# Importance of the Carboxy-Terminal 25 Amino Acid Residues of Lung Collectins in Interactions with Lipids and Alveolar Type II Cells†

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ABSTRACT: Surfactant proteins A and D (SP-A and SP-D) are structurally related members of the collectin family found in the alveolar compartment of the lung. SP-A binds dipalmitoylphosphatidylcholine (DPPC) and galactosylceramide (GalCer), induces liposome aggregation, and regulates the uptake and secretion of surfactant lipids by alveolar type II cells in vitro. SP-D binds phosphatidylinositol (PI) and glucosylceramide. The purpose of this study was to identify a critical stretch of primary sequence in the SP-A region $Cys^{204}$−$Phe^{228}$ and the SP-D region $Cys^{331}$−$Phe^{355}$ that is involved in protein-specific lipid and type II cell interactions. Chimeras *ad1* and *ad2* were constructed with rat SP-A/SP-D splice junctions at $Cys^{218}$/$Gly^{346}$ and $Lys^{203}$/$Cys^{331}$, respectively. Chimera *ad1* but not *ad2* retained DPPC liposome binding activity. Both chimeras retained significant binding to GalCer liposomes. Chimera *ad1* did not bind to PI, whereas chimera *ad2* acquired a significant PI binding. Both chimeras failed to induce liposome aggregation and to interact with alveolar type II cells. In addition, monoclonal antibody 1D6 that blocks specific SP-A functions did not recognize either chimera. From these results, we conclude that (1) the SP-A region $Leu^{219}$−$Phe^{228}$ is required for liposome aggregation and interaction with alveolar type II cells, (2) the SP-A region $Cys^{204}$−$Cys^{218}$ is required for DPPC binding, (3) the SP-D region $Cys^{331}$−$Phe^{355}$ is essential for minimal PI binding, and (4) the epitope for mAb 1D6 is located at the region contiguous to the SP-A region $Leu^{219}$−$Phe^{228}$.

Pulmonary surfactant keeps the alveoli from collapsing by lowering surface tension at the air−liquid interface (*1*). Phospholipids are major constituents of surfactant, and dipalmitoylphosphatidylcholine (DPPC)[1] is the most important phospholipid for its biophysical function of surfactant. DPPC acts in conjunction with the hydrophobic surfactant proteins B and C (*2*). Surfactant proteins A (SP-A) and D (SP-D) are now thought to play key roles in the innate immune system of the lung, which is critical in first-line host defense (*1*). These proteins bind a variety of microbial species including Gram-negative bacteria, *Pneumocystis carinii*, and *Herpes simplex* type I (*3*−*6*), and also interact with alveolar macrophages with high affinity (*7*−*9*). SP-A and SP-D belong to the collectin subgroup of the C-type lectin superfamily along with mannose-binding protein (MBP) (*10*). Collectins share a common structural domain arrangement: a cysteine-containing amino terminus; a collagen-like domain; a neck domain; and a carbohydrate recognition domain (CRD). They bind mannose, glucose, and/or *N*-acetylglucosamine (*11*−*13*). Glycosphingolipids are also ligands for the surfactant collectins. SP-A binds to galactosylceramide (GalCer), lactosylceramide, and asialo-$G_{M2}$ (*14, 15*). SP-D binds to glucosylceramide (GlcCer) (*16*). Human and rat MBPs bind to glycolipids containing *N*-acetylglucosamine residues (*17, 18*). We have previously shown that phospholipids are also ligands for collectins. Lung collectins, SP-A and SP-D, bind with high affinity to DPPC and phosphatidylinositol (PI), respectively (*19, 20*). Rat MBPs isolated from serum and liver bind to PI and phosphatidylglycerol and weakly to phosphatidylserine (*21*). Although the collectins show similarity in carbohydrate binding specificity and approximately 40% sequence identity among the CRDs of SP-A and SP-D or MBP (*22*−*24*), the proteins exhibit different phospholipid and glycolipid binding specificities.

SP-A inhibits phospholipid secretion from alveolar type II cells (*25, 26*). It binds to a high-affinity receptor expressed on alveolar type II cells (*27, 28*), and receptor binding activity correlates well with the inhibitory activity of SP-A on lipid secretion (*29*). The phospholipid binding specificity of SP-A may be important for recycling and reutilization of DPPC. SP-A also induces the $Ca^{2+}$-dependent aggregation of liposomes containing DPPC (*30*) and facilitates the formation

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[1] Abbreviations: SP-A, surfactant protein A; wt SP-A, wild-type SP-A; SP-D, surfactant protein D; MBP, mannose-binding protein; CRD, carbohydrate recognition domain; DPPC, dipalmitoylphosphatidylcholine; PI, phosphatidylinositol; GalCer, galactosylceramide; GlcCer, glucosylceramide; PS, phosphatidylserine; ELISA, enzyme-linked immunosorbent assay; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; mAb, monoclonal antibody.

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of tubular myelin phospholipid structures in coordination with SP-B (*31*). In vitro SP-A promotes phospholipid association with alveolar type II cells (*32, 33*). Although compelling evidence has accumulated that SP-A regulates surfactant phospholipid homeostasis in vitro, mice homozygous for null alleles of SP-A are viable and show modest alterations in surfactant phospholipid structure (*34, 35*). The findings with the SP-A null mice may indicate functional redundancy in the surfactant system. In vitro functions of SP-D in phospholipid homeostasis have been unclear. SP-D (−/−) mice developed pulmonary lipidosis without the accumulation of SP-B and SP-C that is a typical characteristic of alveolar proteinosis syndromes (*36*). This latter finding suggests a previously unexpected role for SP-D in surfactant lipid homeostasis in vivo. The exact relationship between the in vitro activities of lung collectins and the in vivo functions is unclear and requires further study to resolve the apparent discrepancies.

