The Mannose-Binding Protein A Region of Glutamic Acid^{185}−Alanine^{221} Can Functionally Replace the Surfactant Protein A Region of Glutamic Acid^{195}−Phenylalanine^{228} without Loss of Interaction with Lipids and Alveolar Type II Cells†

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ABSTRACT: Pulmonary surfactant protein A (SP-A) is a C-type lectin that regulates the uptake and secretion of surfactant lipids by alveolar type II cells and binds dipalmitoylphosphatidylcholine (DPPC) and galactosylceramide (GalCer). We isolated mannose-binding protein A (MBP-A) from rat sera, which is structurally analogous to SP-A, and examined if it was functionally equivalent to SP-A. We found that MBP-A did not possess the ability to interact with lipids and type II cells. The purpose of this study was to investigate the SP-A region involved in binding lipids and interacting with type II cells by using chimeric proteins with MBP-A. Chimeras AM1, AM2, and AM3 were constructed with SP-A/MBP splice junctions at Cys^{218}/Gln^{210}, Lys^{203}/Cys^{195}, and Gly^{194}/Glu^{185}, respectively. All of the chimeras bound DPPC and GalCer with activity comparable to recombinant SP-A. The three chimeras retained the ability to induce phospholipid vesicle aggregation and augment lipid uptake by type II cells, albeit to a lesser extent than wild type SP-A. The chimeras inhibited lipid secretion from type II cells with an IC_{50} of 0.5 μg/mL and competed effectively for SP-A receptor binding. In addition all these chimeras contained the epitope for monoclonal antibody 1D6, which blocks specific SP-A function. From these results, we conclude that the MBP-A region of Glu^{185}−Ala^{221} can functionally replace the homologous SP-A region of Glu^{195}−Phe^{228} without loss of interaction with lipids and type II cells.

Pulmonary surfactant is a mixture of lipids and proteins that alveolar type II cells produce, and it acts to keep alveoli from collapsing during expiration. Surfactant proteins specific to this complex play important roles in the surfactant functions of host defense and modulation of the biophysical properties of the lung (Kuroki & Voelker, 1994). The most abundant hydrophilic surfactant protein is surfactant protein A (SP-A),[1] which exhibits a reduced denatured molecular mass of 26−38 kDa in the rat (Kuroki et al., 1988c). Four domains are readily discernible in this protein: (1) a cysteine-containing amino terminus, (2) a collagen-like domain, (3) a neck domain, and (4) a carbohydrate recognition domain (CRD) (Kuroki & Voelker, 1994). SP-A specifically binds to dipalmitoylphosphatidylcholine (DPPC) (Kuroki & Akino, 1991), a phospholipid essential for reducing the surface tension at the air−liquid interface, and galactosylceramide (GalCer) (Childs et al., 1992; Kuroki et al., 1992a). In the presence of Ca^{2+} SP-A causes phospholipid vesicle aggregation (Hawgood et al., 1985). In vitro studies provide compelling evidence that SP-A acts as an inhibitor of phospholipid secretion by alveolar type II cells (Dobbs et al., 1987; Rice et al., 1987). The protein has also been shown to augment lipid uptake by type II cells (Wright et al., 1987), suggesting that SP-A plays an important role in surfactant phospholipid homeostasis in the alveoli. In addition, SP-A binds to a high-affinity receptor expressed on type II cells (Kuroki et al., 1988a; Wright et al., 1989), and this activity correlates well with the inhibitory effect on lipid secretion (Kuroki et al., 1988b). We have previously shown that the collagenase-resistant fragment of human SP-A inhibits lipid secretion from type II cells (Murata et al., 1993), indicating that the region of the neck plus CRD is responsible for the activity. Monoclonal antibody 1D6, but not antibody 6E3, against rat SP-A blocked the inhibitory effect of the protein on lipid secretion by type II cells (Kuroki et al., 1988c). Antibody 1D6 also attenuated the ability of SP-A to bind to DPPC and GalCer and to cause vesicle aggregation. Antibody 6E3 failed to block the interaction with lipids (Kuroki et al., 1994). Both 6E3 and 1D6 blocked the uptake of lipids by type II cells that is caused by SP-A (Kuroki et al., 1994). An epitope-mapping study using recombinant SP-A proteins revealed that antibodies 6E3 and 1D6 recognized the neck domain and the CRD, respectively (Kuroki et al., 1994). These studies indicate that the CRD is essential for the SP-A functions of lipid binding, liposome aggregation, inhibitory

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[1] Abbreviations: SP-A, surfactant protein A; MBP-A, mannose-binding protein A; CRD, carbohydrate recognition domain; DPPC, dipalmitoylphosphatidylcholine; GalCer, galactosylceramide; ELISA, enzyme-linked immunosorbent assay; $NaDodSO_4$−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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effect on lipid secretion, and augmentation of lipid uptake by type II cells and that the neck domain may also be involved in the SP-A-mediated lipid uptake. Introducing simultaneous mutations into rat SP-A for $Glu^{195} \rightarrow Gln$ and $Arg^{197} \rightarrow Asp$ altered the carbohydrate-binding specificity from mannose > galactose to the converse and attenuated the ability of this mutant SP-A to cause lipid aggregation and to interact with alveolar type II cells (McCormack et al., 1994b). Taken together, these studies suggest that the amino acids in the locus of the small disulfide loop (Cys^{204}−Cys^{218}) within the CRD may be important for multiple functions.

SP-A belongs to the collectin subgroup of the C-type lectin superfamily. The collectins contain a collagen-like domain and include SP-A, surfactant protein D (SP-D), mannose-binding protein (MBP), bovine conglutinin, and protein CL43 (Day, 1994). SP-A forms a bouquet-like structure consisting of an octadecamer composed of six trimeric subunits (Voss et al., 1988), like MBP and complement C1q. The strong sequence conservation in the CRD among collectins and the similarities in carbohydrate-binding specificity and oligomeric structure between SP-A and MBP suggest the possibility that the binding sites for carbohydrates and phospholipids are structurally analogous. The binding sites in the SP-A molecule for lipids and a receptor have not yet been identified. In this study we focused on the structural similarity of SP-A and MBP-A and asked if MBP-A, a predominant serum form of MBP (Drickamer et al., 1986), exhibited analogous functions to SP-A. We constructed three chimeric proteins in which progressively longer carboxy terminal regions of MBP-A were substituted for that of SP-A and examined if these chimeras possessed SP-A functions and the epitope for monoclonal antibody 1D6. We found that the carboxy terminal MBP region of Glu^{185}−Ala^{221} can functionally replace the SP-A region of Glu^{195}−Phe^{228} without loss of inteaction with lipids and type II cells.

