Introduction of Mannose Binding Protein-Type Phosphatidylinositol Recognition into Pulmonary Surfactant Protein A[†]

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Received February 12, 1999; Revised Manuscript Received April 2, 1999

ABSTRACT: Pulmonary surfactant protein A (SP-A) and mannose-binding protein A (MBP-A) are collectins in the C-type lectin superfamily. These collectins exhibit unique lipid binding properties. SP-A binds to dipalmitoyl phosphatidylcholine (DPPC) and galactosylceramide (GalCer) and MBP-A binds to phosphatidylinositol (PI). SP-A also interacts with alveolar type II cells. Monoclonal antibodies (mAbs PE10 and PC6) that recognize human SP-A inhibit the interactions of SP-A with lipids and alveolar type II cells. We mapped the epitopes for anti-human SP-A mAbs by a phage display peptide library. Phage selected by mAbs displayed the consensus peptide sequences that are nearly identical to ^{184}TPVNYT-NWYRG^{194} of human SP-A. The synthetic peptide GTPVNYTNWYRG completely blocked the binding of mAbs to human SP-A. Chimeric proteins were generated in which the rat SP-A region Thr^{174}-Gly^{194} or the human SP-A region Ser^{174}-Gly^{194} was replaced with the MBP-A region Thr^{164}-Asp^{184} (*rat ama4* or *hu ama4*, respectively). The mAbs failed to bind *hu ama4*. *Rat ama4* bound to an affinity matrix on mannose-sepharose but lost all of the SP-A functions except carbohydrate binding and Ca^{2+}-independent GalCer binding. Strikingly, the *rat ama4* chimera acquired the PI binding property that MBP-A exhibits. This study demonstrates that the amino acid residues 174−194 of SP-A and the corresponding region of MBP-A are critical for SP-A-type II cell interaction and Ca^{2+}-dependent lipid binding of collectins.

Pulmonary surfactant proteins A (SP-A)[1] and D (SP-D) and mannose-binding protein (MBP) belong to the collectin subgroup of C-type lectin superfamily (*1*, *2*). 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). SP-A and MBP form bouquet-like structures consisting of four to six trimeric subunits (*3*, *4*), whereas SP-D is a dodecamer that forms a cruciform structure (*5*). The collectins exhibit specificity for mannose, glucose, and/or *N*-acetylglucosamine sugars (*6*−*8*). They also bind to certain glycosphingolipids; human MBP binds to *N*-acetylglucosamine-terminated glycosphingolipids, and rat MBP-A, a predominant form of serum MBP, binds neoglycolipids containing terminal *N*-acetylglucosamine residues (*9*, *10*). SP-A binds to galactosylceramide (GalCer), lactosylceramide and asialo-G_{M2} (*11*, *12*). SP-D binds to glucosylceramide (*13*). We have previously shown that phospholipids are also ligands for these collectins; SP-A and SP-D specifically bind dipalmitoyl phosphatidylcholine (DPPC) and phosphatidylinositol (PI), respectively (*14*, *15*). Rat MBPs were isolated from serum and liver bind PI, and liver MBP also binds to phosphatidylglycerol and weakly to phosphatidylserine (*16*). The phospholipid binding specificity of SP-A may be important for recycling and reutilization of DPPC, the lipid component essential for surface activity of pulmonary surfactant. The physiological relevance of the interactions of SP-D and MBP with PI remains unclear.

Compelling evidence has been accumulated that the collectins play important roles in the innate immune system, which is critical in the first line host defense. They bind a wide range of microbial species including Gram-negative bacteria, *Pneumocystis carinii*, and Herpes simplex type I (*17*−*20*). They recognize distinct but overlapping ligands of carbohydrates and lipids that decorate the cell wall of pathogens. In addition, the collectins interact with alveolar macrophages (*21*−*23*). SP-A and MBP bind to the C1q receptor (*24*, *25*). MBP has been known to activate complement pathway (*26*, *27*).

The in vitro functions of SP-A have all of the characteristics that are expected as a regulatory protein involved in surfactant homeostasis in the alveoli. SP-A inhibits the secretion of phosphatidylcholine from alveolar type II cells

[†] Supported in part by Grant-in-Aid (Grants 10557058, 09670159, and 09470147) for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, and NIH Grant HL45286.

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[1] Abbreviations: SP-A, surfactant protein A; SP-D, surfactant protein D; MBP-A, mannose binding protein A; wt rSP-A, wild-type recombinant rat SP-A; wt hSP-A, wild-type recombinant human SP-A; *hu ama4*, chimeric construct of human SP-A and rat [T164-D184] MBP-A; *rat ama4*, chimeric construct of rat SP-A and rat [T164-D184] MBP-A; AM3, chimeric construct of rat SP-A and rat [E185-A221] MBP-A; mAbs, monoclonal antibodies; DPPC, dipalmitoyl phosphatidylcholine; PI, phosphatidylinositol; GalCer, galactosylceramide.

10.1021/bi990353e CCC: $18.00
Published on Web 05/08/1999

(*28*, *29*). It binds to alveolar type II cells with high affinity (*21*, *30*), and a receptor-binding activity correlates well with the inhibitory activity of SP-A on lipid secretion (*31*). SP-A enhances the uptake of liposomes by type II cells (*32*) and the lipid uptake stimulated by SP-A is preferential to liposomes containing DPPC (*33*). It promotes the association of lipid aggregates with the surface of type II cells (*34*). The protein also causes aggregation of liposomes (*35*) and facilitates the formation of an unusual phospholipid structure called tubular myelin, in coordination with surfactant protein B (*36*). Despite numerous in vitro functions of SP-A that implicate it in surfactant homeostasis, mice homozygous for null alleles of SP-A are viable and exhibit only modest alterations in surfactant phospholipid structure (*37*, *38*). This latter result may simply reflect functional redundancy within the surfactant system. However, the relationship between the in vivo functions of SP-A and the in vitro activities is currently unclear.

Anti-human SP-A (hSP-A) monoclonal antibodies (mAbs) PC6 and PE10 (*39*) have been shown to recognize the peptide portion of hSP-A within the CRD. Both PC6 and PE10 block the inhibitory effect of hSP-A on lipid secretion and attenuate the binding of the protein to alveolar type II cells (*40*, *41*). These studies suggest that the epitope for the mAbs is involved in the expression of SP-A functions. A previous study (*41*) suggested that an area contiguous to or near the region containing Glu^{202}-Met^{207} of hSP-A might be important, since antibody against the synthetic peptide containing this region inhibited the binding of mAb PE10 to hSP-A and also attenuated the binding of [^{125}I]SP-A to type II cells and the inhibitory effect of hSP-A on lipid secretion. However, the interpretation of the results obtained by this approach would be restricted if the binding of anti-peptide antibody to hSP-A causes steric hindrance of an essential region or an epitope when it interacts with a ligand. In this study, we further investigated the effect of mAbs on the interactions of hSP-A with lipids and mapped the epitope for mAbs using a phage display peptide library. We further generated a chimeric protein in which the SP-A region containing the mAbs epitope is replaced with the corresponding MBP-A region and examined the interactions of the chimera with lipids and alveolar type II cells. The results demonstrate that the SP-A region containing mAbs epitope is essential for SP-A-type II cell interactions and that this region is critical for the binding of collectins to phospholipids.

EXPERIMENTAL PROCEDURES

Lipids. Phosphatidylcholine (PC) from egg yolk, phosphatidylinositol (PI) from bovine liver, phosphatidylglycerol (PG) from egg yolk, phosphatidylserine (PS) from bovine brain, dipalmitoyl phosphatidylcholine (DPPC), and galactosylceramide (GalCer) was 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.

Antibodies. Monoclonal antibodies (mAbs), PE10 and PC6, against human SP-A were prepared as described previously (*39*). Both PE10 and PC6 recognize the peptide portion of SP-A CRD (*40*, *41*). Monoclonal antibody 6E3 against rat SP-A was prepared as described previously (*42*). Polyclonal antisera against human SP-A (hSP-A) and rat serum MBP were also raised in New Zealand White rabbits as described previously (*16*, *43*). All antibodies were purified by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia Biotech Inc.).