Although lung collectins possess homologous structures, they exhibit quite different properties of binding lipids and interacting with alveolar type II cells. SP-A/SP-D chimeras, in which progressively longer carboxy-terminal regions of rat SP-A were replaced with the corresponding rat SP-D regions, were constructed in a previous study (*37*). The chimera *ad3*, in which the SP-D region $Glu^{321}$−$Phe^{355}$ was substituted for the SP-A region $Glu^{195}$−$Phe^{228}$, lost all of the SP-A functions and acquired the PI but not the GlcCer binding property. The chimera *ad5* that consisted of the SP-A region $Asn^{1}$−$Met^{134}$ and the SP-D region $Cys^{266}$−$Phe^{355}$ binds to PI and GlcCer. This previous study indicates that the SP-A region $Glu^{195}$−$Phe^{228}$ is required for interactions with DPPC, GalCer, and alveolar type II cells, and that the SP-D region $Glu^{321}$−$Phe^{355}$ is essential for the binding to PI, but the longer SP-D region of $Cys^{266}$−$Phe^{355}$ is required for optimal lipid binding of SP-D. In the present work, we extend the study with SP-A/SP-D chimeras. This study narrows down the essential region for DPPC and type II cell interaction and reveals that the structural requirement for the binding of SP-A to lipids is different from that for liposome aggregation and type II cell interaction. The study further shows that the shorter SP-D region of $Cys^{331}$−$Phe^{355}$ confers the PI binding property in the context of the SP-A region $Asn^{1}$−$Lys^{203}$.

## EXPERIMENTAL PROCEDURES

*Lipids*. Phosphatidylcholine (PC) from egg yolk, phosphatidylinositol (PI) from bovine liver, phosphatidylglycerol (PG) from egg yolk, phosphatidylserine (PS) from bovine brain, dipalmitoylphosphatidylcholine (DPPC), and galactosylceramide (GalCer) were purchased from Sigma. Cholesterol was obtained from Serdary Research Laboratories. The 1-palmitoyl-2-[$^3$H]palmitoyl-L-3-phosphatidylcholine ([$^3$H]-DPPC) was purchased from NEN Life Science Products.

*DNA Construction of SP-A and Chimeric Proteins.* The isolation and sequencing of the 1.6 kilobase cDNA for rat SP-A and the 1.2 kilobase cDNA for rat SP-D were described previously (*22, 23*). We constructed three chimeras in which the rat SP-A CRD region was replaced with the corresponding rat SP-D CRD region. The chimeras used in this study are schematically represented in Figure 1. Chimera *ad1* consists of $Asn^{1}$−$Cys^{218}$ of rat SP-A and $Gly^{346}$−$Phe^{355}$ of rat SP-D. Chimera *ad2* consists of $Asn^{1}$−$Lys^{203}$ of rat SP-A and $Cys^{331}$−$Phe^{355}$ of rat SP-D. The cDNAs for the chimeras were constructed by the PCR and the overlap extension method (*38*) using the cDNAs for SP-A and SP-D as the templates. *Eco*RI and *Xma*I sites were incorporated into flanking 5′ and 3′ primers, respectively. Two primers used at the SP-A/SP-D splicing junctions were 5′-AATGATAGGGGCTGCGGAGAGCAGCGCCTG-3′ and 5′-CAGGCGCTGCTCTCCGCAGCCCCTATCATT-3′ for chimera *ad1*; 5′-CAGGGCAAAGAAAAGTGTGTGGAGATCTTC-3′ and 5′-GAAGATCTCCACACACTTTTCTTTGCCCTG-3′ for chimera *ad2*. The SP-A sense and SP-D antisense primers used were 5′-TCTAGAGAATTCTGTGGCAGAAGCCACTGG-3′ and 5′-TAACCCGGGGCTAAGCTCCTGGCCCAG-3′, respectively. Both constructions of chimeras were inserted into pVL1392 plasmid vector. A combination of restriction enzyme mapping and DNA sequencing confirmed the structure of the recombinant plasmids.

*Expression of Recombinant Proteins.* The recombinant proteins were expressed in the baculovirus−insect cell expression system, as described by O'Reilly et al. (*39*). *Spodoptera frugiperda* (Sf9) cells were cotransfected with linearized virus DNA (BaculoGold, Pharmingen) and the pVL1392 vector containing the cDNAs for SP-A and the chimeras. Plaques containing recombinant baculoviruses were isolated and amplified to approximately $5 \times 10^{-7}$ plaque-forming units/mL. The recombinant proteins were expressed in *Trichoplusia ni* cells that had been infected at a viral multiplicity of 2. All recombinant proteins were purified from the serum-free culture media by affinity chromatography using mannose−Sepharose 6B, as described previously (*37*).

*Binding of Chimeric Proteins to Mannose−Sepharose Beads.* Fifty microliters of mannose−Sepharose beads and 50 $\mu$L of the purified recombinant proteins (100 ng) were mixed and incubated at 4 °C for 1 h in 5 mM Tris buffer (pH 7.4) containing 5 mM $CaCl_2$. The mixture was then centrifuged at 1000*g* for 10 min to separate unbound proteins from bound proteins. The bound proteins were eluted from the matrix by mixing with 50 $\mu$L of 5 mM Tris buffer (pH 7.4) containing 5 mM EDTA. Bound and unbound proteins were determined by ELISA, as described previously (*37*). The binding efficiencies to the beads were determined as the percent of the bound proteins in total proteins.

*Protein Analysis.* The recombinant proteins were analyzed by electrophoresis in 13% polyacrylamide gels in the presence of SDS (*40*). Protein concentrations were determined by the bicinchoninic protein assay kit (BCA, Pierce). Size-fractionation of chimeric proteins was performed by gel filtration chromatography using Sephacryl S300 (1.5 × 100 cm column) in 10 mM Tris buffer (pH 7.4) containing 0.15 M NaCl. Chimeric protein in each fraction was detected by sandwich ELISA using anti-SP-A polyclonal antibody. Blue dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), and aldolase (158 kDa) were used as standards.

