Analysis of Chimeric Proteins Identifies the Regions in the Carbohydrate Recognition Domains of Rat Lung Collectins That Are Essential for Interactions with Phospholipids, Glycolipids, and Alveolar Type II Cells*

Hitomi Sano, Yoshio Kuroki†, Toshio Honma, Yoshinori Ogasawara, Hitoshi Sohma, Dennis R. Voelkers, and Toyoaki Akino

From the Department of Biochemistry, Sapporo Medical University School of Medicine, Sapporo 060, Japan and the §Department of Medicine, National Jewish Medical and Research Center, Denver, Colorado 80206

Pulmonary surfactant proteins A (SP-A) and D (SP-D) are collectins in the C-type lectin superfamily. SP-A binds to dipalmitoylphosphatidylcholine and galactosylceramide, and it regulates the uptake and secretion of surfactant lipids by alveolar type II cells. In contrast, SP-D binds to phosphatidylinositol (PI) and glucosylceramide (GlcCer). We investigated the functional region in the carbohydrate recognition domain of rat SP-A and SP-D that is involved in binding lipids and interacting with alveolar type II cells by using chimeric proteins. Chimeras ad3, ad4, and ad5 were constructed with SP-A/SP-D splice junctions at Gly194/Glu321, Gln173/Thr300, and Met134/Cys261, respectively. All three chimeras lost SP-A-specific functions. Chimeras ad3, ad4, and ad5 bound to PI with increasing activity. In contrast, chimeras ad3 and ad4 did not bind to GlcCer, whereas ad5 avidly bound this lipid. From these results, we conclude that 1) the SP-A region of Glu195–Phe228 is required for lipid and type II cell interactions, 2) the SP-D region of Cys261–Phe355 is required for optimal lipid interactions, and 3) the structural requirement for the binding of SP-D to PI is different from that for GlcCer.

Pulmonary surfactant is a complex mixture of lipids and proteins that acts to keep alveoli from collapsing during expiration (1). Surfactant protein A (SP-A) is the most abundant hydrophilic surfactant protein (2). SP-A binds to dipalmitoylphosphatidylcholine (DPPC) (3), a lipid component essential for the biophysical function of surfactant, and initiates aggregation of liposomes containing DPPC in the presence of calcium (4). SP-A also binds to the glycosphingolipids galactosylceramide (GalCer), lactosylceramide, and asialo-gangliotriaosylceramide (5, 6). The specific interaction of SP-A with alveolar type II cells has also been documented. SP-A inhibits the secretion of surfactant phospholipids (7, 8) and enhances the uptake of liposomes by type II cells (9). In addition, SP-A binds to alveolar type II cells with high affinity (10, 11), and a receptor binding activity correlates well with the inhibitory effect of SP-A on lipid secretion (12).

The in vitro functions of SP-A have all of the characteristics expected of a regulatory protein involved in surfactant homeostasis within the alveolar compartment of the lung. However, mouse strains with null alleles for SP-A are viable and appear to have only modest alterations in surfactant properties (13). The possibility of functional redundancy between SP-A and other molecules remains to be elucidated. In addition to its interaction with lipid and type II cell ligands, SP-A interacts with macrophages (14) and a large spectrum of microorganisms, including viruses (15) and Pneumocystis carinii (16), and is now thought to be an important component of the innate (antibody-independent) immune system of the lung. SP-A-deficient mice have been shown to be susceptible to group B streptococcal infection (17). A clear understanding of the interaction of SP-A with its multiple ligands is likely to provide important insight into its physiological roles in surfactant dynamics and host defense.

Surfactant protein D (SP-D) is also a hydrophilic protein of the extracellular alveolar compartment produced by type II cells (18, 19). It has a molecular mass of 43 kDa under denaturing and reducing conditions (20). SP-D also binds specific lipids, but its specificity is distinct from that of SP-A. SP-D binds phosphatidylinositol (PI) (21, 22) and glucosylceramide (GlcCer) (23). The role of SP-D in lipid metabolism in the surfactant system has not yet been clearly defined. A growing body of evidence also implicates SP-D as an important element of the innate immune system of the lung. The protein binds viruses (24), bacteria, fungi (25), and P. carinii (26). SP-D also binds to different classes of molecules on macrophages. One of the SP-D-binding proteins found on the surface of alveolar macrophages has been purified (27). A clear definition of the ligands for SP-D and their binding sites on the protein is essential for understanding how this protein functions within the alveolar compartment.