EXPERIMENTAL PROCEDURES

Isolation and Purification of Rat SP-A and MBP-A. Surfactant was isolated from lung lavage fluids of Sprague−Dawley rats that had been given an intratracheal instillation of silica (Dethloff et al., 1986), by the method of Hawgood et al. (1985). The surfactant was delipidated by extraction with 1-butanol, and SP-A was then purified from the butanol-insoluble materials as described previously (Kuroki et al., 1988a). Briefly, after the protein was suspended in 5 mM Tris buffer (pH 7.4) and dialyzed against the same buffer, the suspension was centrifuged at 150 000g_{av} for 1 h and the supernatant was applied to an affinity column of mannose-Sepharose 6B prepared by the method of Fornstedt and Porath (1975). The SP-A that bound to the affinity matrix in the presence of 2 mM $CaCl_2$ was eluted with 5 mM Tris buffer (pH 7.4) containing 2 mM EDTA and further purified by gel filtration over Bio-Gel A15m (Bio-Rad).

MBP was isolated from sera of Sprague−Dawley rats using an affinity column of mannose-Sepharose 6B by a method based on that described by Kozutsumi et al. (1980). Briefly, the pooled rat sera was mixed with an equal volume of 20 mM Tris buffer (pH 7.4) containing 2.5 M NaCl and 40 mM $CaCl_2$ and incubated for 2 h at 4 °C followed by centrifugation at 10 000g_{av} for 10 min. The supernatant was applied to a mannose-Sepharose 6B column equilibrated with 20 mM Tris buffer (pH 7.4) containing 1.25 M NaCl and 20 mM $CaCl_2$. After the column was washed, the proteins which had bound to the affnity matrix were eluted with 20 mM Tris (pH 7.4) containing 1.25 M NaCl and 2 mM EDTA. The calcium concentration of the eluate was adjusted to 20 mM using 0.5 M $CaCl_2$ and applied to another mannose-Sepharose 6B column. Serum mannose-binding protein was finally eluted with 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl and 2 mM EDTA.

Lipids. DPPC, phosphatidylcholine (PC), and phosphatidylglycerol (PG) from egg yolk, phosphatidylserine (PS) from bovine brain, and GalCer were purchased from Sigma. Cholesterol was purchased from Serdary Research Laboratories. The 1-palmitoyl-2-[^{3}H]palmitoyl-L-3-phosphatidylcholine ([^{3}H]DPPC) was obtained from DuPont NEN.

Iodination of SP-A and MBP-A and Binding of ^{125}I-Labeled Proteins to Lipids. SP-A and MBP-A were iodinated by the method of Bolton and Hunter (1973) using the Bolton−Hunter reagent (Amersham). More than 94% of the radioactivity was precipitated with 10% (w/v) trichloroacetic acid. The specific activity of [^{125}I]SP-A or [^{125}I]MBP-A used ranged between 270 and 420 cpm/ng.

The binding of [^{125}I]SP-A or [^{125}I]MBP-A to DPPC or GalCer which was developed on thin layer chromatograms or coated onto microtiter wells was carried out as described previously (Kuroki & Akino, 1991; Kuroki et al., 1992a).

DNA Construct. The isolation and sequencing of the 1.6-kilobase cDNA for rat SP-A was previously reported (Sano et al., 1987). The cDNA of rat MBP-A in pUC 8 plasmid vector was a generous gift from Dr. Kurt Drickamer. The mutant cDNAs were produced by the polymerase chain reaction and the technique of overlap extension (Ho et al., 1989) using the cDNAs for SP-A and MBP-A as templates. The cDNAs were inserted into a pVL 1392 plasmid vector using the *Eco*RI and *Xma*I sites. Mutations were confirmed by sequencing the coding region for each mutant cDNA fragment using the Sequenase kit based on the dideoxynucleotide termination method of Sanger et al. (1977).

Expression and Isolation of Recombinant Proteins. The expression of recombinant proteins in the baculovirus system was carried out as described by O'Reilly et al. (1992). Monolayers of *Spodoptera frugiperda* (Sf-9) cells were cotransfected with linearized virus DNA (Baculogold, Pharmingen) and the pVL 1392 plasmid vector containing the cDNAs for SP-A and mutant proteins. Recombinant mutant viruses were isolated by plaque purification. Viral titers were amplified to approximately $3-4 \times 10^7$ plaque-forming units/mL. Recombinant proteins were expressed into serum-free insect media by infection of monolayers of Sf-9 cells with viral stock at a multiplicity of 2. Recombinant proteins were purified from the culture media by affinity chromatography on mannose-Sepharose 6B. The recovered recombinant proteins were dialyzed against 5 mM Tris buffer (pH 7.4) and stored at −20 °C.

Binding of the Proteins to Liposomes. The binding of the proteins to multilamellar liposomes containing DPPC or GalCer was performed by the sedimentation method with a minor modification, as described previously (McCormack et al., 1994b). To prepare multilamellar liposomes, DPPC or the lipid mixture composed of GalCer:phosphatidylserine: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 37 °C for 1 h and then vortexed vigorously for 5

min. The multilamellar liposomes (100 μg) and the protein solution (0.2 μg of protein/tube) in 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl, 2 mM $CaCl_2$, and 20 mg/mL bovine serum albumin (buffer A) were separately centrifuged at 10 000g_{av} for 10 min. Each liposome pellet was then suspended in 50 μL from the supernatant of the protein solution. The lipid−protein mixture was incubated for 1 h at room temperature and then put on ice and incubated for 15 min. The mixture was centrifuged at 10 000g_{av} for 10 min at 4 °C. The supernatant was stored (unbound fraction), and the precipitate was washed once with 50 μL of ice-cold buffer A. After centrifugation, the supernatant was combined and the pellet was suspended with 100 μL of buffer A. The amount of protein in each fraction was determined by sandwich ELISA for SP-A or MBP using polyclonal antibodies against each protein. Liposome binding is defined as (SP-A(or MBP)$_{pellet}$/SP-A(or MBP)$_{pellet+supernatant}$) × 100. Control experiments where liposomes were deleted from the incubation mixture were also performed.

