 32 kDa, as described (McCormack et al., 1994a). The migrations of SP-$A^{hyp,E195A}$, SP-$A^{hyp,R197G}$, SP-$A^{hyp,E202A}$, SP-$A^{hyp,N214A}$, and SP-$A^{hyp,D215A}$ were similar or indistinguishable from that of the wild type recombinant protein SP-A^{hyp}. Under nonreducing conditions, rat SP-A migrated as a dimer and a series of disulfide-dependent oligomers. The SP-A^{hyp} and the mutant SP-As appeared as monomers and faint higher-order species. Larger multimeric species were most clearly apparent after transfer to nitrocellulose membranes and reaction with the HRP-conjugated polyclonal anti-SP-A antibody (Figure 6B). The immunoblot also demonstrated that the immunoreactivities of the individual mutant SP-As were comparable to that of SP-A^{hyp} and to each other.

Relative binding activities of the mutant proteins for carbohydrate-linked beads were compared using a competitive binding assay, and the results are shown in Figure 7. Variant SP-As (200 ng) were incubated with mannose-conjugated Sepharose beads and 0−2 M mannose for 1 h at 4 °C. Following centrifugation, SP-A was measured separately in the pellet and supernatant fractions, and the percent of SP-A bound was determined according to the equation $SP\text{-}A_{pellet}/(SP\text{-}A_{pellet} + SP\text{-}A_{supernatant})$. Nonspecific binding which occurred in the presence of EDTA was subtracted from the total binding. In the absence of free mannose, the rank order of binding to the beads was SP-$A^{hyp,R197G}$ (9.8 ± 1.2%) > SP-A^{hyp} (9.3 ± 0.2%) > SP-$A^{hyp,E202A}$ (5.3 ± 0.9%) > SP-$A^{hyp,N214A}$ (7.8 ± 0.7%) > SP-$A^{hyp,D215A}$ (2.6 ± 0.1%) > SP-$A^{hyp,E195A}$ (2.3 ± 0.9%). To demonstrate that the association of SP-A with the beads was carbohydrate-dependent, binding was also analyzed in the presence of increasing concentrations of the monosaccharide competitor. At the highest concentration of free mannose tested, the binding of the SP-A^{hyp} was inhibited to 2.8 ± 1.6% and the binding of all mutant SP-As was inhibited to less than 2% of the added protein. These results indicate that Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215} are important for the binding of SP-A to carbohydrate while Arg^{197} does not appear to play a major role.

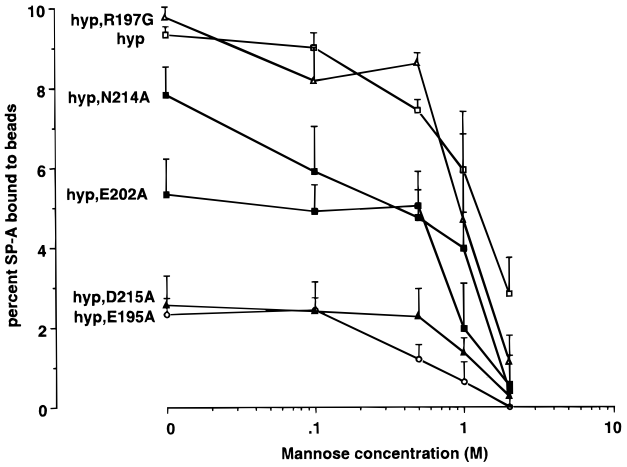

FIGURE 7: Monosaccharide competition for binding of mutant recombinant proteins to mannose−Sepharose beads. Mannose−Sepharose beads (50 μL bed volume) were incubated with 200 ng aliquots of SP-A^{hyp}, SP-$A^{hyp,E195A}$, SP-$A^{hyp,R197G}$, SP-$A^{hyp,E202A}$, SP-$A^{hyp,N214A}$, SP-$A^{hyp,D215A}$, and free mannose at varying concentrations (0−2 M) for 1 h at 4 °C. After centrifugation, the percent of SP-A bound was determined by ELISA of supernatant and pellet fractions. Data are the mean ± se of three experiments.