SP-A and SP-D belong to the collectin subgroup of the C-type lectin superfamily, along with mannose-binding protein (MBP), bovine conglutinin, and the protein CL43 (28). These proteins possess characteristic primary structures that are organized into four domains: 1) a cysteine-containing amino terminus, 2) a collagen-like domain, 3) a neck domain, and 4) a carbohydrate recognition domain (CRD) (2). SP-D forms a bouquet-like structure consisting of an octadecamer composed of six trimeric subunits (29), whereas SP-D is a dodecamer that forms a cru-
ciform structure (30). Although lung collectins possess homologous structures and exhibit similar carbohydrate binding specificities, they show quite different properties of binding lipids and interacting with alveolar type II cells. Previous studies have suggested that the CRD of SP-A or SP-D plays a critical role in interactions with lipids and type II cells (31–34). However, the regions of the CRDs that are essential for lipid binding and type II cell interactions have not yet been fully identified. The purpose of this study was to investigate the functional region in the CRD of rat SP-A or SP-D for the interactions with lipids and type II cells by using chimeric proteins of SP-A and SP-D. We constructed SP-A/SP-D chimeras in which progressively longer carboxyl-terminal regions of SP-D were substituted for the corresponding SP-A regions and examined whether these chimeras retained the SP-A functions or acquired the SP-D functions. We also determined whether anti-rat SP-A monoclonal antibodies that affect the SP-A functions recognized the SP-A/SP-D chimeras. In this report, we show that the SP-A region of Glu195–Phe228 is required for the interactions recognized the SP-A/SP-D chimeras. In this report, we

FUNCTIONAL REGIONS IN THE CRDS OF RAT SP-A AND SP-D

EXPERIMENTAL PROCEDURES

Isolation and Purification of Rat SP-A—Surfactant was isolated from lung lavage fluids of Sprague-Dawley rats (4) that had been given an intratracheal instillation of silica in saline 4 weeks before the lavage (35). The surfactant was delipidated by extraction with 1-butanol. SP-A was isolated and purified from the delipidated surfactant by mannose-Sepharose 6B column chromatography followed by gel filtration over Bio-Gel A5m as described previously (10).

Lipids—DPPC, phosphatidylcholine from egg yolk, PI, phosphatidylycerol from egg yolk, phosphatidylserine (PS) from bovine brain, GalCer, and GlicCer were purchased from Sigma. Cholesterol was obtained from Serdary Research Laboratories. The 1-palmitoyl-2-[14]H]palmitoyl-1-3-phosphatidylcholine ([14]H)PC was purchased from NEN Life Science Products.

DNA Construction of SP-A, SP-D, and Chimeric Molecules—The isolation and sequencing of the 1.6-kilobase cDNA for rat SP-A was described previously (36). The isolation of the 1.2-kilobase cDNA for rat SP-D was also previously reported (37).

We constructed three chimeric proteins with SP-A and SP-D in which progressively longer carboxyl-terminal regions of the SP-D CRD were substituted for the corresponding CRD regions of SP-A. The chimeric molecules used in this study are schematically represented in Fig. 1. Chimera ad3 consists of Asn1–Gly194 of SP-A and Glu321–Phe355 of SP-D. Chimera ad4 consists of Asn1–Gln173 of SP-A and Thr207–Phe233 of SP-D. Chimera ad5 consists of Asn1–Met134 of SP-A and Cys261–Phe355 of SP-D. The cDNAs for chimeric molecules were amplified by the polymerase chain reaction and the overlap extension method (38) using the cDNAs for SP-A and SP-D as the templates. The primers used at the SP-A/SP-D splicing junctions were 5′-AATCTGCTCCAGGAGCCCAACAAAT-3′ and 5′-ATTGGTGTGGGCTCTCTTG-GGTACCAGTT-3′ for chimera ad3; 5′-ATGGTGAACAGCAGGAGGGCAAGTTCT-3′ and 5′-GAATGGGCTCTGCTTCGTCTCATCAATCT-3′ for chimera ad4; 5′-ACCATTAAAGATGTCGAGCGGCGCCGAG-3′ and 5′-TCCGGCTTCTGTGCAATCTCGTGTTTAAATGG-3′ for chimera ad5. An SP-A sense and an SP-D antisense primers were used 5′-TCTAGAAGATCTTGTCGAGAACCCCTG-3′ and 5′-AAAGCCGGGCTAATCTGGCCAGCAATCG-3′, respectively. The EcoRI and XmnI sites were incorporated into the flanking 5′ and 3′ primers, respectively. All of the constructions were inserted into plasmid pVL1392 plasmid vector using the EcoRI and XmnI sites. The recombinant plasmids constructed were confirmed by a combination of restriction enzyme mapping and DNA sequencing.