Liposome Aggregation. Liposome aggregation was performed by the method based on that described by Hawgood et al. (1985). The unilamellar liposomes were prepared from the multilamellar liposomes composed of DPPC:egg PC:PG (7:2:1, w/w/w) by probe sonication for 5 min. Native or recombinant proteins (10 or 20 μg/mL) in 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl were preincubated for 3 min with 200 μg/mL unilamellar liposomes. After equilibration, turbidity was measured at 400 nm 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 turbidity was further measured until a time of 10 min.

Primary Culture of Alveolar Type II Cells and Secretion of Phosphatidylcholine. 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 (Dobbs & Mason, 1979). Type II cells were cultured with [^{3}H]choline (0.5 μCi/mL) overnight in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum. Secretion of radiolabeled phosphatidylcholine was performed using MBP and the recombinant proteins as antagonists of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (10^{-7}M)-stimulated surfactant secretion as described previously (Kuroki et al., 1988c). In some experiments, the medium containing 0.2 M mannose was used during secretion experiments. Following incubation for 3 h, radiolabeled phospholipid was extracted from the cells and media. Surfactant secretion is expressed as percent secretion ((radioactivity in medium/radioactivity in medium plus radioactivity in cells) × 100).

Binding of SP-A to Type II Cells. The binding study was performed by determining the ability of chimeric proteins to compete with rat [^{125}I]SP-A for receptor occupancy on type II cells as described previously (Kuroki et al., 1988b; McCormack et al., 1994b). Briefly, monolayers of type II cells (2×10^6/dish) cultured overnight after isolation were incubated with DMEM containing 10% (v/v) fetal bovine serum, 0.5 μg/mL [^{125}I]SP-A, and the indicated concentrations of unlabeled proteins for 3 h at 37 °C in the presence of 0.2 M mannose. The cells were washed four times on ice with 50 mM Tris buffer (pH 7.4) containing 0.1 M NaCl, 2 mM $CaCl_2$, and 1 mg/mL bovine serum albumin and then dissolved in 2 mL of 0.1 N NaOH. The radioactivity bound to the cells was determined using a γ-radiation counter.

Uptake of Phospholipid Liposomes by Alveolar Type II Cells. Uptake of phospholipids by freshly isolated type II cells was performed by the method based on that described by Wright et al. (1987). Type II cells (2×10^6 cells) were incubated with 0.5 mL of DMEM containing 10 mM *N*(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) (pH 7.4), radiolabeled phospholipid liposomes (100 μg/mL) composed of DPPC:egg PC:PG (7:2:1, w/w/w) and a trace amount of [^{3}H]DPPC, and the proteins (5 or 20 μg/mL) at 37 °C for 1 h. After incubation, cells and media were separated by centrifugation at 160*g* for 5 min at 4 °C. The medium was removed, and the cells were resuspended in 1 mL of ice-cold phosphate-buffered saline containing 1 mg/mL bovine serum albumin. The washing steps were repeated three times. Before the final centrifugation, the cell suspension was transferred to a fresh Eppendorf tube. The final cell pellet was analyzed for radioactivity.

Monoclonal Antibody Binding Assay. Monoclonal antibodies to rat SP-A were prepared as described previously (Kuroki et al., 1988c). All monoclonal antibodies recognized epitopes on the polypeptide portion of SP-A and had a nearly equivalent affinity for the SP-A antigen. The epitopes for antibodies 1D6 and 6E3 were found to be located at the CRD and the neck domain, respectively (Kuroki et al., 1994).

The binding of monoclonal antibodies 1D6 and 6E3 to SP-A, MBP, and recombinant proteins was examined by ELISA. Fifty microliters of the purified protein (1 μg/mL) was coated onto microtiter wells and incubated with monoclonal antibody 1D6 or 6E3 or polyclonal antibody to SP-A or MBP (10 μg/mL) at 37 °C for 1 h after the nonspecific binding had been blocked with phosphate-buffered saline containing 0.1% (v/v) Triton X-100 and 3% (w/v) skim milk. The wells were washed followed by their incubation with horseradish peroxidase-labeled anti-mouse IgG or anti-rabbit IgG. *o*-Phenylenediamine was used as the substrate for the peroxidase reaction. The antibody binding to the proteins was measured by absorbance at 490 nm.

Other Methods. Protein concentrations were estimated by the method of Lowry et al. using bovine serum albumin as the standard. (1951). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis ($NaDodSO_4$−PAGE) was performed by the method of Laemmli (1970).

RESULTS

Electrophoretic Analysis of Native SP-A and MBP-A Isolated from Rats. The native forms of purified SP-A and MBP were analyzed by $NaDodSO_4$−PAGE (Figure 1A,B, lanes a, f, g, and l). Native SP-A migrated as a triplet at 26, 32, and 38 kDa, and native MBP showed the protein band at 29 kDa, as previously described (Kuroki et al., 1988a; Drickamer et al., 1986). The amino terminal sequence of MBP was SGSQTXEE (X: not determined), which was identical to Ser^1−Glu^8 of rat MBP-A described by Drickamer et al. (1986) and Ikeda et al. (1987). For both proteins, the major iodinated forms correlated well with underivatized proteins (Figure 1C).

Interaction of MBP with Lipids and Type II Cells. SP-A has been shown to bind DPPC and GalCer (Kuroki & Akino, 1991; Kuroki et al., 1992a). In this study we examined if MBP bound to these lipids. The lipid-binding study was performed using a TLC plate and microtiter wells. [^{125}I]-