The recombinant proteins were then tested for binding to MSG adsorbed to microtiter plates (Figure 8). The SP-A^{hyp} bound to MSG in a dose- and calcium-dependent fashion that was similar to that for rat SP-A, with a K_d of 1.35 ± 0.48 μg/mL (2.08 ± 0.74 nM, based on an approximate SP-A mass of 500 kDa). The mutant forms of SP-A generally manifested an increased or decreased total binding to MSG, rather than an altered affinity. For each mutant protein, comparisons with SP-A^{hyp} were therefore based on the total binding and the dose dependence of binding was determined to ensure that all values were obtained from saturated portions of the binding curve. The SP-$A^{hyp,E195Q,R197D}$ was analyzed first, since prior analyses had shown that this mutant protein retained carbohydrate binding activity (McCormack et al., 1994b). The binding of SP-$A^{hyp,E195Q,R197D}$ was specific and saturable and was very similar to the binding of SP-A^{hyp} (Figure 8A). At 20 μg/mL, the amount of SP-$A^{hyp,E195Q,R197D}$ bound was 135 ± 25% of SP-A^{hyp} ($n = 3$, p = ns) (Figure 8B). These results illustrate that the CRD of SP-A can tolerate rather extreme mutations involving charge change without a loss of MSG binding activity. To separately assess the contribution of the 195 and 197 amino acid positions to carbohydrate binding, we characterized mutants in which Glu^{195} and Arg^{197} were individually substituted with alanine and glycine, respectively. The binding of SP-$A^{hyp,E195A}$ to MSG was reduced compared to that of SP-A^{hyp}, representing only 21.8 ± 7.6% of the SP-

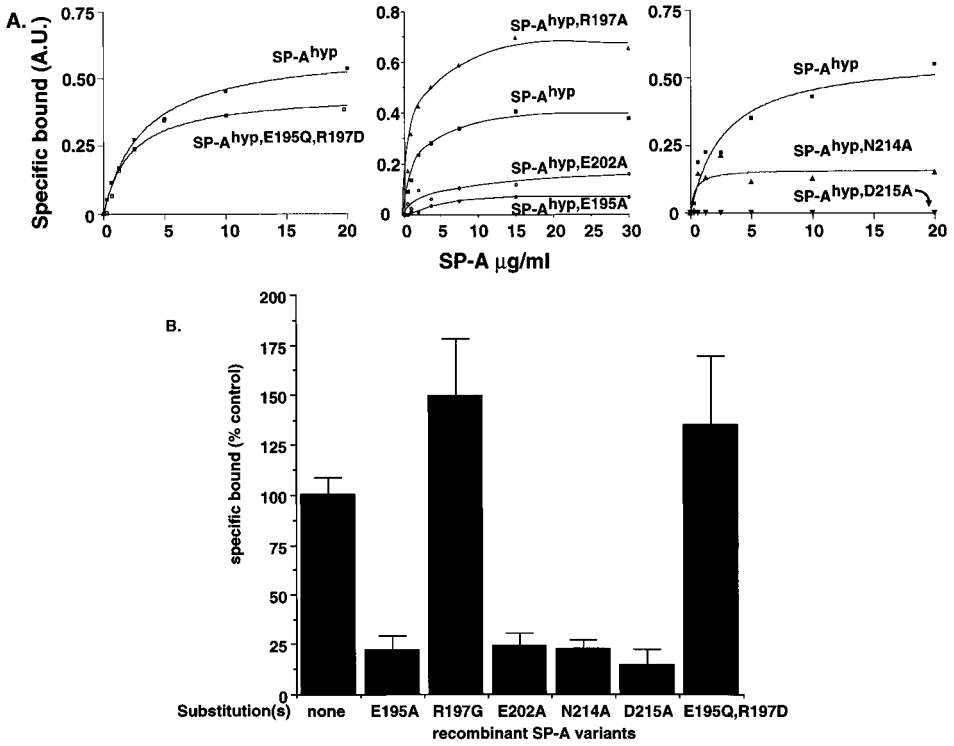

FIGURE 8: Direct binding of mutant recombinant SP-A to solid phase MSG. MSG purified from rat *P. carinii* (10 μg/mL) was adsorbed to microtiter plates. Wild type recombinant SP-A^{hyp} and the CRD mutant recombinants SP-A^{hyp,E195A}, SP-A^{hyp,R197G}, SP-A^{hyp,E202A}, SP-A^{hyp,N214A}, SP-A^{hyp,D215A}, and SP-A^{hyp,E195Q,R197D} were added to the wells, and binding was determined as in Figure 2. Dose-dependent specific binding of each mutant was compared with the SP-A^{hyp} (A). Data are from one representative experiment of three experiments. Specific binding of each mutant was also compared at saturating concentrations (20 μg/mL), expressed as the percentage of SP-A^{hyp} binding (B). Data in panel B are the mean ± se from three or four experiments.