Expression and Isolation of Recombinant Proteins—The recombinant proteins were expressed in the baculovirus system using the methods described by O’Reilly et al. (39). Monolayers of Spodoptera frugiperda (SF-9) cells were cotransfected with linearized virus DNA (BaculoGold, Pharmingen) and the pVL 1392 plasmid vector containing the cDNAs for SP-A, SP-D, and the chimeras. Recombinant plaques were isolated and viral titers were amplified to approximately 5 × 10^8 to 5 × 10^9 plaque-forming units/ml. The recombinant viruses were used to infect monolayers of SF-9 cells in serum-free insect medium at a multiplicity of 2. The culture medium was then collected and dialyzed against 5 mM Tris buffer (pH 7.4) containing 0.15 mM NaCl and 1 mM EDTA to remove monosaccharides. The culture medium was then applied to an affinity column of mannose-Sepharose 6B in the presence of 5 mM CaCl_2. The recombinant proteins were eluted with 5 mM Tris buffer (pH 7.4) containing 10 mM EDTA. The purified recombinant proteins were dialyzed against 5 mM Tris buffer (pH 7.4) and stored at −20 °C.

Protein concentrations were estimated by the method of Lowry et al. (40) using bovine serum albumin as the standard. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (41).

Antibody Binding Assay—Monoclonal antibodies were prepared against rat SP-A as described previously (42). The monoclonal antibodies recognized epitopes in the polypeptide portion of SP-A and had nearly equivalent affinity for the SP-A antigen. The epitopes for antibodies 1D6 and 6E3 have been identified to be located at the CRD and the neck domain, respectively (32).

The binding of antibodies to SP-A, SP-D, or the chimeric proteins was examined by enzyme-linked immunosorbent assay (ELISA). Aliquots of the purified proteins (50 μl, 1 μg/ml) in 5 mM Tris buffer (pH 7.4) were
coated onto the microtiter wells and incubated with polyclonal antibody to SP-A or SP-D, or monoclonal antibody 1D6 or 6E3 (50 μg, 10 μg/ml) at 37 °C for 1 h after the nonspecific binding sites were blocked with phosphate-buffered saline containing 0.1% (v/v) Triton X-100 and 3% (w/v) skim milk. The wells were washed and incubated with horseradish peroxidase-labeled anti-rabbit IgG (1:1000, 100 μg/ml of bovine serum albumin, various concentrations of SP-A or the chimeric proteins (0–20 μg/ml) were added and incubated for 1 h at room temperature. After the incubation, the wells were washed three times with ice-cold buffer of 50 mM Tris pH 7.4 containing 1 mM NaCl, 5 mM CaCl2, and 1 mg/ml of bovine serum albumin. Monoclonal antibody 6E3 (10 μg/ml) was then added and incubated for 1 h, followed by the incubation with horseradish peroxidase-labeled anti-mouse IgG.

The binding of the proteins to lipids was determined by measuring the absorbance at 492 nm using o-phenylenediamine as a substrate for the peroxidase reaction. The binding of SP-D to the lipids was examined in the same manner as described using anti-SP-D polyclonal antibody and horseradish peroxidase-labeled anti-rabbit IgG.

**Binding of Chimeras to Multilamellar Liposomes—DPPC, PI, or the mixture composed of GalCer:PS:cholesterol (7:2:1, w/w/w) or GlCer:PS:cholesterol (3:4:3, w/w/w) was dried under nitrogen and hydrated in 20 mM Tris buffer (pH 7.4) containing 0.1 M NaCl at 37 °C for 1 h and vortexed vigorously for 5 min to prepare multilamellar liposomes.**

The protein solutions (0.2 μg/μl) were allowed to block binding sites of the lipids. Tris buffer (pH 7.4) containing 0.15 mM NaCl, 5 mM CaCl2 and 2% (w/v) bovine serum albumin, various concentrations of SP-A or the chimeric proteins (0–20 μg/ml) were added and incubated for 1 h at room temperature. After the incubation, the wells were washed three times with ice-cold buffer of 50 mM Tris (pH 7.4) containing 0.15 mM NaCl, 5 mM CaCl2, and 20 mg/ml of bovine serum albumin (binding buffer). Then the multilamellar liposomes—

**Binding of Chimeras to Multilamellar Liposomes—DPPC, PI, or the mixture composed of GalCer:PS:cholesterol (7:2:1, w/w/w) or GlCer:PS:cholesterol (3:4:3, w/w/w) was dried under nitrogen and hydrated in 20 mM Tris buffer (pH 7.4) containing 0.1 M NaCl at 37 °C for 1 h and vortexed vigorously for 5 min to prepare multilamellar liposomes.**