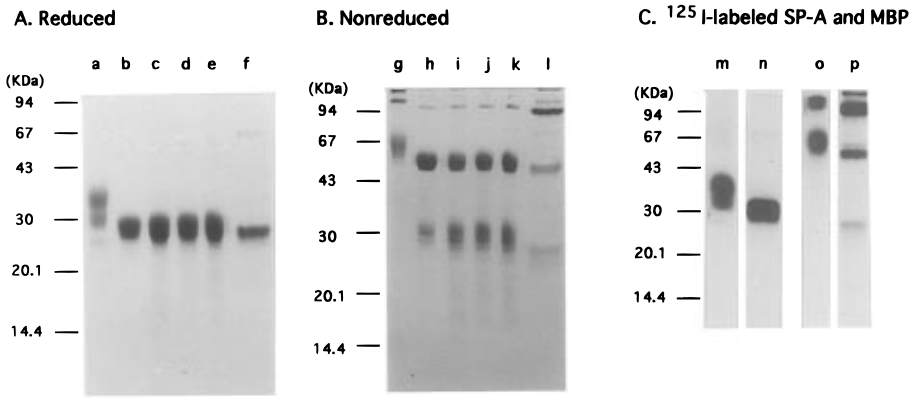

FIGURE 1: Electrophoretic analysis of SP-A and MBP-A isolated from rats and recombinant proteins. Proteins were separated on 13% $NaDodSO_4$−polyacrylamide gel electrophoresis under reducing conditions (panel A) and nonreducing conditions (panel B) and stained with Coomassie brilliant blue. Preparations (5×10^5 cpm) of ^{125}I-labeled SP-A and MBP were also analyzed in 13% $NaDodSO_4$−polyacrylamide gel electrophoresis under reducing conditions (panel C, lanes m and n) and nonreducing conditions (panel C, lanes o and p) and autoradiographed. Lanes a, g, m, and o, SP-A; lanes b and h, wild type SP-A; lanes c and i, chimera AM1; lanes d and j, chimera AM2; lanes e and k, chimera AM3; lanes f, l, n, and p, MBP-A.

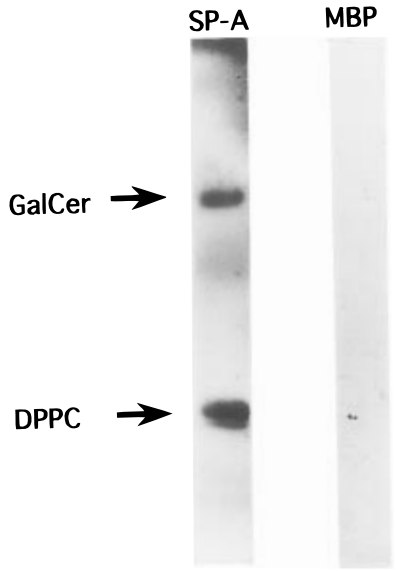

FIGURE 2: [^{125}I]MBP-A fails to bind DPPC and GalCer developed on TLC plate. Dipalmitoylphosphatidylcholine (DPPC) (5 μg) and galactosylceramide (GalCer) (5 μg) were separated by thin layer chromatography (TLC) with a solvent system of chloroform: methanol:water (65:35:5, v/v/v), and the binding of [^{125}I]SP-A (SP-A) and [^{125}I]MBP-A (MBP) was performed at 1 μg/mL iodinated ligands as described under Experimental Procedures.

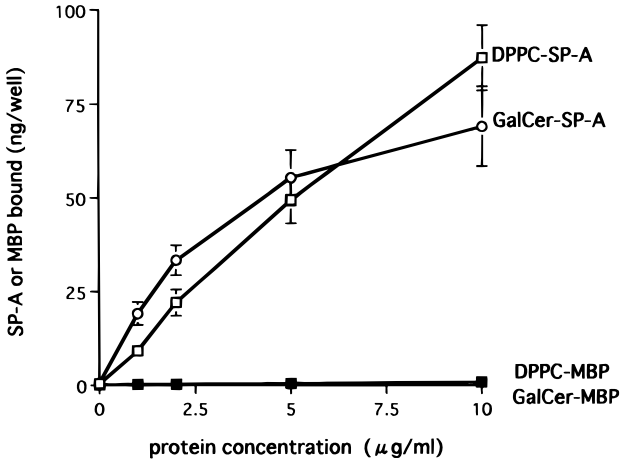

FIGURE 3: Binding of [^{125}I]SP-A and [^{125}I]MBP-A to DPPC and GalCer coated onto microtiter wells. One microgram of DPPC (□, ■) or GalCer (○, ●) was coated onto microtiter wells and the indicated concentrations of [^{125}I]SP-A (□, ○) or [^{125}I]MBP-A (■, ●) were incubated with lipids at room temperature for 1 h. After the incubation, the wells were washed and the amount of SP-A or MBP-A bound to the lipids was determined as described under Experimental Procedures. The data shown are mean ± SD, $n = 3$.

SP-A bound to DPPC and GalCer developed on the TLC plate (Figure 2) and bound to these lipids coated onto the microtiter wells in a concentration-dependent manner (Figure 3). In contrast, [^{125}I]MBP exhibited no binding to these lipids (Figures 2 and 3). The interactions of MBP with liposomes and alveolar type II cells were also examined. MBP failed to aggregate liposomes and interact with type II cells (see below). The data clearly demonstrate that MBP is not functionally equivalent to SP-A in lipid and type II cell binding and suggest that MBP lacks the necessary structural determinants for such interactions. These basic observations led us to prepare the chimeric molecules of SP-A with MBP-A to determine the domain of SP-A required for the above-described functions.

Construction of Chimeric Proteins and Proteins Expressed in Insect Cells. Since the epitope-mapping study for monoclonal antibody 1D6 and the $Glu^{195} \rightarrow Gln$ and $Arg^{197} \rightarrow Asp$ mutations suggest that the carboxy terminal region near the small disulfide loop may be important for SP-A functions, we constructed three chimeric proteins with SP-A and MBP-A in this study. The chimeric molecules used are schematically represented in Figure 4. Chimera AM1 consists of Asn^1−Cys^{218} of SP-A and Gln^{210}−Ala^{221} of MBP. Chimera AM2 consists of Asn^1−Lys^{203} of SP-A and Cys^{195}−Ala^{221} of MBP. Chimera AM3 consists of Asn^1−Gly^{194} of SP-A and Glu^{185}−Ala^{221} of MBP. The recombinant proteins were analyzed by $NaDodSO_4$−PAGE. Recombinant wild type SP-A and chimeras produced by the baculovirus expression system migrated as broad bands at 27−32 kDa under reducing conditions (Figure 1A). Analysis under nonreducing conditions revealed that the wild type SP-A and chimeric molecules migrated as oligomers (Figure 1B). The recombinant forms of SP-A typically migrated faster than SP-A derived from lung lavage as a consequence of different post-translational modifications. The recombinant wild type SP-A retains all of the functions of its native counterpart (McCormack et al., 1994a).

