Epitope Mapping for Monoclonal Antibodies Identifies Functional Domains of Pulmonary Surfactant Protein A That Interact with Lipids*

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Pulmonary surfactant protein A (SP-A) contains 4 domains: a disulfide forming amino terminus, a collagen-like domain, a carbohydrate recognition region, and a carbohydrate recognition region. The protein binds the lipids dipalmitoylphosphatidylcholine and galactosylceramide and induces aggregation of phospholipid vesicles. SP-A also inhibits lipid secretion and enhances the uptake of phospholipid by alveolar type II cells. Previously described monoclonal antibody 1D6 blocks the inhibitory effect of SP-A on lipid secretion by type II cells, but antibody 6E3 has no effect. In the present study we mapped the epitopes for monoclonal antibodies 1D6 and 6E3 by enzyme-linked immunosorbent assay of recombinant proteins expressed using the baculovirus system, and investigated the domain that is responsible for the SP-A interactions with lipid. Monoclonal antibody 1D6 bound to mutant SP-A in which the neck region of the molecule was deleted or substituted with that of mannos-binding protein A, but 6E3 failed to bind to these mutants. In contrast, 1D6 did not bind to a chimera in which the carbohydrate recognition domain (CRD) was substituted with that of surfactant protein D (SP-D). In addition, 1D6 failed to recognize antigen in cells infected with type I1 cells, but antibody 6E3 has no effect. This study demonstrated that the CRD; and 3) the neck domain of SP-A may also be involved in the process of SP-A-mediated uptake of phospholipids by alveolar type II cells.

Pulmonary surfactant is a complex mixture of lipids and proteins that function to keep alveoli from collapsing at the end of expiration. Surfactant protein A (SP-A) is a major glycoprotein component of surfactant, with a reduced denatured molecular mass of 26–38 kDa in the rat (1). The polymorphism of this protein is mainly due to the differential glycosylation of 26-kDa species (2). The cDNAs for SP-A from human, rat, dog, rabbit, and mouse have been isolated and sequenced (3–6). In vitro studies with SP-A have provided compelling evidence to demonstrate that it can function as an inhibitor of phospholipid secretion by alveolar type II cells (7, 8) via interaction with a high affinity cell surface receptor (9–12). SP-A (13) binds to dipalmitoylphosphatidylcholine (DPPC) and galactosylceramide (GalCer) (14–17) and facilitates phospholipid uptake by type II cells (18). SP-A preferentially enhances DPPC uptake by type II cells, and it facilitates the incorporation of this lipid into lamellar bodies (19). Since DPPC is the principal component responsible for the biophysical properties of surfactant, SP-A may play an important role in phospholipid homeostasis in the alveolar space. SP-A can also accelerate calcium-induced aggregation of phospholipid vesicles (20) and form tubular myelin-like structures in concert with SP-B when these proteins are added to artificial phospholipid mixtures (21).

SP-A is a member of the C-type lectin superfamily and along with mannos-binding proteins A and C, surfactant protein D (SP-D), conglutinin (22), and CL-43 (23) it forms the collectin (Group III) subgroup (24). These proteins possess characteristic structural features of: 1) an NH₂-terminal domain containing cysteine(s) involved in interchain disulfide bond formation; 2) a collagenous domain that is rich in hydroxyproline; 3) a neck domain; and 4) a carbohydrate recognition domain (CRD). The minimal CRD has been identified as the COOH terminus from Gly¹⁰⁷ to Ala²⁰¹ in rat mannos-binding protein A (MBP-A) (25, 26), which corresponds to that from Gly¹¹⁵ to Phe²³⁹ in the rat SP-A. The structural determinants of SP-A responsible for its multiple functions have not been fully mapped, although some chemical modification and proteolytic degradation studies have provided important insights. The integrity of disulfide bonds is required for SP-A to inhibit surfactant secretion (1) and to

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1 The abbreviations used are: SP-A, surfactant protein A; DPPC, dipalmitoylphosphatidylcholine; GalCer, galactosylceramide; CRD, carbohydrate recognition domain; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; MBP-A, mannos-binding protein A; PG, phosphatidylglycerol; PC, phosphatidylcholine.
aggregated phospholipid (27). Removal of the amino terminus and collagen-like domain markedly attenuates but does not completely eradicate the inhibition of secretion (28) or the binding of the protein to lipids (14).