A^{hyp} bound at the highest concentration tested ($p < 0.01$) (Figure 8B). In contrast, the binding of SP-A^{hyp,R197G} was greater than that of SP-A^{hyp}, reaching 149 ± 29% of SP-A^{hyp} at 20 μg/mL ($p < 0.01$) (Figure 8B). These data indicate that Glu^{195} but not Arg^{197} is essential for binding to MSG. The substitution of glutamic acid at position 202 by alanine also blocked binding (Figure 8A). The amount of SP-A^{hyp,E202A} bound to MSG at the highest concentration of SP-A tested (20 μg/mL) was only 23.7 ± 7.3% of SP-A^{hyp} ($p < 0.01$) (Figure 8B). Finally, the motif tryptophan-asparagine-aspartic acid (W-N-D) is present near the COOH-terminal end of the CRD of most of the reported C-type lectins (Drickamer, 1988). Alanine substitutions for the conserved asparagine at position 214 and aspartic acid at position 215 also blocked binding of SP-A to MSG (Figure 8A). Total binding of SP-A^{hyp,N214A} and SP-A^{hyp,D215A} to MSG was only 22.5 ± 4.7% ($p < 0.01$) and 14.9 ± 7.9% ($p < 0.01$) of SP-A^{hyp} at 20 μg/mL, respectively (Figure 8B). Collectively, these data provide strong evidence that the CRD of SP-A mediates binding to MSG.

Asparagine-Linked Oligosaccharides of SP-A. The N-linked oligosaccharides of SP-A have been reported to mediate binding to some microorganisms (van Iwaarden et al., 1992). To explore the role of carbohydrate domains of SP-A in the attachment to *P. carinii*, we tested the binding of a nonglycosylated recombinant SP-A to MSG adsorbed to microtiter plates. N-linked oligosaccharide attachment to SP-A was prevented by Asn^{1} → Thr and Asn^{187} → Ser amino acid substitutions in the consensus sequences for glycosylation by site-directed mutagenesis, as described previously (McCormack et al., 1994a). The SP-A^{hyp,N1T,N187S} comigrated as a tight band with SP-A^{hyp,TM} (recombinant SP-A synthesized in the presence of tunicamycin) on reducing SDS−PAGE gels, consistent with the absence of N-linked oligosaccharides (McCormack et al., 1994a). The binding of SP-A^{hyp,N1T,N187S} was very similar to that for SP-A^{hyp} throughout the range of SP-A concentrations tested (Figure 9A). At 20 μg/mL, binding of SP-A^{hyp,N1T,N187S} was 86.0 ± 14.1% of that of SP-A^{hyp} ($n = 3$, $p =$ ns) (Figure 9B). These data indicate that the N-linked oligosaccharides of SP-A do not play a major role in the attachment to MSG.

DISCUSSION

The purpose of this study was to examine the structural basis of the binding of SP-A to MSG. SP-A had previously been shown to bind to the surface of *P. carinii*, and ligand immunoblots of whole organism membrane preparations identified MSG as the putative binding site (Zimmerman et al., 1992). In this study, we used purified MSG as the substrate for binding reactions and mutant SP-As to dissect the protein domains which mediate the binding interaction. We found that SP-A binds to MSG via the carbohydrate recognition domain, at least in part through binding to N-linked carbohydrates of MSG.

We found that the binding of rat SP-A to both *P. carinii* and MSG was saturable and Ca^{2+}-dependent, suggesting that binding occurs through a specific mechanism which is similar for the whole organism and the purified protein. The binding of SP-A to MSG was at least partially carbohydrate-dependent, since the interaction was inhibited by coincubation with monosaccharides or by pretreatment of MSG with *N*-glycanase. These results provided evidence that the carbohydrate moiety of MSG is an important ligand for SP-A

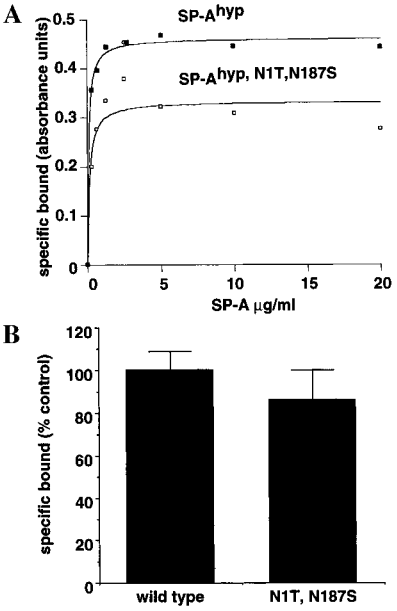

FIGURE 9: Role of the attached oligosaccharides of SP-A in the binding to solid phase MSG. Recombinant SP-A with mutations that prevented the attachment of N-linked oligosaccharides (SP-$A^{hyp,N1T,N187S}$) was incubated with MSG-coated microtiter plates. Binding was determined as in Figure 2. Dose-dependent specific binding of each mutant was compared with the wild type recombinant SP-A^{hyp} (A). Data are a representative experiment of three experiments. Specific binding of the nonglycosylated mutant was also compared at saturating concentrations of SP-A (20 μg/mL), expressed as the percentage of SP-A^{hyp} binding (B). Data in panel B are the mean $\pm$ se for three or four experiments.

and a rationale for the use of mutant forms of SP-A to address the structural basis of the interaction.