Interaction of Chimeras with Lipids. The ability of chimeric proteins to bind liposomes containing DPPC or

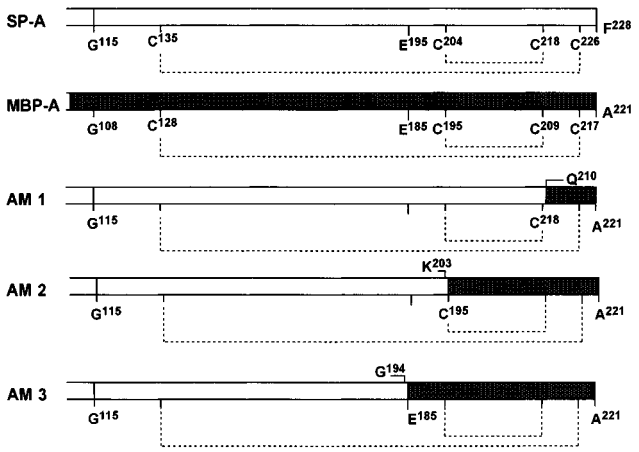

FIGURE 4: Schematic representation of CRDs of chimeric proteins. The structures of carbohydrate recognition domains (CRD) of SP-A, MBP-A, and chimeras (AM1, AM2, and AM3) are shown. The open regions are from SP-A, and the filled regions are from MBP-A. The dashed lines represent the disulfide bonds.

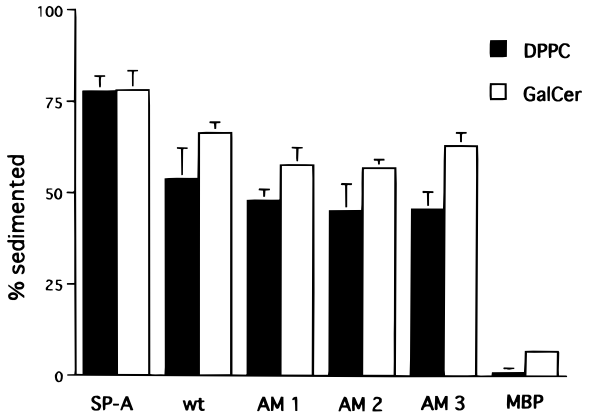

FIGURE 5: SP-A/MBP-A chimeras, but not MBP-A, bind to liposomes containing DPPC or GalCer. Multilamellar liposomes (100 μg/tube) containing DPPC (■) or GalCer (□) were mixed with 0.2 μg of rat SP-A (SP-A), wild type SP-A (wt), chimera AM1, chimera AM2, chimera AM3, or MBP-A and incubated at room temperature for 1 h. The proteins bound to liposomes were sedimented at 10 000g_{av}, and the amount of proteins in the supernatant and pellet 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 ± SD, $n = 3$.

GalCer was examined by sedimentation. A significant amount of native SP-A and the wild type SP-A were cosedimented with multilamellar liposomes containing DPPC or GalCer (Figure 5). When the percentage of the protein sedimented in the absence of lipids (nonspecific binding) was subtracted, 54% and 66% of the wild type SP-A was cosedimented with the liposomes containing DPPC and GalCer, respectively. The DPPC binding of the recombinant wild type and chimeric SP-A proteins ranged from 45% to 48%, and the GalCer binding ranged from 57% to 63%. In contrast MBP gave negligible binding to the liposomes (Figure 5). These results clearly indicate that the chimeric proteins, but not MBP, are capable of binding liposomes containing DPPC or GalCer.

Next, we investigated the abilities of MBP and the chimeras to cause phospholipid vesicle aggregation. Native and wild type recombinant SP-A induced phospholipid vesicle aggregation in the presence of Ca^{2+} in a time- and

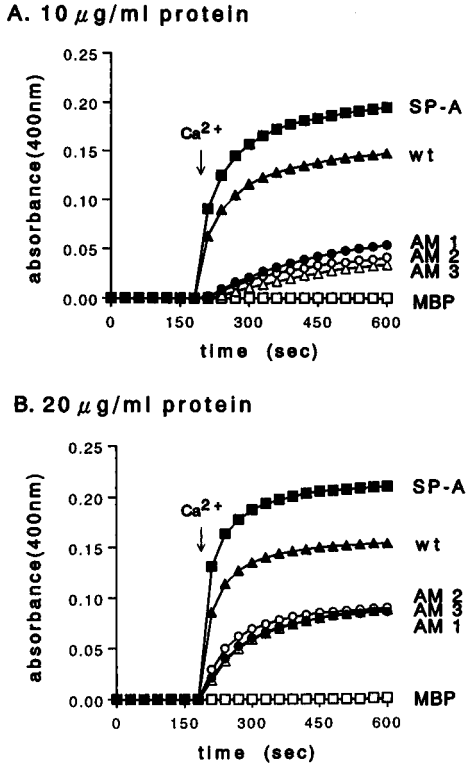

FIGURE 6: SP-A/MBP-A chimeras retain the ability to cause phospholipid vesicle aggregation. Unilamellar liposomes (200 μg/mL) were preincubated in the presence of 10 μg/mL (panel A) or 20 μg/mL (panel B) SP-A (■), MBP-A (□), wild type SP-A (▲), chimera AM1 (●), chimera AM2 (○), or chimera AM3 (△). Following the initial absorbance reading at 400 nm, $CaCl_2$ was added to a final concentration of 5 mM at a time of 180 s, and light scattering was further measured until a time of 600 s. Data presented are a representative of three experiments.

concentration-dependent manner (Figure 6), while MBP failed to induce liposome aggregation at concentrations of both 10 and 20 μg/mL. All of the chimeras were able to cause vesicle aggregation, albeit to a lesser extent when compared to the wild type SP-A. The maximal turbidity at concentrations of 10 and 20 μg/mL of the chimeras was 23−36% and 56−58%, respectively, of those observed for the wild type recombinant SP-A. The chimeras did not show any increases of turbidity in the absence of phospholipid liposomes (data not shown). The data clearly indicate that these chimeras retain the ability to induce phospholipid vesicle aggregation.

Interaction of Chimeras with Alveolar Type II Cells. Since the binding of SP-A to lipids is considered to be essential to initiate lipid uptake by type II cells, we next examined if the chimeras augmented lipid uptake. The wild type SP-A facilitated the uptake of lipids into type II cells (Figure 7); 4.9% and 13.1% of liposomes containing DPPC were taken up at concentrations of 5 and 20 μg/mL, respectively. All the chimeras stimulated lipid uptake although the degree of the stimulation was less than that of the wild type SP-A. The amount of lipids incorporated into type II cells in the presence of MBP-A was essentially the same level as the control.