Screening of Peptide Ligands for mAbs PE10 and PC6 with a Phage Display Peptide Library. The phage display peptide library kit (New England Biolabs Inc.), which is based on a combinatorial library of random peptide 12-mers fused to a minor coat protein (pIII) of the filamentous coliphage M13, was used in this study. First, 50 μL of protein A-Sepharose CL 4B (50% aqueous suspension) was incubated in 1 mL of blocking buffer (0.1 M NaHCO3 containing 5 mg/mL BSA, 0.02% (w/v) NaN3). In the meantime, 1.4×10^{11} phage (10 μL) were combined with 300 ng of antibody PE10 or PC6 to a final volume of 200 μL with 50 mM Tris buffer (pH 7.4) containing 0.15 M NaCl and 0.1% Tween 20 (TBST) and incubated at room temperature for 20 min. The phage-antibody mixture was then transferred to the eppendorf tube containing the resin of protein A-Sepharose that had been washed with TBST and was mixed gently and incubated at room temperature for 15 min. The resin was pelleted by a low-speed microcentrifuge and washed 10 times with TBST. The bound phage was finally eluted by suspending the pelleted resin in 1 mL of 0.2 M glycine-HCl buffer (pH 2.2) containing 1 mg/mL BSA. The eluted supernatant obtained by a microcentrifuge was transferred to a new eppendorf tube and was immediately neutralized by the addition of 150 μL of 1 M Tris (pH 9.1). Eluate phage was amplified in *Escherichia coli* ER2537 host cells, and titer of amplified phage was determined. These steps were repeated twice more.

The final eluate of phage was used to infect ER2537 cells. Serial dilutions (usually $10^{-2}-10^{-6}$) of infected cells were then inoculated on LB agar plates, which were incubated overnight at 37 °C. Approximately 20 plaques were picked up from plates having no more than ~100 plaques and dispersed in 1 mL of diluted ER2537 culture and incubated at 37 °C with shaking for 5 h. DNA from the isolated phage was prepared and sequenced automated cycle sequencing with dye-labeled dideoxynucleotide using −96 gIII primer (5′- CCCTCATAGTTAGCGTAACG-3′).

Competition of Synthetic Peptide with SP-A for Antibody Binding. A peptide GTPVNYTNWYRG (peptide A) corresponding to the amino acid residues 183−194 of hSP-A and a control peptide MESPPDYSAAPRGRFG (peptide C) was synthesized by TANA Laboratories and purified by reversed-phase HPLC Inertsil C_{18} column (GL Science, Japan). Amino acid sequence was confirmed by ABI 477A amino acid sequencer (Applied Biosystem Inc.). Fifty microliters of 1 μg/mL wt hSP-A was coated onto microtiter wells (Immunlon 1B) (Dynex Laboratories, Inc.), and nonspecific binding was blocked with PBS containing 0.1% Triton X-100 and 3% (w/v) skim milk. Monoclonal antibody PC6 or PE10 (50 μL of 0.2 μg/mL) was incubated at 37 °C for 90 min in the presence of 50 μL of 0.01−10 μg/mL synthetic peptide A or C. After washing the wells, anti-mouse IgG conjugated with HRP (1:1500 dilution) was added into the wells and incubated at 37 °C for 60 min. The binding of monoclonal antibody to solid phase wt hSP-A was finally detected using *o*-phenylenediamine as a substrate.

DNA Constructs. The isolation and sequencing of the 1.6 kb cDNA for rat SP-A (*44*) and 1.13 kb cDNA for human

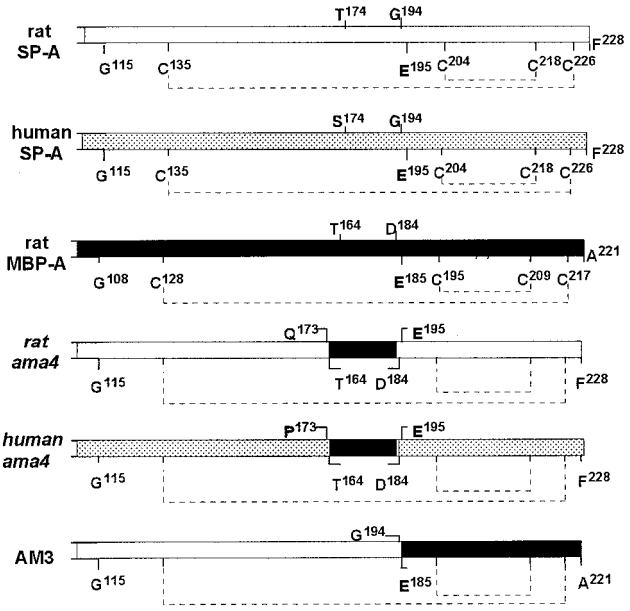


FIGURE 1: Schematic representation of the CRDs of chimeric proteins. The structures of the CRDs of rat SP-A, human SP-A, rat MBP-A, rat SP-A/rat MBP-A chimera *rat ama4*, human SP-A/rat MBP-A chimera *human ama4*, and rat SP-A/rat MBP-A chimera AM3 are shown. The regions that are *unfilled* are from rat SP-A, the regions that are *stippled* are from human SP-A, and the regions that are *black* are from rat MBP-A. The dashed lines represent the intramolecular disulfide bonds. Chimera AM3 has been characterized as decribed previously (*46*).

SP-A (*43*) have been previously reported. Two primers (5′-TCTAGAGAATTCTGTGGCAGAAGCCACTGG-3′ as a sense primer and 5′-TCTAGACCCGGGTGGGACAC-CGAGCTACAG-3′ as an antisense primer) were used for constructions of rat SP-A cDNAs. *Eco*RI and *Xma*I sites were incorporated into flanking 5′ and 3′ primers, respectively. Two primers (5′-ACTAGATCTACCCAAGCAGCTGGAG-GC-3′ as a sense primer and 5′-TAACCCGGGTGGGGAA-GAGTCAGGGCC-3′ as an antisense primer) were used for constructions of human SP-A cDNAs (*43*). *Bgl*II and *Xma*I sites were incorporated into flanking 5′ and 3′ primers, respectively. A 0.79 kb cDNA for rat MBP-A was isolated from rat liver mRNAs by reverse transcription-polymerase chain reaction (RT-PCR) using two primers (5′- ACCAT-GCTCCTGCTTCCA-3′ as a sense primer and 5′- TTGAG-AAACCAGTAACTG −3′ as an antisense primer), which were designed according to the oligonucleotide sequence of rat MBP-A described by Drickamer et al. (*45*). The cDNA for wt MBP-A expression was constructed by fusing the bases 10−712 (amino acid residues 1−221) in the original MBP-A DNA sequence (*45*) with the upstream region of rat SP-A bases 30−120 containing a signal sequence and the downstream region of rat SP-A bases 803−1198 (*44*).

We constructed three chimeras in which the regions of MBP-A CRD were substituted for the corresponding CRD regions of SP-A. The chimeric molecules used in this study are schematically represented in Figure 1. Chimera rat *ama4* (*rat ama4*) consists of Asn^{1}-Gln^{173} of rat SP-A, Thr^{164}-Asp^{184} of rat MBP-A, and Glu^{195}-Phe^{228} of rat SP-A. Chimera human *ama4* (*hu ama4*) consists of Glu^{1}-Pro^{173} of human SP-A, Thr^{164}-Asp^{184} of rat MBP-A, and Glu^{195}-Phe^{228} of human SP-A. Chimera AM3 consists of Asn^{1}-Gly^{194} of rat SP-A and Glu^{185}-Ala^{221} of rat MBP-A, as described previously (*46*). The cDNAs for chimeras were constructed by the PCR and the overlap extension method (*47*). All the constructions were inserted into pVL1392 plasmid vector. The recombinant plasmids constructed were confirmed by a combination of restriction enzyme mapping and DNA sequencing.