*Binding of Chimeras to Multilamellar Liposomes.* The binding study using multilamellar liposomes was carried out as described previously (*37*). DPPC, PI, or the lipid mixture composed of GalCer/PS/cholesterol (7:2:1, w/w/w) was dried under nitrogen and hydrated in 20 mM Tris buffer (pH 7.4) containing 0.1 M NaCl at 48 °C for 1 h. The lipids were then vortexed vigorously for 5 min to prepare multilamellar

liposomes. Each protein was diluted at 4 $\mu$g/mL in 20 mM Tris buffer (pH 7.4) containing 0.1 M NaCl, 5 mM $CaCl_2$, and 20 mg/mL bovine serum albumin (binding buffer). The multilamellar liposomes (100 $\mu$g/tube) and the protein solutions (0.2 $\mu$g/tube) were next separately centrifuged at 12 000 rpm at room temperature for 10 min. Fifty microliters of the supernatant from the protein solution was added to the liposome pellet. The mixture of the lipid and the protein was suspended and incubated for 1 h at room temperature. The mixture was then chilled on ice for 15 min and centrifuged at 12 000 rpm at 4 °C for 10 min. After the centrifugation, the supernatant was stored, and the pellet was washed once with 50 $\mu$L of ice-cold binding buffer and centrifuged again. The supernatant was finally combined, and the pellet was suspended with 100 $\mu$L of the binding buffer. The amount of protein in each fraction was determined by ELISA, as described previously (*37*). Liposome binding was defined as percent sedimentation [(the protein content in pellet/the protein content in pellet plus supernatant) × 100]. Control experiments without liposomes (nonspecific sedimentation) were also performed. The results are expressed as specific sedimentation, obtained by subtracting nonspecific sedimentation from total sedimentation. Nonspecific sedimentations of wt SP-A, chimera *ad1*, chimera *ad2*, and wt SP-D were 3.1 ± 2.7%, 1.8 ± 1.38%, 2.4 ± 1.57%, and 0.45 ± 0.33% (mean ± SE, $n = 3-5$), respectively.

*Phospholipid Liposome Aggregation.* Phospholipid liposome aggregation was performed by a modified method based on that described by Hawgood et al. (*30*). Unilamellar liposomes composed of DPPC/egg PC/PG (7:2:1, w/w/w) were prepared by probe sonication. The liposomes (200 $\mu$g/mL) and the recombinant proteins (20 and 80 $\mu$g/mL) in 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl were mixed and preincubated for 3 min. $CaCl_2$ was then added to a final concentration of 5 mM. The absorbance at 400 nm was measured using a Hitachi U-2000 spectrophotometer at room temperature.

*Lipid Association with Alveolar Type II Cells.* Alveolar type II cells were isolated from the lungs of male Sprague−Dawley rats by tissue dissociation with elastase digestion and purification on metrizamide density gradients (*41*). Association of phospholipids with freshly isolated alveolar type II cells was performed by a modified method (*37*) based on that described by Wright et al. (*32*). Type II cells ($2 \times 10^6$ cells) were incubated with radiolabeled phospholipid liposomes (100 $\mu$g/mL) composed of DPPC/egg PC/PG (7:2:1, w/w/w) and a trace amount of [$^3$H]DPPC in the presence of 5 or 20 $\mu$g/mL recombinant proteins at 37 °C for 1 h. After the incubation, the cells and the media were separated by centrifugation at 1500 rpm for 5 min at 4 °C. The cells were washed 3 times with phosphate-buffered saline containing 1 mg/mL bovine serum albumin, and the radioactivity associated with the cells was counted.

*Inhibition of Lipid Secretion and Receptor Binding.* Lipid secretion from type II cells was performed by analysis of the cellular phosphatidylcholine pool labeled with [$^3$H]-choline, as described previously (*42*); 5 or 10 $\mu$g/mL wt SP-A or chimera and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) ($10^{-7}$ M) as an antagonist were used. SP-A binding to alveolar type II cells was measured using rat $^{125}$I-SP-A. SP-A receptor recognition of the chimeras was determined by measuring the competition for $^{125}$I-SP-A binding (*37*, *43*).

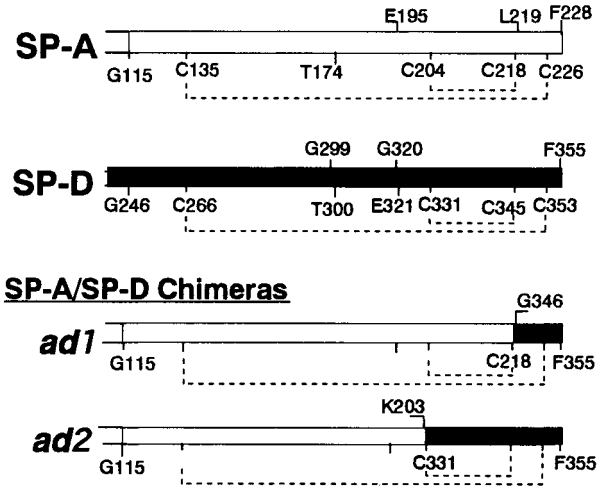


FIGURE 1: Schematic representation of the CRDs of SP-A/SP-D chimeras. The structures and splice junctions of the carbohydrate recognition domains (CRD) of SP-A, SP-D, and chimeras (*ad1* and *ad2*) are shown. The open regions are from rat SP-A, and the filled regions are from rat SP-D. The dashed lines represent the intramolecular disulfide bonds.

*Monoclonal Antibody Binding to Chimeric Proteins.* Monoclonal antibodies (mAbs) to rat SP-A were prepared as described previously (*42*). mAbs 1D6 and 6E3 have been shown to recognize the CRD and the neck domain, respectively (*44*). The binding of mAbs to wt SP-A and chimeras was examined by ELISA. Fifty microliters of the purified recombinant proteins (1 $\mu$g/mL) was coated onto microtiter wells. After nonspecific binding was blocked with phosphate-buffered saline containing 3% (w/v) skim milk and 0.1% (v/v) Triton X-100, the wells were incubated with 10 $\mu$g/mL mAb 1D6 or 6E3, or anti-rat SP-A or anti-rat SP-D polyclonal antibody at 37 °C for 90 min. The wells were then washed and incubated with HRP-labeled anti-mouse IgG or anti-rabbit IgG. The binding of antibodies to the recombinant proteins was determined by measuring the absorbance at 492 nm using *o*-phenylenediamine as a substrate for the peroxidase reaction.