We also examined whether MBP inhibited lipid secretion by type II cells. MBP failed to inhibit TPA-stimulated lipid secretion by type II cells even at a concentration of 10 μg/mL in both the absence and the presence of excess mannose (Figure 8), demonstrating that MBP does not possess the

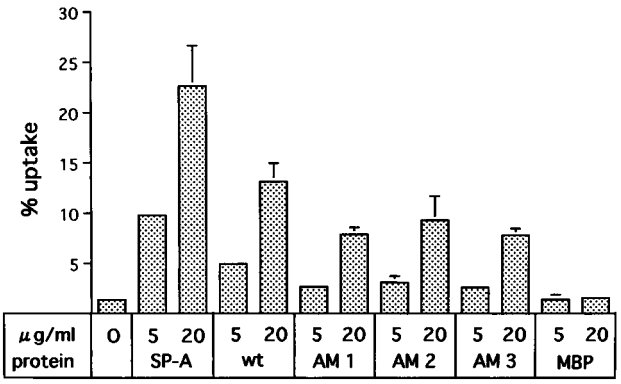

FIGURE 7: SP-A/MBP chimeras retain the ability to augment lipid uptake by type II cells. Unilamellar liposomes containing [^{3}H]DPPC and rat SP-A (SP-A), rat MBP-A (MBP-A), wild type SP-A (wt), or chimera AM1, AM2, or AM3 at the indicated concentrations were incubated with freshly isolated alveolar type II cells at 37 °C for 1 h. The cells were then washed, and the radioactivities associated with cells were counted in a scintillation counter as described under Experimental Procedures. The results are expressed as percent of radioactivities associated with cells in total radioactivities of the incubation mixture. The data presented are mean ± SD, $n = 3$.

region responsible for inhibitor activity. We next examined if the chimeras containing the MBP region inhibited lipid secretion. All three chimeras inhibited the TPA-stimulated lipid secretion from type II cells in a concentration-dependent manner (Figure 8A). The IC_{50} for inhibition of [^{3}H]-phosphatidylcholine secretion was approximately 0.5 μg/mL. Inclusion of 0.2 M mannose in the medium failed to alter the inhibitory activity of these chimeras on lipid secretion (Figure 8B). The results clearly indicate that the MBP region of Glu^{185}−Ala^{221} can replace the SP-A region of Glu^{195}−Phe^{228} without loss of inhibitory activity on lipid secretion.

Competition experiments with [^{125}I]SP-A for type II cell binding were also performed in the presence of 0.2 M mannose (Figure 9). All the chimeras were as effective as the wild type SP-A in competing with rat [^{125}I]SP-A for receptor occupancy. The binding of labeled SP-A was reduced to 28−29% of control binding at 40 μg/mL of the chimeras. MBP-A failed to compete with labeled SP-A for receptor binding. These results clearly show that the inhibitory effect of the chimeras on lipid secretion from type II cells is mediated by the same receptor mechanism as the native and wild type SP-As.

Binding of Monoclonal Antibodies to the Chimeras. A previous study has revealed that the epitopes for monoclonal antibodies 1D6 and 6E3 are located at the CRD (Gly^{115}−Phe^{228}) and the neck domain, respectively (Kuroki et al., 1994). To investigate whether monoclonal antibodies recognized the chimeras containing the MBP region, monoclonal antibody binding to the proteins coated onto microtiter wells was measured by ELISA. Neither antibody 1D6 nor antibody 6E3 recognized MBP (Figure 10). Both antibodies bound to the three chimeras as well as to native and wild type SP-As. Anti-MBP antibody did not bind to the chimeras AM1−3. We further investigated a possibility that the 1D6 epitope could be a three-dimensional epitope that occurs in both MBP and SP-A. A dot blot analysis revealed that antibody 1D6 failed to bind to MBP adsorbed onto nitrocellulose membrane although the antibody bound to SP-A and the chimeras (data not shown). The data demonstrate that

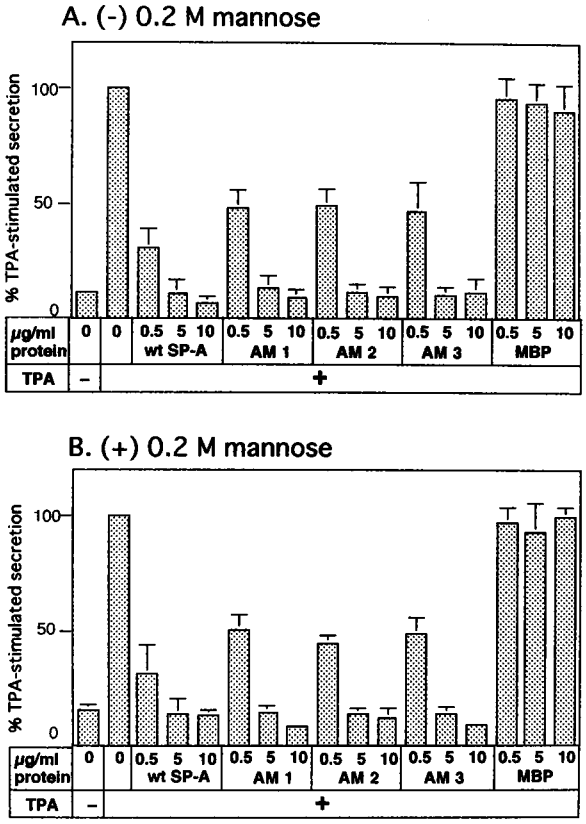

FIGURE 8: SP-A/MBP-A chimeras can inhibit lipid secretion from type II cells. Alveolar type II cells were isolated from rats, and cellular phosphatidylcholine was labeled with [^{3}H]choline overnight as described under Experimental Procedures. The secretagogue TPA (10^{-7} M) and the indicated concentrations of rat MBP-A (MBP), wild type SP-A (wt), or chimera AM1, AM2, or AM3 were added and incubated for 3 h at 37 °C in 1.6 mL of DMEM with (panel B) or without (panel A) 0.2 M mannose. Secretion is defined as (radioactivity in the media)/(radioactivity in the media + cells) (% secretion). Percent secretion stimulated by TPA in the absence or the presence of 0.2 M mannose was 12.53 ± 5.23% (mean ± SD, $n = 3$) or 9.26 ± 5.97%, respectively. Results are expressed as percent of the TPA-stimulated secretion. Data presented are mean ± SD ($n = 3$) with duplicate determinations in each experiment.

the chimeras contain the epitopes for antibodies 1D6 and 6E3.