The logical focus of the mutagenesis studies was the carbohydrate binding site of the CRD of SP-A. The CRD is composed of 114 amino acids, including 18 residues which are highly conserved and 14 residues which are invariant among the C type lectins (Drickamer, 1988). The crystal structure of SP-A has not been solved, and the identities of the amino acids of SP-A which mediate binding to carbohydrate are not known. Candidate ligand binding residues were modeled on the basis of the crystal structure of the homologous lectin mannose-binding protein A (MBP) complexed with an oligosaccharide (Drickamer, 1992; Weis et al., 1992). There are five amino acids near the short disulfide loop of SP-A which correspond to the conserved carbohydrate coordination site of MBP: Glu^{195}, Arg^{197}, Glu^{202}, Asn^{214}, and Asp^{215}. We had previously shown that the tandem substitutions $Glu^{195} \rightarrow$ Gln and $Arg^{197} \rightarrow$ Asp within the CRD (SP-$A^{hyp,E195Q,R197D}$) converted the carbohydrate ligand preference of SP-A so it favored mannose over galactose (McCormack et al., 1994a), confirming that this region is important for carbohydrate binding by SP-A. In the present study, the individual contribution of the five targeted amino acids of the CRD to the binding of SP-A to carbohydrates and MSG was determined by substitutions of the five residues with alanine or glycine. The small, neutral side chains of these amino acids do not support hydrogen bonding or charged interactions with carbohydrate ligands. The alanine and glycine mutations had little or no effect on the migration of the proteins on SDS−PAGE gels or the recognition of the proteins by the polyclonal anti-rat SP-A antibody. The alanine substitutions for Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215} but not the glycine substitution for Arg^{197} reduced the binding of SP-A to the mannose−Sepharose beads. It is not surprising that mutation R197A had little effect on carbohydrate binding since this residue is poorly conserved in SP-As of various species and is occupied by alanine in the human type (White et al., 1985). These data confirmed that the Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215} residues of SP-A are essential for the binding of SP-A to carbohydrate. The alanine-substituted mutant proteins were then used as probes to determine the role of the CRD of SP-A in the binding to MSG. Each of the mutations which inhibited carbohydrate binding to the mannose−Sepharose beads also blocked the binding to MSG. The R197G mutation did not block binding to MSG, and in fact slightly enhanced binding. The SP-$A^{hyp,E195Q,R197D}$ bound to MSG to nearly the same extent as SP-A^{hyp}, despite the previously demonstrated alteration in carbohydrate binding specificity and reduced affinity for the SP-A receptor on type II cells (McCormack et al., 1994b). This result suggests that the structural requirements for the binding of SP-A to MSG are less stringent than for the binding of SP-A to the type II cell receptor. The ability to increase and decrease the binding affinity of SP-A for MSG by single amino acid substitutions in this narrowly defined region indicates that the CRD mediates the binding of SP-A to MSG. In addition, the concordance between the mutations which block carbohydrate binding and those which reduce binding to MSG provides further evidence that binding to MSG is carbohydrate-dependent.

It is not possible to conclude that the specific residues which were targeted play a direct role in carbohydrate binding, since comparison with the structure of MBP predicts that each of the amino acids also participates in the coordination of calcium. The binding of calcium to SP-A has been shown to produce conformational shifts, which may be important for binding to lipid and carbohydrate ligands (Haagsman et al., 1990). The colocalization of the calcium binding site and the carbohdyrate binding site may explain the finding that some mutations altered total binding rather than binding affinity. We speculate that amino acid substitutions which weaken the calcium binding site perturb the equilibrium between binding and nonbinding conformations of the protein.

A second domain of SP-A that has been implicated in the clearance of microorganisms is composed of the N-linked oligosaccharides of the protein, which have been shown to recognize viruses (Benne et al., 1995; van Iwaarden et al., 1992) and to enhance adherence of *Mycobacterium tuberculosis* to macrophages (Gaynor et al., 1994). In the present study, the deletion of the oligosaccharide domains of the protein by genetic alteration of the consensus sequences for glycosylation had little or no effect on the binding to MSG. These results indicate that the oligosaccharides of SP-A are not required for the binding of SP-A to MSG.

In summary, we have found that SP-A binds specifically to the surface of whole *P. carinii* organisms and to MSG purified from rat *P. carinii*. The binding of SP-A to MSG is inhibited by excess monosaccharides and by removal of N-linked carbohydrates from MSG, indicating a carbohydrate-dependent mechanism. This study provides direct evidence that binding to MSG is mediated by the CRD of SP-A, since several amino acid substitutions within that domain block the interaction between the proteins. A lectin−carbohydrate is further supported by the finding that amino acid substitutions which block SP-A binding to mannose−Sepharose

beads are identical to those that block binding to MSG. The oligosaccharide domains of SP-A do not play an important role in the ligation of MSG.

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