Expression and Isolation of Recombinant Proteins. The production of recombinant proteins in the baculovirus-insect cell expression system was carried out as described by O'Reilly et al. (*48*). Recombinant baculoviruses were produced by cotransfection of *Spodoptera frugiperda* (Sf9) cells with linearized *Autographa californica* virus (Baculogold, Pharmingen) and the pVL1392 vector containing the cDNA for SP-A, MBP-A, or chimera. Plaques containing recombinant baculoviruses were isolated and amplified to $10^{-7}-10^{-8}$ plaque-forming units/mL. Recombinant proteins were expressed into serum-free media by infection of *Trichoplusia ni* cells with viral stock at a multiplicity of 2. The approximate rank order of production levels were wt rSP-A and wt hSP-A > wt MBP-A > *rat ama4*. Recombinant proteins were purified from the culture media by adsorption to mannose-Sepharose 6B, as described previously (*49*). The amounts of wt rSP-A, wt hSP-A, wt MBP-A, and *rat ama4* purified by mannose-affinity column from 100 mL culture media were 1.78 mg, 1.2 mg, 628 μg, and 128 μg (means of 3−4 preparations), respectively.

Isolation of Native Rat SP-A and Protein Labeling. Rat SP-A was purified by affinity chromatography on mannose-Sepharose 6B and gel filtration. Surfactant was isolated from Sprague−Dawley rats that had been given intratracheal instillations of 10 mg/kg of silica 4 weeks before lung lavage, as described previously (*21*). Rat SP-A (rSP-A) and wild-type human SP-A (wt hSP-A) were iodinated by the method of Bolton and Hunter (*50*) using the ^{125}I-Bolton−Hunter reagent (Amersham). More than 86% of the radioactivity was precipitated with 10% (w/v) trichloroacetic acid. The specific activity used ranged 650−1175 cpm/ng. Monoclonal antibodies PE10 and PC6 were biotinylated using *N*-hydroxysulfosuccinimide-biotin (Peirce) according to the manufacturer's instruction.

Binding of Chimeras to Lipids Coated onto Microtiter Wells. The binding of chimeras to lipids coated onto microtiter wells was performed as described previously (*49*). Lipids (1 μg/well) in 20 μL of ethanol were coated on microtiter wells (Immunlon 1B) (Dynex Laboratories, Inc.) and air-dried. After the nonspecific binding was blocked with 50 mM Tris buffer (pH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$, and 20 mg/mL bovine serum albumin, various concentrations of SP-A, MBP-A or the chimeric proteins (0−10 μg/mL) were added and incubated for 1 h at 37 °C. After the incubation, the wells were washed three times with ice-cold buffer of 50 mM Tris (pH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$, and 1 mg/mL of bovine serum albumin (washing buffer). Monoclonal antibody 6E3 (10 μg/mL), which recognizes the neck domain of rat SP-A (*51*), and polyclonal antibody to rat serum MBP (20 μg/mL) were used for the detection of wt rat SP-A (wt rSP-A) and rat SP-A/MBP-A chimeras, and wt MBP-A, respectively. Antibody was added into the wells and incubated for 1 h, followed by the incubation with HRP-labeled anti-mouse IgG or anti-rabbit 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. In some experiments 10 mM EDTA was used instead

of 5 mM $CaCl_2$ when the proteins were incubated with the solid-phase lipids and washed.

To examine the effect of monoclonal antibodies on the binding of human SP-A to lipids, ^{125}I-labeled wild type human SP-A was used according to the method described previously (*51*). [^{125}I]SP-A (0−5 μg/mL) was incubated with DPPC or GalCer coated onto microtiter wells in the absence or the presence of antibodies (100 μg/mL) for 1 h at room temperature. After washing the wells three times, the radioactivity was measured using a γ radiation counter.

Liposome Binding. The binding of the proteins to multilamellar liposomes was performed as described previously (*49*). DPPC, PI, or the lipid mixture composed of GalCer: phosphatidylserine (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 and vortexed vigorously for 5 min to prepare multilamellar liposomes. The protein solutions (0.2 μg/tube) were prepared in 50 μL of 20 mM Tris buffer (pH 7.4) containing 0.1 M NaCl, 5 mM $CaCl_2$ and 20 mg/mL of bovine serum albumin (binding buffer). Next, 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 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/tube) and centrifuged again. The supernatant was finally combined and the pellet was suspended with 100 μL of the binding buffer. The amount of the protein in each fraction was determined by sandwich ELISA using polyclonal antibody against SP-A or serum MBP, as described previously (*16*, *43*). Liposome binding was defined as percent sedimentation [the proteins in pellet/the proteins in pellet plus supernatant × 100]. Control experiments without liposomes were also performed. In some experiments 10 mM EDTA was used instead of 5 mM $CaCl_2$ for the binding buffer.

Liposome Aggregation. Liposome aggregation was carried out by the method (*49*) based on that described by Hawgood et al. (*35*). Unilamellar liposomes composed of DPPC:egg PC:PG (7:2:1, w/w/w) were prepared from the multilamellar liposomes by probe sonication for 5 min. Liposomes (200 μg/mL) and the proteins (10 and 20 μg/mL) in 20 mM Tris (pH 7.4) containing 0.15 M NaCl were preincubated for 3 min. After equilibration, the absorbance at 400 nm was measured using a Hitachi U-2000 spectrophotometer at room temperature. Following the initial absorbance readings, $CaCl_2$ was added to a final concentration of 5 mM at a time of 3 min and the absorbance was further measured until a time of 10 min. In some experiments, monoclonal antibodies PE10 and PC6 (100 μg/mL) were coincubated with wt hSP-A and liposomes to examine the effect of antibody on the SP-A induced liposome aggregation.

Interaction 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 gradients (*52*). Secretion of PC from type II cells was performed after labeling of the cellular [^{3}H]PC pool by culturing freshly isolated type II cells with [^{3}H]choline. For secretion, 12-*o*-tetradecanoylphorbol-13-acetate (TPA) was used as an agonist and recombinant SP−As were used as antagonists, as described previously (*42*). The binding of the chimera to type II cells was also performed by determining the ability of the proteins to compete with rat [^{125}I]SP-A for receptor occupancy, as described previously (*31*, *49*). Uptake of phospholipids by freshly isolated type II cells was performed using [^{3}H]DPPC by the method (49) based on that desrcibed by Wright et al. (*32*).

Protein Analysis. The recombinant proteins were analyzed by electrophoresis in 13% polyacrylamide gels in the presence of SDS (*53*) as well as immunoblotting with anti-SP-A antibodies. Protein concentrations were determined by the bicinchoninic protein assay kit (BCA) (Pierce).

RESULTS

Effects of Anti-Human SP-A Monoclonal Antibodies on Interaction of SP-A with Lipids. Previous studies (*40*, *41*) from this laboratory indicated that anti-human SP-A mAb PE10 blocked the inhibitory effect of hSP-A on lipid secretion from alveolar type II cells and that antibody PE10 attenuated the binding of SP-A to these cells. We examined whether antibodies PE10 and PC6 affect the SP-A interaction with lipids. Both PE10 and PC6 blocked the binding of wt hSP-A to DPPC coated onto microtiter wells (Figure 2A). Control mAb 6B2 did not decrease the hSP-A binding to DPPC. Both PE10 and PC6 also inhibited the SP-A binding to GalCer (Figure 2B). These antibodies decreased the binding of SP-A to DPPC to the levels of 10% of that obtained in the absence of antibodies at 5 μg/mL of SP-A, but the extent of antibody inhibition of the GalCer binding was weaker than that observed for DPPC binding. The antibodies inhibited the binding of hSP-A to GalCer by 74−78% of that obtained in the absence of antibodies at 5 μg/mL SP-A.