DISCUSSION

C-Type animal lectins that require Ca^{2+} to ligate sugars constitute a family with shared functional and structural characteristics (Day, 1994; Drickamer, 1988). They exhibit a common sequence motif, consisting of 14 invariant and 18 highly conserved residues in the CRD. Although the collectin family prefers to bind mannose and glucose, these proteins show distinct properties: SP-A binds DPPC and GalCer (Kuroki & Akino, 1991; Kuroki et al., 1992a), SP-D binds phosphatidylinositol and glucosylceramide (Kuroki et al., 1992b; Ogasawara et al., 1992), and human serum MBP binds *N*-acetylglucosamine-terminated glycoconjugates (Kyogashima et al., 1990). SP-A, but not MBP, shows specific interactions with alveolar type II cells as shown in this study. The expression of distinct functions, in spite of similar carbohydrate-binding specificities between these collectins, indicates the importance of structures other than the common sequence motif.

In this study we investigated the importance of the carboxy terminal region of rat SP-A near the small disulfide loop

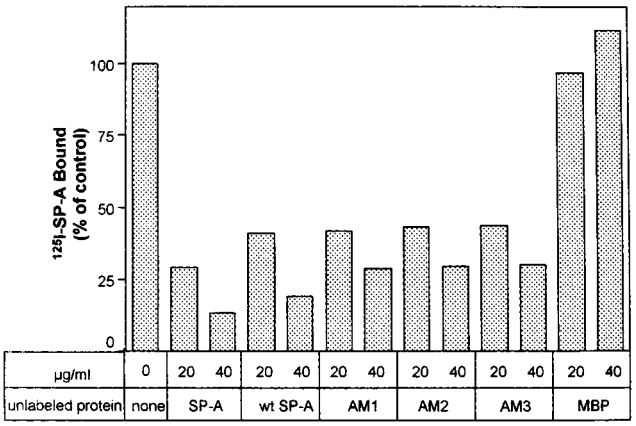

FIGURE 9: SP-A/MBP chimeras compete with rat [^{125}I]SP-A for binding to type II cells. Type II cell monolayers were incubated at 37 °C for 3 h in 1 mL of DMEM containing 10% fetal bovine serum, 0.5 μg/mL rat [^{125}I]SP-A and the indicated concentrations of unlabeled proteins in the presence of 0.2 M mannose. The cell monolayers were washed and harvested as described under Experimental Procedures. The amount of rat [^{125}I]SP-A bound in the absence of unlabeled proteins (control binding) was 21.8 ng/dish (mean of two experiments). The results are expressed as percent of control binding. The data presented are mean with duplicate determinations in two separate experiments.

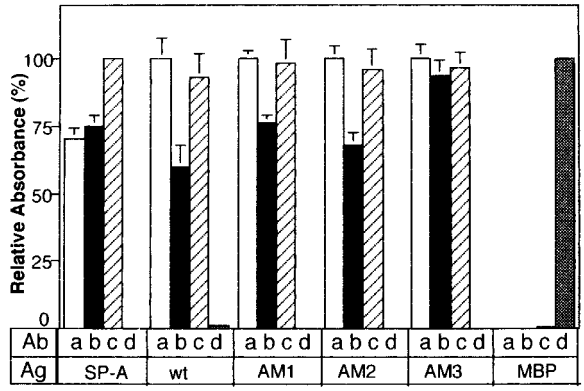

FIGURE 10: Monoclonal antibody binding to SP-A/MBP-A chimeras. The binding of monoclonal antibodies 1D6 and 6E3 to the purified chimeric proteins was examined by ELISA. Aliquots (50 μL, 1 μg/mL) of rat SP-A (SP-A), rat MBP-A (MBP-A), wild type SP-A (wt), and chimeras AM1, AM2, and AM3 were coated onto the microtiter wells. The wells were then incubated with monoclonal antibody 1D6 (a), or 6E3 (b) or polyclonal antibody to SP-A (c) or MBP-A (d) as described under Experimental Procedures. The relative absorbance is expressed as a percent of maximal absorbance at 490 nm for each protein. The mean of maximal absorbance for each protein is SP-A, 0.632; wild type SP-A, 1.542; AM1, 1.294; AM2, 1.524; AM3, 1.642; MBP, 1.917. The data presented are mean ± SD ($n = 3$).

(Cys^{204}−Cys^{218}) in the interaction of SP-A with lipids and alveolar type II cells using the chimeric proteins in which the MBP region was substituted for the SP-A region. The chimeras AM1, AM2, and AM3 bound DPPC and GalCer and caused phospholipid vesicle aggregation. These chimeras were also able to compete for SP-A receptor binding, inhibit lipid secretion from type II cells, and augment lipid uptake by these cells. Since native MBP-A isolated from rat sera did not have any of the above-described SP-A properties, the results clearly indicate that the MBP region of Glu^{185}−Ala^{221} can functionally replace the SP-A region of Glu^{195}−Phe^{228}. One interpretation of the results is that the amino acids required for the SP-A function are located between Gly^{115} and Gly^{194}. Alternatively the MBP region of Glu^{185}−Ala^{221} may assume a conformation in the chimera that it does not have in the native state that confers new function. The MBP region of Glu^{185}−Ala^{221}, homologous to the SP-A region of Glu^{195}−Phe^{228}, may acquire SP-A function in the context of the SP-A region of Gly^{115}−Gly^{194}. Since anti-MBP antibody does not recognize the chimeras, but antibody 1D6 does recognize the chimeras, the MBP region of Glu^{185}−Ala^{221} appears to no longer exhibit the correct three-dimensional MBP-A structure in the context of the SP-A region of Gly^{114}−Gly^{194}. However, the MBP-A domain may adopt the SP-A structure.