## RESULTS

*Characterization of Chimeric Recombinant Proteins.* We constructed chimeras in which regions of the SP-D CRD were substituted for the corresponding regions of SP-A as shown schematically in Figure 1. Chimera *ad1* consists of $Asn^1-Cys^{218}$ of rat SP-A and $Gly^{346}-Phe^{355}$ of rat SP-D. Chimera *ad2* consists of $Asn^1-Lys^{203}$ of rat SP-A and $Cys^{331}-Phe^{355}$ of rat SP-D.

Wild type (wt) SP-A, chimeras *ad1* and *ad2*, and wt SP-D were expressed using the baculovirus expression system, and purified by affinity chromatography on mannose−Sepharose 6B. The binding properties of the purified recombinant proteins to mannose−Sepharose beads were examined and were not significantly different. The percentages of the protein bound to the beads were 79.2 ± 7.9% (mean ± SE, $n = 3$), 84.9 ± 5.1%, 61.5 ± 9.7%, and 65.3 ± 12.2% for wt SP-A, chimeras *ad1* and *ad2*, and wt SP-D, respectively. When each recombinant protein was purified from the serum-free culture media by a mannose−Sepharose 6B column after 3 days incubation of *Trichoplusia ni* cells infected with recombinant baculovirus, the protein contents in the fraction

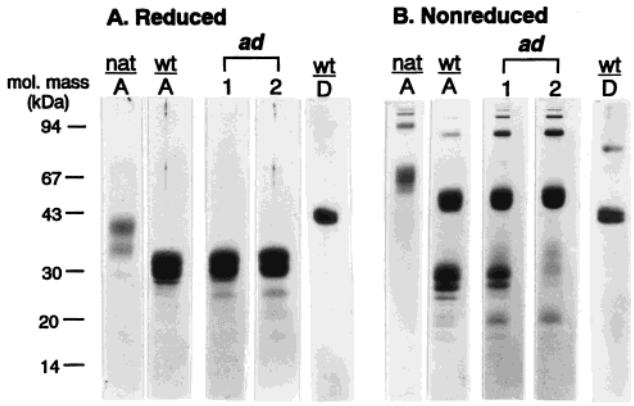


FIGURE 2: Electrophoretic analysis of SP-A and recombinant proteins. Proteins were separated on an SDS−polyacrylamide gel (13%) by electrophoresis under reducing conditions (panel A) and nonreducing conditions (panel B) and stained with Coomassie brilliant blue. Abbreviations: nat/A, native SP-A; wt/A, wild-type SP-A; ad/1, chimera *ad1*; ad/2, chimera *ad2*; wt/D, wt SP-D.

that passed through the column and in the eluted fraction were determined by sandwich ELISA. The percentages of the protein in the media adhered to the mannose−Sepharose column were 71.7%, 83.7%, and 85.3% of wt SP-A, chimera *ad1*, and chimera *ad2*, respectively. The results indicate that there are not significant differences in carbohydrate binding among the recombinant proteins. The preservation of lectin activity indicates that the recombinant proteins exhibit no global defects in protein folding.

The purified recombinant proteins and native rat SP-A were analyzed by SDS−polyacrylamide gel electrophoresis. Native rat SP-A migrated at 26−38 kDa under reducing conditions (Figure 2A, nat/A). The recombinant proteins produced in insect cells typically migrated faster than native SP-A due to the differences in glycosylation and hydroxylation between insect cells and mammalian cells, as described previously (*45*). wt SP-A and chimeric proteins migrated as bands at 27−32 kDa under reducing conditions (Figure 2A, wt/A and *ad*/1 and -2). wt SP-D migrated as a band at approximately 40−43 kDa under reducing conditions. Analysis under nonreducing conditions revealed that wt SP-A and chimeras migrated as oligomers (Figure 2B). wt SP-D appeared mainly as a monomer with small populations of oligomers under nonreducing conditions, as described previously (*37*). Both wt SP-A and wt SP-D retain all of the functions of their native counterparts (*45*, *46*).

The chimeric proteins were also analyzed by gel permeation chromatography (Figure 3). The protein in each fraction was detected by sandwich ELISA. Recombinant wild-type SP-A eluted as a broad asymmetric peak at positions between blue dextran and ferritin. Most of wt SP-A eluted before the peak of aldolase. The highest peaks of chimeras *ad1* and *ad2* eluted at a postion between blue dextran and thyroglobulin. They also were exhibited as asymmetric peaks, suggestive of multiple components. Although monomeric and dimeric forms of the recombinant proteins were observed by gel electrophoresis under nonreducing and denaturing conditions, the forms of the proteins observed under nondenaturing conditions were highly oligomeric.

*Interactions of Chimeric Proteins with Lipids.* We examined the binding of chimeras *ad1* and *ad2* to DPPC liposomes. The protein contents of the bound and unbound

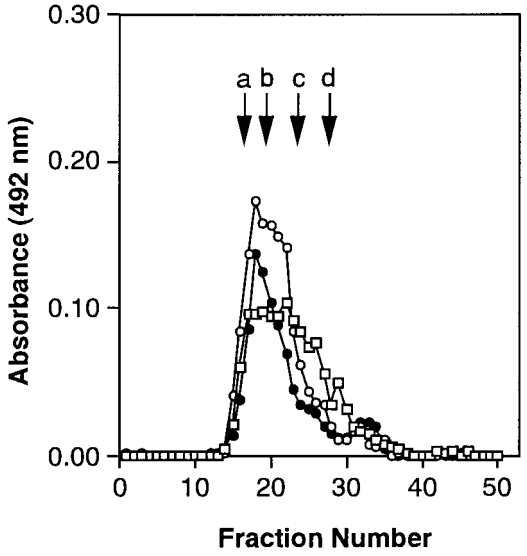


FIGURE 3: Gel filtration chromatography of recombinant proteins. Wild-type SP-A (□), chimera *ad1* (○), and chimera *ad2* (●) were subjected to Sephacryl S300 column chromatography (1.5 × 100 cm column). Each fraction (3 mL) was analyzed using an SP-A ELISA as described under Experimental Procedures. The arrows show the peak positions of the eluted standards: a, blue dextran; b, thyroglobulin; c, ferritin; d, aldolase.