We also examined the effect of mAbs on SP-A-induced liposome aggregation. wt hSP-A induced phospholipid liposome aggregation in the absence and the presence of control mAb in a Ca^{2+}- and a time-depenedent manner (Figure 2C). mAbs PE10 and PC6 blocked SP-A-mediated liposome aggregation. These data indicate that two mAbs against hSP-A inhibit the interaction of hSP-A with lipids to nearly equivalent levels.

We next asked whether mAbs PE10 and PC6 recognized the same epitope. Because SP-A forms oligomers under nondenaturing condition (*4*), it is possible that two different mAbs do not effectively compete with each other for antigen protein even if they recognize the same epitope. Thus, wt hSP-A was electrophoressed under denaturing and reducing conditions and transferred to PVDF membranes, and competition experiments were performed using biotinylated mAbs (Figure 2D). After PVDF membranes were preincubated in the absence or the presence of control antibody (anti-rat SP-A mAb 6E3), mAb PE10 or PC6, the membranes were probed with biotinylated PE10 or PC6. Biotinylated mAbs bound to the glycosylated and nonglycosylated forms of SP-A monomer. Preincubation of mAbs PE10 and PC6 diminished the binding of biotinylated antibodies to SP-A monomers, indicating that both PE10 and PC6 competed for sites that are physically close to each other. The result raises the possibility that both PE10 and PC6 recognize the same epitope.

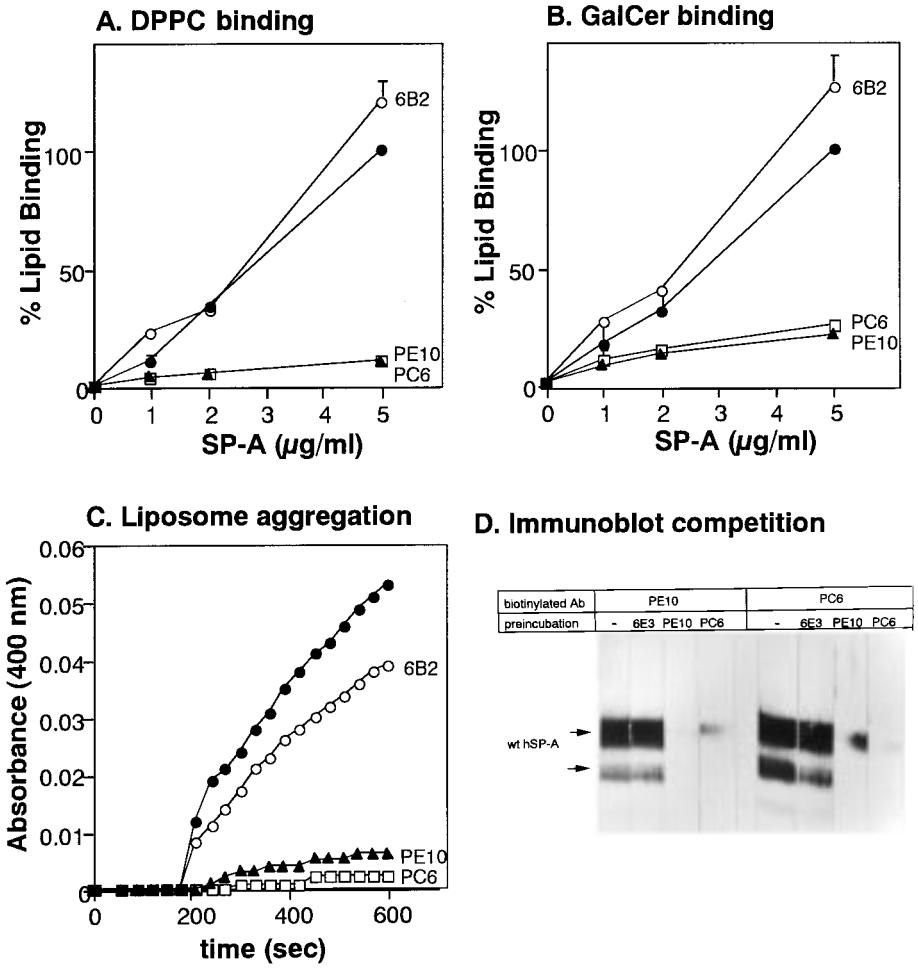


FIGURE 2: Effect of anti-human SP-A monoclonal antibodies PC6 and PE10 on interaction of SP-A with lipids. (Panels A and B) Effect on lipid binding. One microgram per well of dipalmitoyl phosphatidylcholine (DPPC) (panel A) or galactosylceramide (GalCer) (panel B) was coated onto microtiter wells, and the indicated concentrations of ^{125}I-wt hSP-A was incubated at room temperature for 1 h in the absence (●) or the presence of 100 μg/mL anti-hSP-A monoclonal antibodies PC6 (□) or PE10 (▲) or 100 μg/mL control monoclonal antibody 6B2 (○). After the incubation, the wells were washed, and the amount of SP-A that bound to lipids was determined as described in the Experimental Procedures. The results are expressed as a percentage of [^{125}I]SP-A binding at 5 μg/mL in the absence of antibody. Values are mean + SE of three experiments. (Panel C) Effect of monoclonal antibodies on SP-A-induced liposome aggregation. Unilamellar liposomes (200 μg/mL) composed of DPPC:egg PC:PG (7:2:1, w/w/w) and wt hSP-A (10 μg/mL) were preincubated in the absence (●) or the presence of 100 μg/mL monoclonal antibodies PC6 (□) and PE10 (▲) or 100 μg/mL control monoclonal antibody 6B2 (○), and $CaCl_2$ was added to a final concentration of 5 mM after 3 min. The change in absorbance at 400 nm was measured as described in the Experimental Procedures. (Panel D) Immunoblot competition between monoclonal antibodies. Ten nanograms of wt hSP-A was electrophoressed under reducing condition and transferred onto PVDF membrane. The membrane was preincubated with or without 50 μg/mL of anti-hSP-A monoclonal antibody PC6 or PE10 or anti-rat SP-A monoclonal antibody 6E3 at room temperature for 1 h. After the incubation, the membrane was washed and then incubated with biotinylated PE10 or PC6 at room temperature for 90 min. The membrane was finally incubated with HRP-labeled avidin D (1:1000 dilution) for 20 min, followed by the peroxidase reaction using diaminobenzidine.

The present results, taken together with the previous studies (*40*, *41*), suggest that the epitope for mAbs PE10 and PC6 may be involved in the interaction of hSP-A with lipids and alveolar type II cells.

Identification of Monoclonal Antibody Epitope. To identify the amino acid sequence of the epitope for mAbs, mAb-binding epitopes were selected using a phage display peptide library. Figure 3 shows 15 and 16 of the 20 sequences for mAbs PE10 and PC6, respectively, from the peptide library, which exhibit an obvious consensus pattern matching a region of hSP-A. The consensus peptide sequences, PVNYT--YRG for mAb PE10 and TP−NYTN−YR for mAb PC6, closely resemble ^{184}TPVNYTNWYRG^{194} of hSP-A.

The synthetic peptide A corresponding to the amino acid sequences Gly^{183}-Gly^{194} of hSP-A was prepared and we investigated whether the peptide competed with hSP-A for mAb binding. When 0.01−10 μg/mL peptide and mAb PE10 or PC6 were incubated with wt hSP-A coated onto microtiter wells, the peptide A competed with solid-phase hSP-A for mAb binding in a concentration-dependent manner (Figure 4A). Ten micrograms per milliliter peptide A completely blocked the binding of mAbs to wt hSP-A. In contrast, the peptide C did not decrease the mAb binding to hSP-A.

The hSP-A recombinant chimeric protein (*hu ama4*), in which the hSP-A region Ser^{174}-Gly^{194} was replaced with the corresponding MBP-A region Thr^{164}-Asp^{184}, was produced (Figure 1). The mAb binding to the chimera was examined by dot blot with enhanced luminol reagent (Du Pont NEN) because the expression level of *hu ama4* was low (Figure 4B). Anti-human SP-A polyclonal antibody retained the binding activity to the antigen in the culture medium from cells infected with the recombinant virus directing the synthesis of *hu ama4*. However, neither mAbs PE10 nor PC6 bound to the *hu ama4* antigen.