The protein solutions (0.2 μg/μl) were allowed to block binding sites of the lipids. Tris buffer (pH 7.4) containing 0.15 mM NaCl, 5 mM CaCl2 and 2% (w/v) bovine serum albumin, various concentrations of SP-A or the chimeric proteins (0–20 μg/ml) were added and incubated for 1 h at room temperature. After the incubation, the wells were washed three times with ice-cold buffer of 50 mM Tris (pH 7.4) containing 0.15 mM NaCl, 5 mM CaCl2, and 20 mg/ml of bovine serum albumin (binding buffer). Then the multilamellar liposomes (100 μg) and the protein solutions were separately centrifuged at 12,000 rpm at room temperature for 10 min. The supernatant of the protein solution (50 μl) was added to the liposome pellet. The mixtures were suspended and incubated for 1 h at room temperature. The mixture was then put on ice for 15 min and then centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant was stored, and the resultant pellet was once washed with ice-cold binding buffer (50 μl) and centrifuged again. The supernatant was then combined, and the pellet was suspended with 100 μl of the binding buffer. The amount of the proteins in each fraction was determined by competitive ELISA as described below. Liposome binding was defined as percentage of sedimentation (the proteins in pellet/the proteins in pellet plus supernatant × 100). Control experiments without liposomes were also performed.

**Competitive ELISA—Because monoclonal antibody 6E3 binds all three chimeras to the same extent (see “Results”), we developed a competitive ELISA to determine the amount of chimeras in liposome pellets and the supernatants. The wild type (wt) recombinant SP-A (0.5 μg/μl) and SP-A/SP-D chimeras (3–5 μg/μl) were coated onto microtiter wells. After the nonspecific binding was blocked with phosphate-buffered saline containing 3% (w/v) skim milk, 0.1% (v/v) Triton X-100 (blocking buffer), the various dilutions of samples or standard wt-SP-A (50 μg/well), and antibody 6E3 (1 μg/ml, 50 μg/ml) were added to the wells and simultaneously incubated at 37 °C for 90 min. After the wells were washed, horseradish peroxidase-labeled anti-mouse IgG (1:1000, 100 μg/ml) was added and incubated for 37 °C for 60 min. The peroxidase reaction was finally developed using o-phenylenediamine and the absorbance was measured at 492 nm.

To determine the amount of SP-D, wt-SP-D (0.2 μg/ml) as the solid phase antigen, anti-SP-D polyclonal antibody (1 μg/ml), and anti-rabbit IgG were used instead of solid phase wt-SP-A, antibody 6E3, and anti-mouse IgG, respectively.

**Liposome Aggregation—Liposome aggregation was performed by a modified method based on that described by Hawgood et al. (4).** Unilamellar liposomes were prepared from the multilamellar liposomes composed of DPPC:egg phosphatidylcholine:phosphatidylglycerol (7:2:1, w/w/w) by probe sonication for 5 min. Unilamellar liposomes (200 μg/ml) and the purified proteins (20 or 80 μg/ml) in 20 mM Tris buffer (pH 7.4) containing 0.1 M NaCl were preincubated for 5 min. After equilibration, absorption was measured at 400 nm using a Hitachi U-2000 spectrophotometer at room temperature. Following the initial absorbance reading, CaCl2 was added to a final concentration of 5 mM after 5 min, and the absorbance was further measured until a time of 10 min.

**Isolation of Alveolar Type II Cells and Uptake of Phospholipid Liposomes—Alveolar type II cells were isolated from the lungs of male Sprague-Dawley rats by tissue dissociation with elastase digestion and purification on metrizamide gradients (43). Uptake of phospholipids by freshly isolated type II cells was performed by the method described by Wright et al. (9). Briefly, freshly isolated type II cells (2 × 106 cells) were incubated with radiolabeled phospholipid liposomes (100 μg/ml) composed of DPPC:egg phosphatidylcholine:phosphatidylglycerol (7:2:1, w/w/w) and a trace amount of [3H]DPPC, in the presence of the indicated amounts of proteins (5 or 20 μg/ml) at 37 °C for 1 h in 0.5 ml of Dulbecco’s modified Eagle’s medium containing Hepes (10 mM) (pH 7.4).

Following the incubation, cells and media were separated by centrifugation at 1500 rpm for 5 min at 4 °C. The cells were resuspended with 1 ml of fresh cold phosphate-buffered saline containing 1 mg/ml of bovine serum albumin. The washing steps were repeated three times. Before the final centrifugation, the cell suspension was transferred to a fresh tube. The final cell pellet was analyzed for radioactivity.