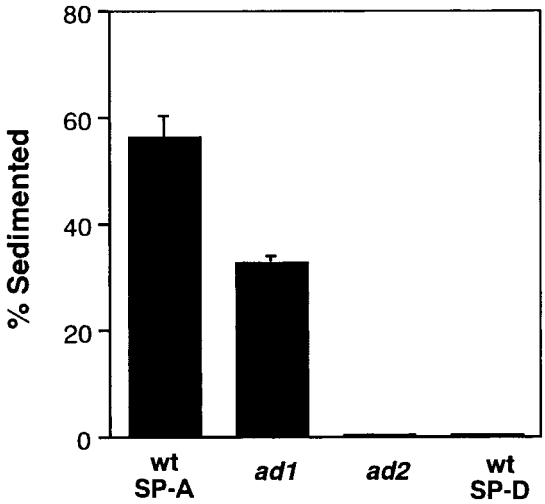


FIGURE 4: Binding of chimeric proteins to DPPC. Multilamellar liposomes (100 $\mu$g/tube) containing DPPC were mixed with 0.2 $\mu$g of wt SP-A, chimera *ad1* or *ad2*, or wt SP-D and incubated at room temperature for 1 h. The amount of protein that cosedimented with liposomes was determined by ELISA, as described under Experimental Procedures. The results show specific sedimentation that was determined by subtracting values obtained when liposomes were omitted (nonspecific sedimentation) from total sedimentation. The data shown are mean ± SE of five experiments.

fractions were determined by ELISA. The wt SP-A bound avidly to DPPC liposomes (Figure 4), and 56% of the protein cosedimented with lipid. Chimera *ad1* retained a significant level of DPPC binding with 30% of the protein cosedimenting with lipid. However, chimera *ad2* lost essentially all DPPC binding and exhibited a characteristic similar to wt SP-D (Figure 4).

SP-A induces aggregation of phospholipid liposomes containing DPPC in the presence of $Ca^{2+}$ (*30*), and we examined the ability of chimeras *ad1* and *ad2* to induce liposome aggregation. wt SP-A at concentrations of 10 and 20 $\mu$g/mL induced liposome aggregation in the presence of

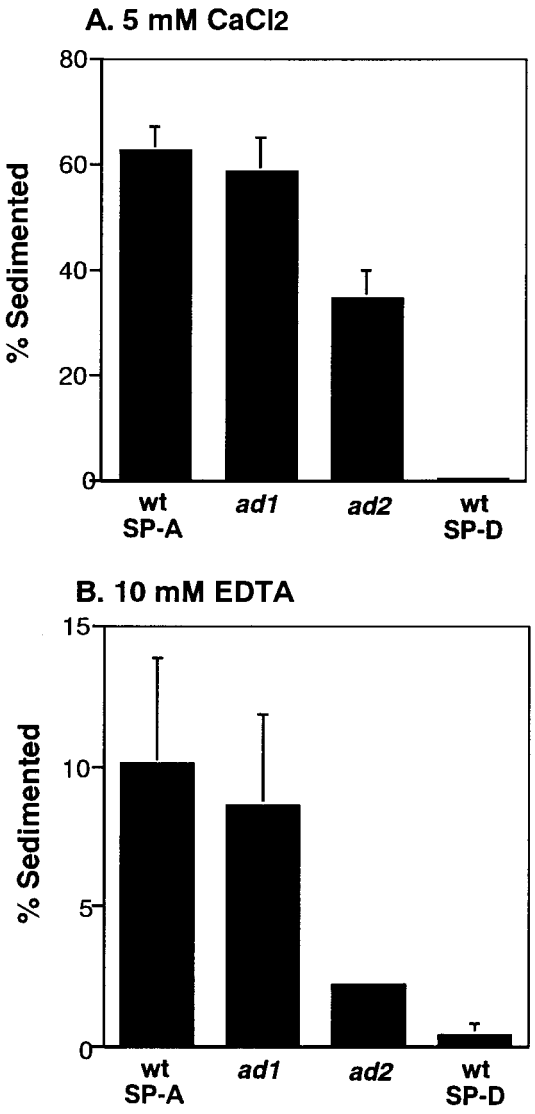


FIGURE 5: Binding of chimeric proteins to GalCer. Multilamellar liposomes (100 $\mu$g/tube) containing the lipid mixture GalCer/PS/cholesterol (7:2:1) were combined with 0.2 $\mu$g of wt SP-A, chimera *ad1* or *ad2*, or wt SP-D and incubated at room temperature for 1 h in the presence of 5 mM $CaCl_2$ (A) or 10 mM EDTA (B). The amount of protein that cosedimented with liposomes was determined by ELISA, as described under Experimental Procedures. The results show specific sedimentation that was determined by subtracting values obtained when liposomes were omitted (nonspecific sedimentation) from total sedimentation. The data shown are mean ± SE of three experiments.

5 mM $Ca^{2+}$, while chimeras *ad1* and *ad2* failed to cause aggregation of liposomes containing DPPC even at 80 $\mu$g/mL.

In contrast to their interaction with DPPC binding, both chimeras bound GalCer. Chimera *ad1* bound to liposomes containing GalCer in the presence of 5 mM $CaCl_2$ at a level (59%) comparable to that of wt SP-A (62%) (Figure 5A). Chimera *ad2* retained significant binding (35%) to GalCer but was lower than SP-A or *ad1*. The wt SP-D showed almost no binding to GalCer, as described previously (*37*).