A. mAb PE10	B. mAb PC6
	(human SP-A)
185-**PVNYTNWYRG**-194	184-**TPVNYTNWYR**-193
NC**PLNYTYTYRQ**	SF**P**PD**Y**WRH**YR**M
H**P**PD**Y**NVI**YR**TP	CQI**P**HS**Y**GG **YR**S
DS**PLNYTYTYRQ**	VL**P**HT**Y**SQ **YR**L
EA**P**LQ**Y**SY**SYR**A	SALS**YT**TV**YR**SP
YQ**PFNY**SVL**YR**A	HQQQQD**N** SS **YR**Q
A**P**QT**Y**QTS**YR**SP	GS**TP**QH**YT**QL**YR**
MHW**PVDYSSTYR**	SPI**P**HS**Y**GGHL**R**
RF**PVSYTQSYR**T	YRS**PPN**RWIQ**YR**
WLH**PLSYTEAYR**	QN**P**ES**F**HE **YR**TN
VNQ**PVDYSSTYR**	IP**H**A**P**RS**Y**VTR**Y**
SNR**PVEYTHVYR**	TG**P**LH**Y**SSQN**R**H
A**PVSYDSHYRG**R	YY**P**LD**Y**SRH**YR**M
VNQ**PSNYTQIYR**	YPS**PPN**RLIQ**YR**
SNR**PLDYSHVYR**	DH**P**P**W**YTWL**YR**
YS**PVTYTK YR**LP	TG**TP**PFQCAS**YR**
	NQE**P**G**NY**AST**YR**

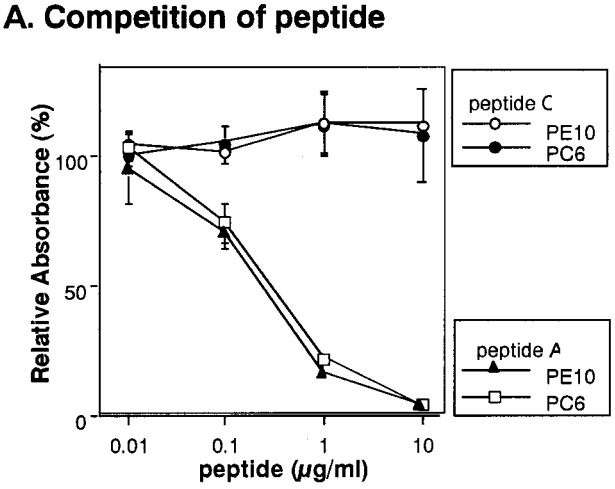
FIGURE 3: Monoclonal antibody-binding sequences selected using a phage display peptide library. Twenty clones from PE10- or PC6-binding phages were sequenced as described in the Experimental Procedures. Fifteen and 16 of the 20 sequences for monoclonal antibodies PE10 and PC6, respectively, showed an obvious consensus pattern matching the hSP-A region of Thr^{184}-Gly^{194}. Residues identical to the corresponding hSP-A sequences are in bold.

These data clearly demonstrate that the epitope for mAbs PE10 and PC6 lies within the hSP-A region of Thr^{184}-Gly^{194} and suggest that the region containing the epitope for mAbs may be involved in the hSP-A functions.

Characterization of Chimeric Proteins. We constructed chimeras in which the regions of MBP-A CRD were substituted for the corresponding CRD regions of SP-A, as shown schematically in Figure 1. Chimera rat *ama4* (*rat ama4*) consists of Asn^{1}-Gln^{173} of rat SP-A, Thr^{164}-Asp^{184} of rat MBP-A and Glu^{195}-Phe^{228} of rat SP-A. Chimera human *ama4* (*hu ama4*) consists of Glu^{1}-Pro^{173} of human SP-A, Thr^{164}-Asp^{184} of rat MBP-A and Glu^{195}-Phe^{228} of human SP-A. Chimera AM3 consists of Asn^{1}-Gly^{194} of rat SP-A and Glu^{185}-Ala^{221} of rat MBP-A, as described previously (*46*). Chimera *hu ama4* or *rat ama4* does not contain the epitope for anti-hSP-A mAbs or the corresponding region of rat SP-A.

All recombinant proteins were expressed using the baculovirus expression system and purified by affinity chromatography on mannose-Sepahrose 6B. Relative binding efficiencies of the recombinant proteins for mannose-Sepharose beads were compared. After 25 μL of beads and the purified proteins (100 ng) were incubated at 4 °C for 1 h in the presence of 5 mM $CaCl_2$, bound and unbound proteins were separated by centrifugation and those amounts were determined by ELISA. The binding efficiencies to the beads were 75% for native rSP-A, 43% for wt rSP-A, 43% for wt hSP-A, 53% for wt MBP-A, and 57% for *rat ama4* (means of two experiments). The results indicate that there are not significant differences in carbohydrate binding among the recombinant proteins. The preservation of lectin activity indicates that the proteins exhibit no significant defect in protein folding.

The purified proteins were analyzed by SDS−PAGE (Figure 5). Recombinant proteins migrated as bands at 27−34 kDa under reducing conditions. They formed oligomers



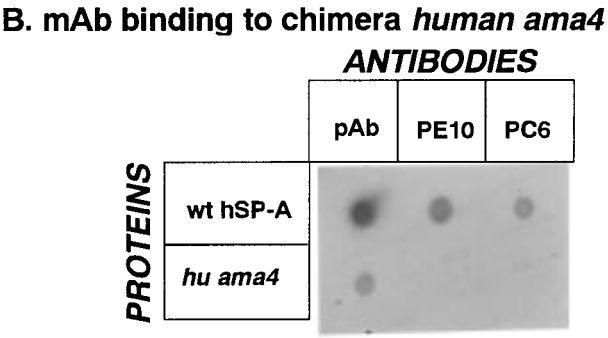


FIGURE 4: The epitope for PC6 and PE10 lies within Thr^{184}-Gly^{194} of human SP-A. (Panel A) Competition of synthetic peptide with hSP-A for antibody binding. The synthetic peptide A (GTPVNYTNWYRG) corresponding to the amino acid sequences Gly^{183}-Gly^{194} of hSP-A and the control synthetic peptide C (MESPPDYSAAPRGRFG) were prepared. Monoclonal antibody PC6 (□, ●) or PE10 (▲, ○) (50 μL of 0.2 μg/mL) was incubated with wt hSP-A coated onto microtiter wells (50 μL of 1 μg/mL) in the presence of 50 μL of the indicated concentrations of synthetic peptide A (▲, □), or C (○, ●) at 37 °C for 90 min, and the binding of monoclonal antibody was detected using HRP-labeled anti-mouse IgG, followed by the peroxidase reaction with *o*-phenylendiamine. The results are expressed as a percentage of control absorbance (monoclonal antibody binding in the absence of synthetic peptide). Values are mean ± SE from three experiments. (Panel B) Binding of monoclonal antibodies to chimera *human ama4*. The monoclonal antibody binding assay for *human ama4* was carried out by dot-blot assay. Two microliters of the culture medium from cells infected with the recombinant virus directing the synthesis of wild-type human SP-A (wt hSP-A) and chimera *human ama4* (*hu ama4*) was applied onto a nitrocellulose sheet. The nitrocellulose sheet was incubated with anti-hSP-A polyclonal antibody (pAb), antibody PC6 (PC6) or antibody PE10 (PE10) at 20 μg/mL followed by anti-rabbit IgG or anti-mouse IgG conjugated with HRP. The binding of antibody was detected using enhanced luminol reagent. When cotrol monoclonal antibody 6B2 was incubated, no positive reaction was observed (data not shown).

under nonreducing conditions. wt hSP-A was highly oligomeric, as described previously (*43*). The recombinant SP−As produced in insect cells typically migrated faster than SP-A derived from lung lavage due to different posttranslational modifications as previously described (*43*, *49*, *54*). The recombinant wild-type SP-A retains all of the functions of its native counterpart (*43*, *49*, *54*).