We examined the intrinsic fluorescence spectra of recombinant wild type SP-A and chimera AM3 in the absence or presence of Ca^{2+} and unilamellar liposomes containing DPPC and PG. Rat SP-A possesses tryptophan residues at positions 191 and 213 and the AM3 chimera contains tryptophan residues at SP-A-191 and MBP-A-204. When emission spectra were recorded from 300 to 450 nm at the excitation wavelength of 280 nm, the emission peaks of recombinant proteins appeared at 344 nm in the absence of Ca^{2+}. The wavelength of the emission peaks of both proteins was shorter by 1.2−1.7 nm in the absence of lipids when Ca^{2+} was added at a concentration of 1 mM (data not shown). The fluorescence spectra also exhibited a blue shift of the emission peaks of both recombinant proteins by 1.4−2.0 nm in the presence of 100 μg/mL lipids with the addition of Ca^{2+}. These results demonstrate that similar conformational changes occurred in chimeras as well as SP-A in the presence of Ca^{2+} and are consistent with the results obtained from the assays for the SP-A functions.

We have previously shown that the collagenase-resistant fragment (the neck domain plus CRD) of human SP-A inhibited lipid secretion from type II cells (Murata et al., 1993), indicating that the region of the neck plus CRD is responsible for this activity. Epitope mapping for monoclonal antibody 1D6 also identified the CRD as the functional domain that interacts with lipids and type II cells since antibody 1D6 blocked the SP-A functions of lipid binding, vesicle aggregation, lipid uptake by type II cells, and inhibitor activity on lipid secretion (Kuroki et al., 1988c, 1994). We have also shown that antibody 1D6 failed to bind to cell lysate of Sf-9 cells infected with the recombinant virus encoding SP-A in which the small disulfide loop was deleted (SP-A$^{hyp,\Delta Cys^{204}-Cys^{218}}$), although antibody 6E3 which recognizes epitopes in the neck domain bound to the cell lysate as the antigen (Kuroki et al., 1994). Since antibody 1D6 blocked the SP-A functions, the previous data suggest that the SP-A functions are largely attributable to amino acid residues within the steric inhibitory footprint of 1D6 bound to the region of the small disulfide loop. In this study we found that antibody 1D6 recognized all three chimeras AM1−3 but not MBP coated onto microtiter wells or nitrocellulose. The results suggest that the epitope for antibody 1D6 is in part located at the SP-A region of Gly^{115}−Gly^{194}. Antibody 1D6 may also recognize an epitope that Glu^{195}−Phe^{228} of SP-A or Glu^{185}−Ala^{221} of MBP-A confers in the context of the SP-A region of Gly^{115}−Gly^{194}. A simple explanation is that the complete epitope straddles the splice junction in the AM3 chimera. This is consistent with the result from the functional assays of chimeras. Our current data indicate that the SP-A domain Glu^{195}−Phe^{228} and the MBP-A domain Glu^{185}−Ala^{221} containing the small disulfide loop are interchangeable with regard to the SP-A functions

and the binding of anti-SP-A monoclonal antibody.

The crystal structure of a MBP complexed with an oligosaccharide has revealed that amino acid residues that ligate both a calcium ion and a saccharide are located in the carboxy terminal 25 amino acids of the CRD (Weis et al., 1992). Amino acid residues Glu^{185} and Asn^{187} of MBP-A near the small disulfide loop, which are calcium ligands that also form hydrogen bonds to the 3′-hydroxyl of mannose, are conserved in CRDs that preferentially bind mannose, while in lectins that prefer to bind galactose, Gln and Asp are consistently conserved at these positions. Introducing mutations into the cDNAs of MBP-A and SP-A to encode for the substitutions $Glu^{185} \rightarrow$ Gln, $Asn^{187} \rightarrow$ Asp and $Glu^{195} \rightarrow$ Gln, $Arg^{197} \rightarrow$ Asp, respectively, altered the carbohydrate-binding specificity (Drickamer, 1992; McCormack et al., 1994b). A study using the SP-A mutant ($SP\text{-}A^{hyp,Gln^{195},Asp^{197}}$) with the substitutions of $Glu^{195} \rightarrow$ Gln and $Arg^{197} \rightarrow$ Asp revealed that both amino acid residues Glu^{195} and Arg^{197} of rat SP-A are essential for vesicle aggregation, regulation of lipid secretion, and facilitated uptake of phospholipid by type II cells but not for DPPC binding (McCormack et al., 1994b). The chimera AM3, in which amino acid residue Asn^{187} of rat MBP-A was substituted for Arg^{197} of rat SP-A, retained mannose binding and the other SP-A functions in this study. This indicates that Glu^{185} and Asn^{187} of MBP-A can replace Glu^{195} and Arg^{197} of SP-A. Since MBP-A, like SP-A, possesses the carbohydrate-binding specificity with higher affinity for mannose than galactose, these studies support the idea that the carbohydrate-binding specificity is important for the SP-A functions of vesicle aggregation and interaction with type II cells.

All the chimeras were nearly as effective as recombinant SP-A in binding liposomes, competing with [^{125}I]SP-A for receptor occupancy and inhibiting lipid secretion from type II cells. However, these chimeras exhibited reduced activities of phospholipid vesicle aggregation and stimulation of lipid uptake by type II cells. These reduced lipid interactions were almost equivalent among the three chimeras with progressively longer carboxy terminal regions of MBP-A. Substituting the MBP region of Gln^{210}−Ala^{221} for the homologous SP-A region of Leu^{219}−Phe^{228} reduced the SP-A activity by 40−70%. The results suggest that the SP-A region of Leu^{219}−Phe^{228} is participating in phospholipid vesicle aggregation and lipid uptake by type II cells. Replacing the SP-A region of Leu^{219}−Phe^{228} with the MBP region of Gln^{210}−Ala^{221} may also confer a conformation in the chimera that partly impairs these activities but not other SP-A functions.

In summary, we have used novel chimeric proteins synthesized by molecular genetic techniques to clarify the region specific for SP-A function. This study provides evidence that MBP does not possess the ability to interact with DPPC, GalCer, and type II cells as does SP-A. However, the MBP-A region of Glu^{185}−Ala^{221} can functionally replace the SP-A region of Glu^{195}−Phe^{228} without loss of interaction with lipids and type II cells. The chimeras also contain the epitope for antibody 1D6. These findings indicate that the SP-A region Gly^{115}−Gly^{194} plays an important role in protein functions either by directly participating in interactions with lipid and type II cell ligands or by stabilizing the interactions specified by Glu^{195}−Phe^{228} of SP-A and Glu^{185}−Ala^{221} of MBP-A. We conclude that the SP-A domain Glu^{195}−Phe^{228} and the MBP-A domain Glu^{185}−Ala^{221} are interchangeable without loss of the SP-A functions.

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