Because SP-A binds to GalCer in a $Ca^{2+}$-dependent and also, in part, a $Ca^{2+}$-independent manner (*14*), we examined the binding of chimeras *ad1* and *ad2* to GalCer in the presence of 10 mM EDTA. Inclusion of EDTA in the binding buffer reduced the GalCer liposome binding. The binding

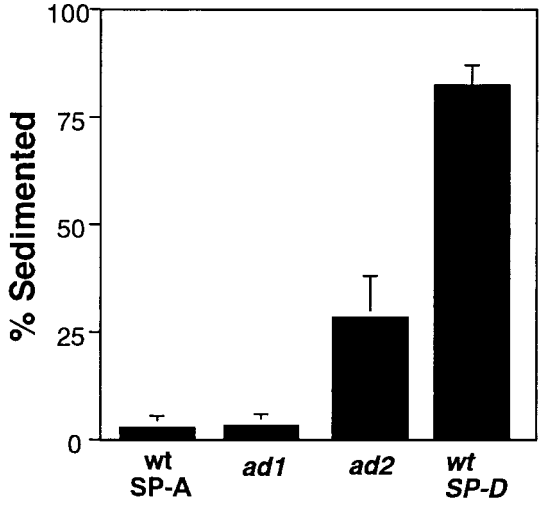


FIGURE 6: Binding of chimeric proteins to PI. Multilamellar liposomes (100 $\mu$g/tube) containing PI were mixed with 0.2 $\mu$g of wt SP-A, chimera *ad1* or *ad2*, or wt SP-D and incubated at room temperature for 1 h. The amount of protein that cosedimented with liposomes was determined by ELISA, as described under Experimental Procedures. The results show specific sedimentation that was determined by subtracting values obtained when liposomes were omitted (nonspecific sedimentation) from total sedimentation. The data shown are mean ± SE of three experiments.

of wt SP-A, chimera *ad1*, or chimera *ad2* to GalCer liposome in the presence of EDTA was 10.1%, 8.6%, or 2.3%, respectively (Figure 5B). The amount of chimera *ad2* cosedimented with GalCer liposome under this condition was significantly higher than that of wt SP-D ($p < 0.02$) (Figure 5B), indicating that chimera *ad2* exhibits very weak $Ca^{2+}$-independent GalCer binding. Taken together, these data indicate that chimeras *ad1* and *ad2* exhibit both $Ca^{2+}$-dependent and $Ca^{2+}$-independent GalCer binding.

A previous study from this laboratory (*37*) showed that the SP-D region of $Glu^{321}$−$Phe^{355}$ is essential for the binding of SP-D to PI, since chimera *ad3* exhibited a significant binding to PI although its binding was weaker than that of chimera *ad5*. Chimera *ad5* bound to PI at a level comparable to wt SP-D (*37*). In the current study, we examined whether substituting the shorter SP-D region of $Gly^{346}$−$Phe^{355}$ or $Cys^{331}$−$Phe^{355}$ for the corresponding SP-A region can introduce SP-D type PI recognition into SP-A. Chimera *ad1* as well as wt SP-A showed a negligible binding to PI liposomes (Figure 6). Chimera *ad2* exhibited significant binding to PI liposomes (Figure 6), although its binding was clearly reduced by comparison with wt SP-D. Twenty-eight percent of chimera *ad2* cosedimented with PI liposomes whereas 85% of wt SP-D bound the lipid under the same conditions. These data indicate that the SP-D region of $Cys^{331}$−$Phe^{355}$ is essential for the binding of SP-D to PI but that this region is not sufficient for maximal PI binding.

*Interaction with Alveolar Type II Cells.* SP-A augments lipid association with alveolar type II cells (*32*, *33*). The ability of chimeras to stimulate liposome association with type II cells was investigated. wt SP-A enhanced lipid association with type II cells in a concentration-dependent manner; 4.6% and 18.1% of liposomes were associated with the cells in the presence of 5 and 20 $\mu$g/mL wt SP-A, respectively. When 5 or 20 $\mu$g/mL chimera *ad1* or *ad2* was incubated with type II cells in the presence of 100 $\mu$g/mL liposomes containing DPPC, none of the chimeras stimulated

lipid association.

SP-A also inhibits lipid secretion from alveolar type II cells (*25, 26*) through the interaction of SP-A with specific receptor expressed on type II cells (*29*). We investigated whether the chimeras inhibit lipid secretion from type II cells in the presence of excess mannose. Previous studies demonstrate that SP-A mediates inhibition of lipid secretion that is insensitive to mannose inhibition. The wt SP-A inhibited TPA-stimulated lipid secretion from type II cells at a level below basal secretion in the presence of 0.2 M mannose. However, the TPA-stimulated lipid secretion observed for chimeras *ad1* and *ad2* ranged from 96% to 110% of control values. Competition experiments with $^{125}$I-SP-A for type II cell binding were also performed in the presence of 0.2 M mannose, since the inhibitory activity on lipid secretion correlates well with the capacity of SP-A to bind a high-affinity receptor expressed on alveolar type II cells (*29*). Both chimeras were ineffective in competing with rat $^{125}$I-SP-A for receptor occupancy, although the binding of labeled SP-A was reduced to 23% of control binding when 40 $\mu$g/mL wt SP-A was present.

Taken together, these results demonstrate that the SP-A region of Leu$^{219}$−Phe$^{228}$ is required for SP-A−type II cell interaction and that our chimeric constructs have lost this activity.