Interaction of Chimera ama4 with Lipids. The role of the amino acid residues 174−194 of SP-A in lipid binding and

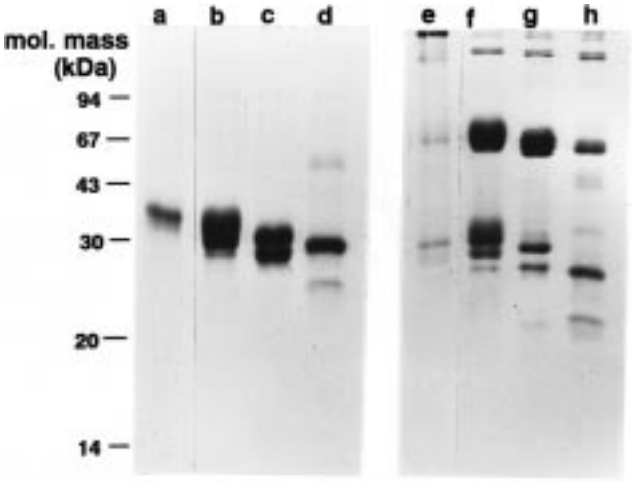


FIGURE 5: Electrophoretic analysis of recombinant proteins. Proteins were subjected to 13% SDS−PAGE under reducing conditions (lanes a−d) and nonreducing conditions (e−h) and visualized by Coomassie Brilliant Blue staining. Wild-type human SP-A (lanes a and e), wild-type rat SP-A (lanes b and f), chimera *rat ama4* (lanes c and g), and wild-type rat MBP-A (lanes d and h) are shown.

liposome aggregation was examined. We first investigated the ability of chimeras *rat ama4* and AM3 to bind lipids coated onto microtiter wells. Chimera AM3 has previously been shown to bind to DPPC and GalCer (*46*). Since antibody 6E3 recognizes the neck domain of rat SP-A and exhibits nearly equivalent binding to wild-type rat SP-A (wt rSP-A) and chimeras with rat SP-A neck (*46*, *49*, *51*), it was used to detect wt rSP-A and chimeras AM3 and *rat ama4*. Anti-MBP polyclonal antibodies were used to detect wt MBP-A. Native and wt rSP-A bound to solid-phase DPPC and GalCer in a concentration-dependent manner but failed to bind PI (Figure 6ABC). Since the binding of SP-A to DPPC is Ca^{2+} dependent (*14*) but SP-A binds to glycolipids, in part, in a Ca^{2+}-independent manner (*12*), we also examined the GalCer binding in the presence of 10 mM EDTA. Inclusion of EDTA in the binding buffer decreased the binding of SP-A to GalCer but SP-A clearly retained some GalCer binding (Figure 6D). Native and wt rSP-A possessed approximately 50% of total GalCer binding in the presence of EDTA. Chimera *rat ama4* failed to bind to DPPC (Figure 6A). The binding activity of this chimera to GalCer in the presence of Ca^{2+} was reduced when compared to wt rSP-A (Figure 6C). Chimera *rat ama4* bound to solid-phase GalCer in a concentration-dependent manner even in the presence of EDTA (Figure 6D). The extent of *rat ama4*'s GalCer binding in the absence and the presence of EDTA is almost equivalent (Figure 6, panels C and D). These results indicate that the *rat ama 4* exhibits specific defects in DPPC binding and Ca^{2+}-dependent binding to GalCer.

We also examined the binding of the proteins to liposomes. When the binding of chimera *rat ama4* to liposomes was examined, the results were consistent with those obtained from solid-phase lipid binding assay. The *rat ama4* lost binding to DPPC liposomes (Figure 7A) but retained some of the GalCer binding in the presence of Ca^{2+} (Figure 7C). In the presence of EDTA, the ability of wt rSP-A to bind GalCer liposome was decreased to the level of that obtained by chimera *rat ama4* (Figure 7D), which is nearly equivalent to that of chimera *rat ama4* in the presence of Ca^{2+} (Figure 7C). The results clearly demonstrate that the rat SP-A region of Thr^{174}-Gly^{194} is essential for DPPC binding and indicate that this region is required for Ca^{2+}-dependent binding to GalCer but is not involved in Ca^{2+}-independent binding to the glycolipid.

Since MBPs isolated from rat sera and liver have been shown to bind PI (*16*), we also examined whether the chimeras bind to PI. The wt MBP-A bound to PI coated onto microtiter wells in a concentration-dependent manner (Figure 6B), and a significant amount of MBP-A protein was also cosedimented with PI liposomes (Figure 7B). Chimera AM3 did not bind to solid-phase PI or PI liposome. Quite remarkably, *rat ama4* bound to both solid-phase PI and PI liposomes at the level comparable to wt MBP-A (Figures 6B and 7B).

The ability of chimera *rat ama4* to induce DPPC dependent liposome aggregation was also examined. The wt rSP-A induced liposome aggregation in a Ca^{2+}- and a concentration-dependent manner. The A400 obtained at 10 and 20 μg/mL wt rSP-A was 0.025 and 0.064, respectively. However, the *rat ama4* did not induce liposome aggregation at all at concentrations of both 10 and 20 μg/mL, like wt MBP-A.

The results demonstrate that the amino acid residues 174−194 of SP-A is required for DPPC binding, Ca^{2+}-dependent GalCer binding, and liposome aggregation. In addition, this study clearly shows that substituting the MBP-A region of Thr^{164}-Asp^{184} for the corresponding region of SP-A introduces MBP-like phospholipid binding specificity into SP-A, demonstrating that the MBP-A region of Thr^{164}-Asp^{184} is required for PI binding. The data also idicate that Glu^{185}-Ala^{221} is not involved in MBP's PI binding.

Interaction of Chimera ama4 with Alveolar Type II Cells. We investigated whether the chimera *ama4* inhibited the secretion of phospholipids from alveolar type II cells. When lipid secretion was stimulated by 10^{-7} M TPA, wt SP-A compeletly inhibited this agonist mediated secretion at 5 μg/mL (10.1% of TPA-stimulated secretion, mean of two separate experiments with duplicate samples). Chimera *rat ama4* failed to attenuate the TPA-stimulated lipid secretion from type II cells (99% of TPA-stimulated secretion at 5 μg/mL and 104% at 10 μg/mL). In addition, excess rat *ama4* did not compete with ^{125}I-rat SP-A for type II cell binding at all. We also examined the effect of the chimera *ama4* on lipid uptake by type II cells. The wt rSP-A augmented the association of lipids with type II cells (percent of cell-associated lipids is 3.6% at 5 μg/mL and 11.4% at 20 μg/mL; mean of two separate experiments with duplicate samples). However, chimera *ama4* failed to promote lipid association with type II cells (percent of cell-associated lipids is 2.1% at 5 μg/mL and 1.7% at 20 μg/mL, when that without proteins is 1.8%).