**Secretion of Phosphatidylcholine from Alveolar Type II Cells—Type II cells (2 × 106 cells) were incubated overnight with [3H]choline (0.5 μCi/ml) in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum. Secretion of radiolabeled phosphatidylcholine was performed using 12-o-tetradecanoylphorbol-13-acetate (TPA) (10−7 M) as the stimulant for the surfactant secretion and the purified proteins as antagonists, as described previously (42). Following a 3-h incubation, radiolabeled phospholipids were extracted from the cells and media, and counted. The lipid secretion from type II cells was expressed as percentage of secretion (radioactivity in medium/radioactivity in medium plus cells × 100).

**Receptor Binding—The binding of chimeras to type II cells was performed by determining the ability of chimeric proteins to compete with rat 125I-SP-A for receptor occupancy on alveolar type II cells, as described previously (12, 44). Briefly, monolayers of freshly isolated type II cells (2 × 106/dish) were cultured overnight, and then were incubated with 0.5 μg/ml rat 125I-SP-A in the presence of 20 or 40 μg/ml unlabeled proteins for 3 h at 37 °C. The cells were washed four times on ice with 50 mM Tris buffer (pH 7.4) containing 0.1 mM NaCl, 2 mM CaCl2, and 1 mg/ml bovine serum albumin. The cells were then dissolved in 2 ml of 0.1 N NaOH and the radioactivity associated with the cells was determined using a γ-counter.

**RESULTS**

**Characterization of Chimeric Recombinant Proteins—Wild type SP-A, SP-D, and SP-A/SP-D chimeras were expressed using the baculovirus expression system. All three chimeras used in this study were purified by affinity chromatography using a matrix of mannnose-Sepharose 6B. The preservation of lectin activity confirms a significant degree of fidelity in the protein folding. The purified recombinant proteins and native rat SP-A were analyzed by SDS-polyacrylamide gel electrophoresis. Native SP-A migrated as a tetradecanoylphorbol-13-acetate (TPA) (10−7 M) as the stimulant for the surfactant secretion and the purified proteins as antagonists, as described previously (42). Following a 3-h incubation, radiolabeled phospholipids were extracted from the cells and media, and counted. The lipid secretion from type II cells was expressed as percentage of secretion (radioactivity in medium/radioactivity in medium plus cells × 100).

**Binding of Anti-SP-A Monoclonal Antibodies to SP-A/SP-D Chimeras—Previous studies revealed that the epitopes for anti-SP-A monoclonal antibodies 1D6 and 6E3 are located at the...
CRD and the neck domain, respectively (32). Antibody 1D6 has been reported to block the binding of SP-A to lipids, the SP-A-induced liposome aggregation, the SP-A-mediated lipid uptake into type II cells, the inhibitory activity of SP-A on lipid secretion, and the receptor binding (12, 32). Antibody 6E3 partially blocked SP-A-receptor interactions and completely blocked lipid uptake by alveolar type II cells. To examine whether monoclonal antibodies to SP-A recognized SP-A/SP-D chimeras, antibody binding to the purified proteins was measured by ELISA. Antibody 1D6 failed to bind to chimera ad3, ad4, or ad5 (Fig. 3). In contrast, antibody 6E3 bound to all of the chimeras, as well as to wild type SP-A. When monoclonal antibodies were tested for the reactivity to purified proteins applied to nitrocellulose membranes, the results were consistent with those obtained by ELISA (data not shown). Anti-SP-A polyclonal antibody bound to chimera ad3 at nearly one-half the level of that of wt SP-A, and it bound very weakly to chimeras ad4 and ad5. Anti-SP-D polyclonal antibody exhibited almost no binding to chimeras ad3 and ad4 but recognized ad5 at a level equivalent to that of wt SP-D. The results indicate that the substitution of the SP-D region Glu321–Phe355 for the SP-A region Glu 195–Phe228 disrupts an epitope for anti-SP-A monoclonal antibody 1D6 and are consistent with our previous results that antibody 1D6 failed to recognize antigen in Sf-9 cells infected with the recombinant virus directing the synthesis of Cys204–Cys218 deletion within the CRD (32). The nearly equivalent binding of 6E3 to chimeras and to wt SP-A suggests that antibody 6E3 is useful as a probe for the detection of chimeras in further experiments. We next directly evaluated whether the SP-A/SP-D chimeras possess the functions of either SP-A or SP-D.