*Monoclonal Antibody Binding.* The epitopes for mAbs 1D6 and 6E3 are located at the CRD and the neck region of rat SP-A, respectively (*44*). The mAb 1D6 blocks the interactions of SP-A with DPPC and GalCer, liposome aggregation, the inhibitory effect of SP-A on lipid secretion, and the stimulation of lipid association with type II cells (*42, 44*). It is likely that the epitope for mAb 1D6 is located at the SP-A region responsible for its multiple functions. The binding of mAbs to the purified chimeras was determined by ELISA (Figure 7). Anti-SP-A polyclonal antibody bound to chimeras *ad1* and *ad2*, giving reactivities of 72 and 42%, respectively. Anti-SP-D polyclonal antibody did not recognize either chimera *ad1* or chimera *ad2*. The mAb 6E3 bound to both chimeras and wt SP-A. In contrast, mAb 1D6 did not bind to chimera *ad1* or chimera *ad2*. These results indicate that the substitution of the SP-D region Gly$^{346}$−Phe$^{355}$ for the SP-A region Leu$^{219}$−Phe$^{228}$ completely disrupts the epitope for mAb 1D6. The data suggest that the carboxy-terminal region of Leu$^{219}$−Phe$^{228}$ is involved in the expression of SP-A specific functions.

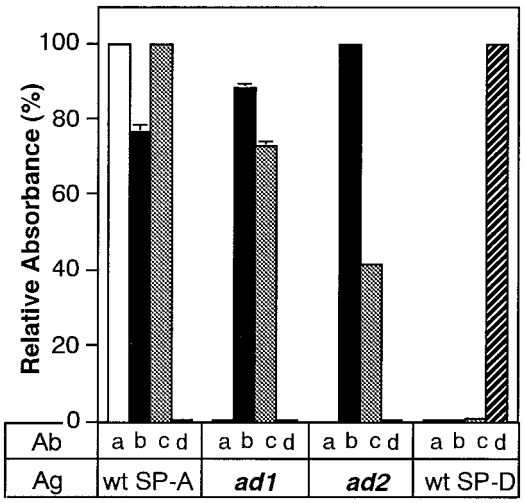


FIGURE 7: Monoclonal antibody binding to SP-A/SP-D chimeras. The antibody binding to the purified chimeric proteins was determined by ELISA. Aliquots (50 $\mu$L, 1 $\mu$g/mL) of wild-type (wt) SP-A, wt SP-D, or chimera *ad1* or *ad2* were coated onto microtiter wells. The wells were then incubated with monoclonal antibody 1D6 (a, white bars) or 6E3 (b, black bars), anti-rat SP-A polyclonal antibody (c, cross-hatched bars), or anti-rat SP-D polyclonal antibody (d, hatched bars) as described under Experimental Procedures. The relative absorbance is expressed as percent of maximal absorbance at 492 nm for each protein. The mean of the maximal absorbance for each protein was as follows: anti-SP-A polyclonal antibody to wt SP-A, 1.805; 1D6 binding to wt SP-A, 1.192; 6E3 binding to chimera *ad2*, 1.027; anti-SP-D polyclonal antibody to wt SP-D, 0.797. The data presented are mean ± SE of three experiments.

## DISCUSSION

Although SP-A and SP-D are homologous and both bind mannose, the lipid binding specificities and the property of interacting with alveolar type II cells are different from each other. This study along with others *(37, 47, 48, 49)* provides evidence that carbohydrate recognition and lipid recognition are distinct functions that are experimentally reasonable. It is probable that multiple protein surfaces, and residues that are distinct from those directly involved in carbohydrate ligation, participate in the lipid binding phenomenon. We now identify an essential region of the carboxy-terminal amino acid residues of lung collectins by determining which chimera loses an SP-A property or acquires an SP-D property.

A previous study (*37*) from this laboratory showed that chimera *ad3*, in which the SP-A region of Glu$^{195}$−Phe$^{228}$ was replaced with the SP-D region of Glu$^{321}$−Phe$^{355}$, lost SP-A specific functions, and that the chimera acquired a PI binding property. The study indicated that the SP-A region of Glu$^{195}$−Phe$^{228}$ is required for binding DPPC and GalCer, inducing liposome aggregation, and interacting with alveolar type II cells; and that the SP-D region of Glu$^{321}$−Phe$^{355}$ is essential for PI binding. In the present study, we focused on amino acid residues 204−228 of rat SP-A and 331−355 of rat SP-D. The purpose of this study was to identify an essential stretch of primary sequence in the carboxy-terminal regions of SP-A and SP-D that is involved in lipid and type II cell interaction. This study shows that substituting the SP-D region Gly$^{346}$−Phe$^{355}$ for the SP-A region Leu$^{219}$−Phe$^{228}$ completely destroys the activities of liposome aggregation, stimulating lipid association with type II cells and inhibiting lipid secretion from type II cells. Replacement of the SP-A region Cys$^{204}$−Phe$^{228}$ with the corresponding SP-D region disrupted the activity of DPPC binding, but the GalCer binding activity was retained. The results demonstrate that the SP-A region of Cys$^{204}$−Phe$^{228}$ is critical for all SP-A specific functions we measured except for mannose−Sepharose and GalCer binding. The region of SP-A from Cys$^{204}$ to Leu$^{219}$ appears sufficient for the specific recovery of DPPC interaction without recovery of type II cell interaction. The findings clearly demonstrate that the structural requirement for SP-A binding to lipids is different from that for interactions with alveolar type II cells. Since chimera *ad3* in the previous study (*37*) failed to bind to GalCer but chimeras *ad1* and *ad2* in this study retained GalCer binding activity, these studies suggest that the SP-A region Glu$^{195}$−Lys$^{203}$ is important for the binding of SP-A to GalCer.