DISCUSSION

The purpose of this study was to map the epitope for anti-hSP-A mAbs PC6 and PE10 and to investigate the importance of this SP-A region in interactions with lipids and alveolar type II cells. The synthetic peptide corresponding to the hSP-A region of Gly^{183}-Gly^{194}, which was designed based on mAb-binding sequences obtained by a phage display peptide library, completely blocked the binding of mAbs to hSP-A. The mAbs failed to recognize the *hu ama4* antigen which lacked the hSP-A region of Ser^{174}-Gly^{194}, but instead contained the MBP-A region of Thr^{164}-Asp^{184}. The

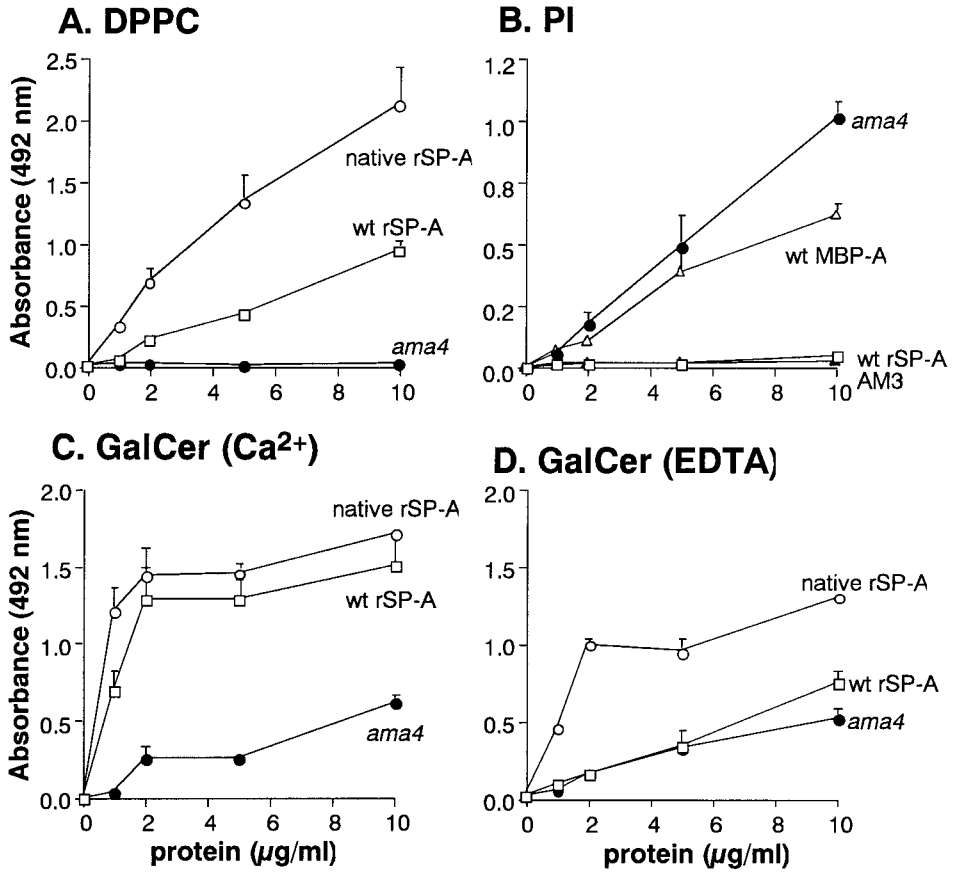


FIGURE 6: The binding of chimeras to DPPC, PI, or GalCer coated onto microtiter wells. One microgram of DPPC (A), PI (B), or GalCer (C and D) was coated onto microtiter wells, and the indicated concentrations of native rat SP-A (native rSP-A, ○), wild-type rat SP-A (wt rSP-A, □), wild-type rat MBP-A (wt MBP-A, △), chimera *rat ama4* (*ama4*, ●), or chimera AM3 (AM3, ▲) were incubated at 37 °C for 1 h in the presence of 5 mM $CaCl_2$ (A, B, and C) or 10 mM EDTA (D). After the incubation, the wells were washed and the binding of the proteins to lipids was determined as described in the Experimental Procedures. Values are mean + SE from three experiments.

present results clearly demonstrate that the epitope for the mAbs lies within the hSP-A region of Thr^{184}-Gly^{194}. Because the previous (*40*, *41*) and the present studies showed that mAbs PC6 and PE10 blocked the binding of hSP-A to DPPC, the SP-A-induced liposome aggregation and the SP-A-mediated regulation of lipid secretion from alveolar type II cells, it is concluded that the epitope for mAbs identified in this study is likely to be important for the SP-A-DPPC and SP-A-type II cell interactions. Chimera *rat ama4*, in which the rSP-A region of Thr^{174}-Gly^{194} is replaced with the MBP-A region of Thr^{164}-Asp^{184}, lacked the rSP-A region corresponding to the epitope for anti-hSP-A mAbs and lost the ability to bind DPPC and to interact with alveolar type II cells. The data obtained from chimera *rat ama4* is consistent with those obtained from epitope mapping for anti-hSP-A mAbs. It is important to emphasize that despite the loss of specific lipid and type II cell interactions, the chimeras recognize mannose ligands on affinity columns. In addition, the CD spectra of chimera *rat ama4* was similar to that of wt rSP-A (estimated α-helix and *β*-sheet contents about 15.1−16.5% and 24.8−27.1%, respectively, by the method Chen et al. (*55*), data not shown), indicating that the insertion of 21 amino acid residues of MBP-A into SP-A does not cause significant defect in protein folding.

A previous study (*41*) from this laboratory suggested that an area contiguous to or near the region containing Glu^{202}-Met^{207} of hSP-A might be important for expressing the biological activities. This conclusion had been drawn from the data that antibody against the synthetic human SP-A peptide E202-N217 (designated P1) containing this region inhibited the binding of mAb PE10 to hSP-A. Anti-P1 antibody also attenuated the binding of [^{125}I]SP-A to alveolar type II cells and reversed the inhibitory effect of hSP-A on lipid secretion, although excess of anti-P1 antibody was required compared to mAb PE10 (*41*). In the present study, the peptide A completely blocked the mAbs' binding to hSP-A (see Figure 4A). In addition, the mAbs failed to bind chimera *hu ama4* (Figure 4B). The current data obtained by the direct competition with the peptide clearly demonstrate that the epitope for the mAbs lies within the hSP-A region corresponding to the peptide A. Thus, the blocking effects of anti-P1 antibody on the mAbs' binding to hSP-A and the SP-A's functions in the previous study (*41*) are likely to be due to the steric hindrance of the hSP-A region Thr^{184}-Gly^{194} by the binding of anti-P1 antibody to the hSP-A region Glu^{202}-Asn^{217}.

Although SP-A binds carbohydrates and phospholipids in a Ca^{2+}-dependent manner (*7*, *14*), it exhibits both Ca^{2+}-dependent and -independent binding to glycolipids. Despite chelation of Ca^{2+} with EDTA, SP-A retains approximately 62% of total glycolipid binding (*12*). In this study, the binding of chimera *ama4* to GalCer was clearly decreased in the presence of Ca^{2+} when compared to wt rSP-A, but it exhibited a significant binding to GalCer at a level comparable to wt rSP-A in the presence of EDTA. The results indicate that the rSP-A region of Thr^{174}-Gly^{194} is required