Interaction of Chimeras with Lipids—We first examined the ability of chimeras to bind lipids coated onto microtiter wells using antibody 6E3 as a probe (Fig. 4). Native SP-A bound to DPPC and GalCer strongly, wt SP-A also bound to these lipids in a concentration-dependent manner (Fig. 4, A and B). The binding of wt SP-A to DPPC was approximately 30% of that of native SP-A at 20 µg/ml, whereas wt SP-A showed binding to GalCer at a level nearly equivalent to native SP-A. The wt SP-D and the chimeras ad3, ad4 and ad5 did not exhibit any binding to DPPC or GalCer (Fig. 4, A and B). The binding of the recombinant proteins to multilamellar liposomes containing DPPC or GalCer was also examined by the sedimentation method (Fig. 5, A and B). As much as 63 or 40% of the wild type SP-A was cosedimented with liposomes containing DPPC or GalCer, respectively. None of the chimeras cosedimented with the DPPC or GalCer liposomes. These results clearly indicate that the chimeras do not show any of lipid binding specificity exhibited by SP-A. Furthermore, the SP-D region of Glu321–Phe355 cannot replace the SP-A region of Glu195–Phe228 with respect to the binding to DPPC or GalCer. The data demonstrate that the SP-A region of Glu195–Phe228 is required for the interaction with DPPC and GalCer.

We next examined whether the chimeras acquired any lipid
binding property of SP-D. When the binding of chimeras to solid phase PI was evaluated, chimeras ad4 and ad5 bound to PI in a concentration-dependent manner (Fig. 4C) \((p < 0.002\) at 20 \(\mu g/ml\), compared with wt SP-A). Chimera ad5 exhibited even stronger binding to PI than wt SP-D. Chimera ad3 showed very little binding to PI, but it showed a reproducibly detectable signal (absorbance at 20 \(\mu g/ml\); 0.102–0.242). Next, the ability of chimeras to bind solid phase GlcCer was examined. Chimera ad5 and wt SP-D exhibited concentration-dependent binding to GlcCer (Fig. 4D) \((p < 0.05\) at 20 \(\mu g/ml\), compared with wt SP-A). Chimera ad3 and the SP-Ax failed to bind to solid phase GlcCer. Chimera ad4 did not show an apparent binding to GlcCer. When the chimeras and PI liposomes were incubated and centrifuged, 81% of wt SP-D was cosedimented with PI liposomes (Fig. 5C). The percentage of chimera ad5 that cosedimented with PI liposomes was even greater than that of wt SP-D \((p < 0.0001\), chimera ad5 versus wt SP-A). Chimera ad4 bound to PI liposomes with activity nearly comparable to wt SP-D \((p < 0.0005\), chimera ad4 versus wt SP-A). A significant amount of ad3 chimera cosedimented with PI liposomes \((p < 0.02\), compared with wt SP-A) but its binding was clearly less than that of chimeras ad4 or ad5. In contrast, chimeras ad3 and ad4 exhibited almost no binding to liposomes containing GlcCer (Fig. 5D). A significant amount of chimera ad5 was cosedimented with GlcCer liposomes \((p < 0.05\), compared with chimera ad3, ad4, or wt SP-A), although the percentage of wt SP-D that cosedimented with GlcCer liposomes was quite low compared to that with PI liposomes. Taken together, the data demonstrate that chimeras ad4 and ad5 exhibit PI binding that is comparable to wt SP-D. For GlcCer binding, the ad5 chimera displays activity that is comparable to wt SP-D, whereas the ad4 and ad3 proteins are essentially inactive.

Because SP-A causes aggregation of phospholipid liposomes containing DPPC in the presence of Ca\(^{2+}\) \((4\), we next investigated the abilities of chimeras to cause liposome aggregation. Native and wild type SP-A at a concentration of 20 \(\mu g/ml\) induced phospholipid vesicle aggregation in a time-dependent manner. In contrast, chimeras ad3, ad4, and ad5 essentially did not cause liposome aggregation even at 80 \(\mu g/ml\) (Fig. 6). These findings clearly indicate that all three chimeras lack the ability of SP-A to induce liposome aggregation.

Interaction of Chimeras with Alveolar Type II Cells—Another measure of the SP-A-specific interaction with lipids is the ability to mediate liposome uptake by freshly isolated alveolar type II cells \((9\). Therefore, we examined whether the chimeras mediated liposome uptake. The wild type SP-A enhanced the uptake of liposomes containing DPPC into type II cells in a concentration-dependent manner (Fig. 7); 3.4 and 7.9% of liposomes were taken up in the presence of 5 and 20 \(\mu g/ml\) of wild type SP-A, respectively. When type II cells were incubated with chimeras or wild type SP-D in the presence of radiolabeled phospholipids, the protein-mediated increases of cell-associated radioactivities were not observed.