Another study from this laboratory (*43*) revealed that the SP-A/MBP-A chimeras, which were composed of the SP-A region $Asn^{1}$−$Cys^{218}$ and the MBP-A region $Gln^{210}$−$Ala^{221}$ (chimera AM1) and of the SP-A region $Asn^{1}$−$Lys^{203}$ and the MBP-A region $Cys^{195}$−$Ala^{221}$ (chimera AM2), retained SP-A functions although MBP-A does not bind DPPC or GalCer. The SP-A/MBP-A chimeras induced liposome aggregation, inhibited lipid secretion, or stimulated lipid association with type II cells. These latter findings demonstrate that the carboxy-terminal MBP-A regions of $Gln^{210}$−$Ala^{221}$ and of $Cys^{195}$−$Ala^{221}$ can functionally replace the corresponding SP-A regions without loss of SP-A function. In the present study, however, the SP-A/SP-D chimeras *ad1* and *ad2*, corresponding to the SP-A/MBP-A chimeras AM1 and AM2, respectively, lost some of the SP-A functions. This study demonstrates that the carboxy-terminal SP-D region of $Cys^{218}$−$Phe^{355}$ or of $Cys^{331}$−$Phe^{355}$ cannot functionally replace the corresponding SP-A regions. Although SP-A, SP-D, and MBP-A exhibit similarities in carbohydrate binding specificity and 48−56% sequence identity between the SP-A region of $Cys^{203}$−$Phe^{228}$ and the corresponding region of SP-D or MBP-A, it is not known why the results obtained from two studies with chimeras are different. In the absence of a crystal structure for SP-A that demonstrates specific contact sites with ligands, the current mapping studies with chimeras provide the best way to probe these interactions.

Chimera *ad1* retained activity of binding DPPC liposomes, whereas this chimera failed to cause aggregation of liposomes containing DPPC. Liposome aggregation is likely to require some form of molecular cross-linking between SP-A and lipid vesicles. Thus, the present results indicate that the SP-A region of $Leu^{219}$−$Phe^{228}$ may not be critical for DPPC binding, but that this region is insufficient for cross-linking of the SP-A/lipid mixture.

Because chimera *ad1* failed to interact with type II cells in this study, we infer that the SP-A region of $Leu^{219}$−$Phe^{228}$ is critical for type II cell interactions. We have recently found that the SP-A region of $Thr^{174}$−$Gly^{194}$ is also required for DPPC and type II cell interactions since chimera *ama4*, in which the rat MBP-A region of $Thr^{164}$−$Asp^{184}$ was substituted for the corresponding rat SP-A region of $Thr^{174}$−$Gly^{194}$, lost all SP-A specific functions except $Ca^{2+}$-independent GalCer binding (*47*). Collectively, these studies indicate that the rat SP-A regions of $Leu^{219}$−$Phe^{228}$ and of $Thr^{174}$−$Gly^{194}$ are both essential for type II cell interaction. It is reasonable to assume that multiple protein surfaces will interact with ligands simultaneously if one considers the three-dimensional aspect of the ligand interactions.

A recent study (*47*) also indicates that the SP-A region of $Thr^{174}$−$Gly^{194}$ is involved in $Ca^{2+}$-dependent GalCer binding because chimera *ama4* lost $Ca^{2+}$-dependent GalCer binding activity but retained the $Ca^{2+}$-independent GalCer binding. In this study chimeras *ad1* and *ad2* exhibited $Ca^{2+}$-dependent binding to GalCer. Since chimeras *ad1* and *ad2* contain the SP-A region of $Thr^{174}$−$Gly^{194}$, the present result is consistent with that obtained from chimera *ama4*.

Substituting the SP-D region $Gly^{346}$−$Phe^{355}$ for the SP-A region $Leu^{219}$−$Phe^{228}$ completely disrupted the epitope for mAb 1D6 since this antibody did not recognize chimera *ad1*. This study demonstrates that the epitope for mAb 1D6 is located at a region contiguous to the carboxy-terminal SP-A region $Leu^{219}$−$Phe^{228}$. The results obtained from functional assays with chimera *ad1* correlate well with those obtained from the mAb binding to this chimera with respect to liposome aggregation and type II cell interaction since mAb 1D6 completely blocked these SP-A functions (*42*, *44*). Although mAb 1D6 blocked the binding of SP-A to DPPC and GalCer (*44*) and this antibody failed to bind chimera *ad1* in the present study, the chimera *ad1* retained the DPPC and GalCer binding activity. The discrepancy between lipid binding of chimera *ad1* and mAb 1D6 recognition of this chimera may be explained by steric hindrance to the region essential for lipid binding due to the binding of mAb 1D6 to the carboxy-terminal region containing $Leu^{219}$−$Phe^{228}$.

Previous work (*37*) provided evidence that the SP-D region of $Glu^{321}$−$Phe^{355}$ is required for the binding of SP-D to PI. In this study, chimera *ad2* avidly bound to PI liposomes although with lower affinity than wt SP-D. The current work now narrows the minimal SP-D structure required for PI binding to the SP-D region of $Cys^{331}$−$Phe^{355}$. Since chimera *ad1* exhibited almost no binding to PI, the SP-D region of $Cys^{331}$−$Cys^{345}$ is proposed to be important for PI binding.

In summary, we focused on the carboxy-terminal SP-A region of $Cys^{204}$−$Phe^{228}$ and the SP-D region of $Cys^{331}$−$Phe^{355}$. The current work now narrows the regions of lung collectins essential for lipids and type II cell interactions. Chimeras *ad1* and *ad2* lost SP-A specific functions for liposome aggregation and type II cell interaction. Chimera *ad1* but not *ad2* bound DPPC liposomes. These chimeras bound to GalCer with rank order of *ad1* > *ad2*. Chimera *ad2* but not *ad1* bound to PI. Neither chimera *ad1* nor chimera *ad2* was recognized by mAb 1D6. From these results, we conclude that (1) the SP-A region of $Leu^{219}$−$Phe^{228}$ is required for phospholipid liposome aggregation, inhibition of lipid secretion, and augmentation of lipid association with type II cells; (2) the SP-A region of $Cys^{204}$−$Cys^{218}$ is critical for DPPC binding; (3) the SP-D region of $Cys^{331}$−$Phe^{355}$ is essential for minimal PI binding; and (4) the epitope for mAb 1D6 is located at the region contiguous to the SP-A region $Leu^{219}$−$Phe^{228}$.

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