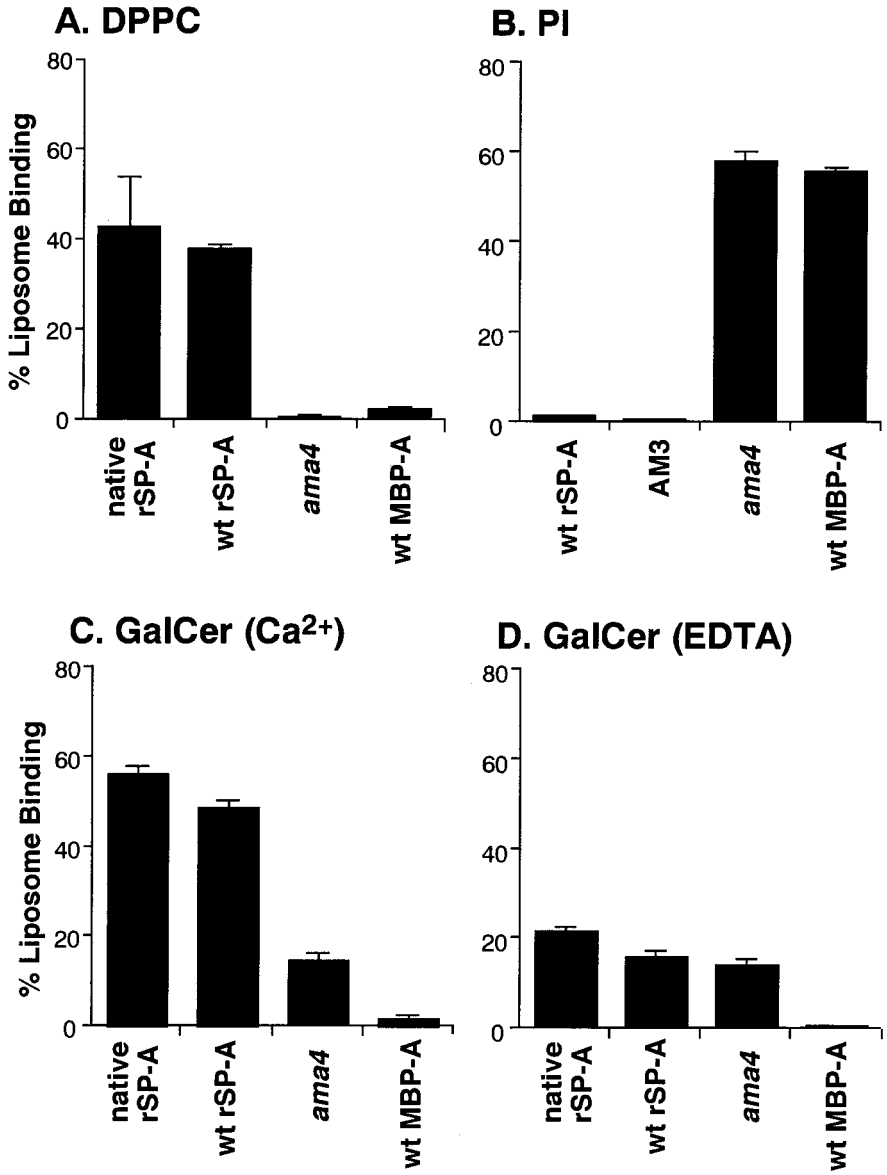


FIGURE 7: The binding of chimeras to multilamellar liposomes. Multilamellar liposomes (100 μg) containing DPPC (A), PI (B), or the lipid mixture composed of GalCer:PS:cholesterol (7:2:1) (C and D) were mixed with 0.2 μg of native rat SP-A (native rSP-A), wild-type rat SP-A (wt rSP-A), wild-type MBP-A (wt MBP-A), chimera *rat ama4* (*ama4*), or chimera AM3 (AM3) and incubated at room temperature for 1 h in the presence of 5 mM $CaCl_2$ (A, B, and C) or 10 mM EDTA (D). The amount of the protein cosedimented with liposomes was determined by sandwich ELISA as described in the Experimetal Procedures. The results show specific sedimentation that was subtracting values obtained when liposomes were omitted (nonspecific sedimentation) from total sedimentation. The data shown are mean + SE from three experiments.

for optimal Ca^{2+}-dependent binding to GalCer but not for Ca^{2+}-independent glycolipid binding. The mAbs PC6 and PE10 almost completely blocked the binding of hSP-A to DPPC but partially inhibited the hSP-A's binding to GalCer by 74−78% (see Figure 2, panels A and B). Since chimera *ama4*, which lacks the rat SP-A region corresponding to the epitope for anti-hSP-A mAbs, retained the Ca^{2+}-independent GalCer binding, the level of GalCer binding (22−26%) that was not blocked by mAbs is likely to be attributable to the region of SP-A involved in Ca^{2+}-independent binding to GalCer.

Strikingly, substitution of the MBP-A region Thr^{164}-Asp^{184} for the rSP-A region Thr^{174}-Gly^{194} introduced MBP-like phospholipid binding specificity into SP-A. The chimera *ama4* bound to PI to a level comparable to wt MBP-A, although this chimera lost DPPC binding and interaction with alveolar type II cells. When the amino acids between the rSP-A region Thr^{164}-Asp^{184} and the corresponding region of MBP-A are compared, eight amino acids out of 21 are identical and 13 out of 21 are homologous. Site-directed mutagenesis within this region may be useful to identify the amino acids that define phospholipid binding specificity of these collectins. The present study unambiguously demonstrates that the MBP-A region Thr^{164}-Asp^{184} is required for PI binding of MBP-A and that the corresponding region of SP-A is essential for interaction of SP-A with PC and type II cells. Since the synthetic peptide corresponding to the hSP-A region Gly^{183}-Gly^{194}, by itself, did not inhibit lipid secretion from type II cells and did not compete with SP-A for PC binding (data not shown), the quaternary structure is likely to be essential.

Both SP-D and MBP bind to PI in a Ca^{2+}-dependent manner (*15, 16*). Since chimera *ad3*, in which the rSP-A region of Glu^{195}-Phe^{228} is replaced with the SP-D region of

Glu^{321}-Phe^{355}, exhibits PI binding (*49*), we investigated whether chimera AM3 (*46*), in which the rSP-A region of Glu^{195}-Phe^{228} is replaced with the MBP-A region of Glu^{185}-Ala^{221}, bound to PI in this study. The results demonstrate that chimera AM3 does not bind solid phase PI or PI liposomes. From the data previously obtained with chimera *ad3*, we conclude that the SP-D region of Glu^{321}-Phe^{355} is required for the binding of SP-D to PI. In contrast, from the data obtained by chimera AM3, it is possible to interpret that the MBP-A region of Glu^{185}-Ala^{221} is not sufficient for the binding to PI. Collectively, these two studies strongly suggest that the mechanism by which SP-D binds to PI is different from that of the MBP. The different binding specificity of SP-D and MBP for phosphoinositides supports this conclusion. MBP binds to phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-bisphosphate (*16*), while SP-D does not bind to these polyphosphoinositides (*15*).

A recent study from this laboratory (*49*) demonstrates that the rat SP-A region of Glu^{195}-Phe^{228} is required for SP-A-lipid and SP-A-type II cell interactions. The present study clearly indicates that the rat SP-A region of Thr^{174}-Gly^{194} is also essential for multiple SP-A functions except carbohydrate binding and Ca^{2+}-independent binding to GalCer. These findings do not conflict with each other, if one assumes that multiple protein surfaces are likely to be involved in ligand association. We are currently undertaking studies to more finely map the important segments of primary sequence within the Glu^{195}-Phe^{228} region of the protein that are important for phospholipid and type II cell recognition. Since anti-rat SP-A mAb 1D6 that recognizes the rat SP-A CRD completely blocked the binding of rat SP-A to GalCer (*51*), the structures required for both Ca^{2+}-dependent and Ca^{2+}-independent binding to GalCer are likely to lie within the SP-A CRD. Thus, the present data also indicate that the rat SP-A CRD regions of Gly^{115}-Gln^{173} and of Glu^{195}-Phe^{228} are likely to be involved in Ca^{2+}-independent GalCer binding. Collectively, the data demonstrate the different structural requirement for Ca^{2+}-independent glycolipid binding from Ca^{2+}-dependent binding.

In conclusion, the present study clearly demonstrates that the epitope for mAbs PC6 and PE10, which block SP-A functions, lies within the hSP-A region of Thr^{184}-Gly^{194}. Substituting the MBP-A region of Thr^{164}-Asp^{184} for the rSP-A region of Thr^{174}-Gly^{194} destroys numerous SP-A functions but spares carbohydrate binding and Ca^{2+}-independent glycolipid binding and introduces the MBP-like phospholipid binding specificity into SP-A. The results clearly demonstrate a critical role for Thr^{184}-Gly^{194} in SP-A function and Thr^{164}-Asp^{184} in MBP-A function.

ACKNOWLEDGMENT

We thank Drs. Shosaku Abe and Hiroki Takahashi (Third Department of Internal Medicine, Sapporo Medical University) for valuable discussions and encouragement and Dr. Toshio Honma (First Department of Surgery, Sapporo Medical University) for preparing the chimeric protein AM3. We are also grateful to Dr. Norio Matsushima (Sapporo Medical University) and Mr. Takanori Tanaka (Hokkaido University) for their valuable comments on the CD spectral data.

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BI990353E