We also investigated whether the chimeras inhibited the secretion of phospholipids from alveolar type II cells. wt SP-A completely inhibited the TPA-stimulated lipid secretion at 5 \(\mu g/ml\). However, none of the chimeras or wt SP-D exhibited the inhibitory activity on lipid secretion from type II cells (Fig. 8). Because the inhibitory activity of SP-A on lipid secretion correlates well with the capacity of SP-A to bind a high affinity receptor on type II cells \((12\), we also performed competition experiments with \[^{125}I\]SP-A for type II cell binding. Although unlabeled wt SP-A reduced the binding of \[^{125}I\]SP-A to 20% of control binding at 40 \(\mu g/ml\), none of the ad chimeras at this concentration competed with the labeled SP-A for receptor occupancy.

These data clearly demonstrate that the SP-D region of Glu\(^{223}\)–Phe\(^{335}\) cannot replace the SP-A region of Glu\(^{195}\)–Phe\(^{228}\) in interactions of SP-A with alveolar type II cells and indicate that the SP-A region of Glu\(^{195}\)–Phe\(^{228}\) is required for SP-A-type II cell interaction.

**DISCUSSION**

The purpose of this study was to investigate the CRD regions of SP-A and SP-D involved in binding lipids and interacting...
Fig. 7. SP-A/SP-D chimeras failed to augment lipid uptake by alveolar type II cells. Freshly isolated alveolar type II cells were incubated with unilamellar liposomes containing [3H]DPPC in the presence of 5 or 20 µg/ml of wt SP-A, chimera ad3, ad4, or ad5, or wt SP-D at 37 °C for 1 h. The cells were then washed, and the radioactivities associated with cells were counted in a scintillation counter as described under “Experimental Procedures.” The results are expressed as percentage of radioactivities associated with cells in total radioactivities of the incubation mixture. The data presented are the means of duplicate determinations in two separate experiments.

FIG. 7. SP-A/SP-D chimeras failed to inhibit lipid secretion from type II cells. Alveolar type II cells were incubated overnight with the medium containing [3H]choline, and cellular phosphatidylcholine was radiolabeled as described under “Experimental Procedures.” The secretagogue TPA (10−7 M) and 5 or 10 µg/ml of wild type SP-A (SP-A), chimera ad3, ad4, and ad5, and wild type SP-D (SP-D) were added and incubated for 3 h at 37 °C. Secretion was defined as percentage of secretion ([radioactivity in the medium]/[radioactivity in the medium + cells] × 100). Results are expressed as percentage of the TPA-stimulated secretion. Data are the means of duplicate determinations in two separate experiments.

Binding of SP-D to GlcCer is critically dependent upon the SP-D region of Cys261–Phe355. This demonstrates that the structural requirement for the binding of SP-D to PI is different from that for the binding of SP-D to GlcCer. Some evidence has accumulated to understand the structure-function relationships of surfactant proteins. Site-directed mutagenesis of SP-A (45) reveals that the N-linked oligosaccharide moiety is not required for type II cell interaction or for phospholipid vesicle aggregation. Altered carbohydrate binding specificity engineered into SP-A or SP-D is accompanied by changes of the functions of these proteins (44, 46). Although the amino-terminal domain and the collagenous domain are essential for the oligomerization of the protein (29, 34, 47) to stabilize these macromolecules, the CRDs have been shown to be functional domains of SP-A and SP-D (31–33). The present study specifies the regions within the CRDs that are critical for the functions of SP-A and SP-D. Although the work in this report is largely limited to well-defined low molecular weight ligands, it may have important consequences for unraveling the interactions of SP-A and SP-D with the surfaces of microorganisms that contain both carbohydrate and lipid determinants available for recognition.

A recent study from this laboratory (48) showed that the SP-A/MBP-A chimera (AM3) composed of the SP-A region Asn1–Gly194 and the MBP-A region Glu185–Ala221 retained SP-A functions, although MBP-A does not possess the ability to interact with DPPC, GalCer, or type II cells as SP-A does. This demonstrates that the MBP-A region of Glu185–Ala221 can functionally replace the SP-A region of Glu195–Phe228. However, the present report reveals that the SP-D region of Glu231–Phe355 cannot replace the homologous SP-A region of Glu195–Phe228 without loss of the SP-A functions. It is unknown why different results are obtained from the replacement studies between the homologous proteins, although lung collectins and MBP-A show similarities in carbohydrate binding specificity and oligomeric structure and 48–53% sequence identity between the SP-A region of Glu195–Phe228 and the corresponding regions of MBP-A or SP-D. An understanding of this discrepancy awaits the resolution of the crystal structures of lung collectins.

Anti-SP-A monoclonal antibody 1D6 did not bind to any of the ad chimeras. Because antibody 1D6 blocks SP-A interactions with lipids and type II cells (12, 32, 42), the loss of the 1D6 epitope by ad chimeras correlates with the dysfunction of the ad chimeras with respect to the SP-A properties. Furthermore, the results from antibody binding and functional assays of chimera ad3, in which the small disulfide loop region (Cys204–
Cys^{215} of SP-A has been replaced with that of SP-D, are consistent with our previous finding that 1D6 binds to epitopes in this region (32). Collectively, these data are consistent with the conclusion that the SP-A region of Glu^{195}–Phe^{228} is required for binding DPPC and GalCer, causing phospholipid liposome aggregation, enhancing lipid uptake, and inhibiting lipid secretion from type II cells.

Antibody 6E3 did not block the binding of SP-A to lipids but did inhibit the SP-A-mediated lipid uptake by type II cells (32). Because antibody 6E3 recognizes the neck domain of SP-A (32), the neck domain may also be involved in the process of the SP-A-mediated uptake of phospholipids. In this study, the chimeras ad3–ad5, which contain the SP-A neck, failed to bind DPPC and to augment lipid uptake by type II cells. Thus, these results indicate that the neck domain of SP-A cannot account for all of the process of SP-A-mediated lipid uptake and support the idea that the binding of SP-A to lipids is an initial step for lipid uptake by type II cells.

A previous study (33) using SP-A/SP-D chimeras revealed that the A1A2A3D4 chimera, in which the whole SP-A CRD (Gly^{152}–Phe^{259}) was replaced with the corresponding domain of SP-D, weakly bound to DPPC liposomes. These findings suggested a positive role for the neck domain in DPPC binding by SP-A. In the present study, the chimeras ad3–ad5 did not bind to DPPC or GalCer. The only structural difference between the A1A2A3D4 chimera and ad chimera is that the whole SP-D CRD is directly connected to the SP-A neck domain in the A1A2A3D4 chimera, but the ad3–ad5 chimeras contain the SP-A neck plus a portion of the SP-A CRD. These observations support the ideas that the CRD of SP-A is the most important domain for the lipid binding property of the protein and that the hydrophobic neck region may contribute to some aspect of lipid binding but cannot account for all of the interaction. These studies are also consistent with the epitope localization and effect of monoclonal antibodies 1D6 and 6E3 on the lipid binding of SP-A (32).

The current results narrow the minimal SP-D structure required for PI binding to the Glu^{214}–Phe^{250} portion of the CRD. In contrast, the minimal region of SP-D required for GlcCer binding extends from Cys^{361} to Phe^{355}. These conclusions are consistent with those made previously (46) suggesting that there are distinct differences between the PI and GlcCer binding sites. The findings highlight chimeras ad3–ad5 as important new tools for examining the mechanism of lipid binding by SP-D. The structures composed of chimera ad3 or ad4 appear to be specific for PI binding without the property of GlcCer binding. It will certainly be very interesting to determine how these chimeric proteins interact with complex biological targets such as bacteria, viruses, and fungi.

In summary, we focused on the CRDs of lung lectins with different lipid binding specificities, expressed novel proteins of SP-A/SP-D chimeras and examined the interaction of the chimeras with lipids and alveolar type II cells. All of the chimeras lacked the specific SP-A interactions with lipids and type II cells. In contrast, the chimeras exhibited partial complete interaction with the SP-D ligand PI depending upon the length of the SP-D sequence. Only one of the chimeras, ad5, retained the GlcCer binding activity of SP-D. From these data, we conclude that 1) the SP-A region of Glu^{195}–Phe^{228} is required for the SP-A functions; 2) the SP-D region of Glu^{215}–Phe^{250} is essential for the binding to PI, and the binding increases with the extension of the region to Cys^{261}–Phe^{255}, and 3) the binding of SP-D to GlcCer is critically dependent upon the SP-D region of Cys^{261}–Phe^{255}, and 4) the structural requirement for SP-D binding to PI is different from that for GlcCer.
Analysis of Chimeric Proteins Identifies the Regions in the Carbohydrate Recognition Domains of Rat Lung Collectins That Are Essential for Interactions with Phospholipids, Glycolipids, and Alveolar Type II Cells

Hitomi Sano, Yoshio Kuroki, Toshio Honma, Yoshinori Ogasawara, Hitoshi Sohma, Dennis R. Voelker and Toyoaki Akino

J. Biol. Chem. 1998, 273:4783-4789.
doi: 10.1074/jbc.273.8.4783

Access the most updated version of this article at http://www.jbc.org/content/273/8/4783

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 26 of which can be accessed free at http://www.jbc.org/content/273/8/4783.full.html#ref-list-1