In an effort to more precisely map structural domains of SP-A that are responsible for specific functions, we have undertaken a program of site-directed mutagenesis and heterologous expression of SP-A using baculovirus vectors. Recent studies reveal that SP-A produced in insect cells by recombinant baculovirus constructs retains significant inhibitory activity upon surfactant secretion by type II cells, and induces lipid aggregation despite imperfect post-translational modification to the protein (29). In this report we have utilized site-directed mutagenesis in conjunction with previously isolated monoclonal antibodies for the purpose of both epitope mapping and structure-function analysis of the protein. Five monoclonal antibodies recognizing peptide epitopes have been prepared against SP-A (1).

Four out of five monoclonal antibodies blocked the inhibitory activity of SP-A on lipid secretion by type II cells, but one antibody (6E3) failed to alter the inhibitory effect of this protein (1). This study was designed to map epitopes for monoclonal antibodies that exhibited different effects upon SP-A functions using recombinant proteins expressed in the baculovirus system. This approach has enabled us to map domains involved in lipid binding, liposome aggregation, and lipid uptake by alveolar type II cells.

**EXPERIMENTAL PROCEDURES**

**Purification and Iodination of Rat SP-A**—Surfactant was isolated from Sprague-Dawley rats given an intratracheal instillation of 10 mg of saline, 4 weeks before lung lavage (30). Native SP-A was isolated and purified from mannose-Sepharose 6B column chromatography using a Mono-Q column by gel filtration over a Bio-Gel A5m column as described previously (9). The 125I-SP-A was prepared by the method of Bolton and Hunter (31) using the Bolton-Hunter reagent (Amersham) as described previously (9). More than 95% of the radioactivity was precipitated by treatment with 10% (w/v) trichloroacetic acid. The specific activity of 125I-SP-A (9). More than 95% of the radioactivity was precipitated by treatment with 10% (w/v) trichloroacetic acid. The specific activity of 125I-SP-A (9). More than 95% of the radioactivity was precipitated by treatment with 10% (w/v) trichloroacetic acid.

**Monoclonal Antibodies to Rat SP-A**—Monoclonal antibodies to rat SP-A were prepared as reported previously (1). Antibodies were purified by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia Biotech Inc.). All monoclonal antibodies recognized epitopes in the polypeptide portion of SP-A, and had nearly equivalent affinity for the SP-A antigen as described previously (1). Monoclonal antibodies 1D6 and 6E3 were used in this study.

**Monoclonal Antibody Binding Assay**—The binding of monoclonal antibodies, 1D6 and 6E3, to the purified recombinant mutant proteins was examined by ELISA. Aliquots of the mutant proteins (50 pl, 1 pg/ml) in 5 mM Tris buffer, pH 7.4, were coated onto the microtiter wells (Immulon 1, Dynatech Laboratories) and air-dried. After preincubation of the wells, nonspecific binding sites were blocked with phosphate-buffered saline (PBS) containing 0.1% (w/v) Triton X-100 and 1% (w/v) bovine serum albumin (the binding buffer). Next, the TLC plate was immersed in the organic solvent, the plate was air-dried and soaked in 50 mM Tris buffer, pH 7.4, containing 0.1 M NaCl, 2 mM CaCl2, and 20 mg/ml bovine serum albumin (the binding buffer). Then, the TLC plate was incubated with 125I-SP-A (0.25 pg/ml) in the presence or absence of monoclonal antibody (20 ng/ml) in the binding buffer for 30 min at room temperature. The plate was then washed with gentle shaking on ice in the washing buffer (50 mM Tris buffer, pH 7.4, containing 0.1 M NaCl, 2 mM CaCl2, and 1 mg/ml bovine serum albumin). The TLC plate was finally air-dried and exposed to x-ray film at ~70°C overnight.

The binding of 125I-SP-A to lipids was also determined using thin-layer chromatography (TLC) on Polygram Sil G (Macherey-Nagel, Dassel, Germany) with a solvent system of n-hexane:isoamyl alcohol (1:1) for lipids and the organic solvent, the plate was air-dried and soaked in 50 mM Tris buffer, pH 7.4, containing 0.1 M NaCl, 2 mM CaCl2, and 20 ng/ml bovine serum albumin (the binding buffer). Then, the TLC plate was incubated with 125I-SP-A (0.25 pg/ml) in the presence or absence of monoclonal antibody (20 ng/ml) in the binding buffer for 30 min at room temperature. The plate was then washed with gentle shaking on ice in the washing buffer (50 mM Tris buffer, pH 7.4, containing 0.1 M NaCl, 2 mM CaCl2, and 1 mg/ml bovine serum albumin). The TLC plate was finally air-dried and exposed to x-ray film at ~70°C overnight.

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of SP-A (5 μg/ml), monoclonal antibodies (50 μg/ml) were added at a time of 5 min and the turbidity was further measured for 10 min.

Uptake of Phospholipid Lipoasome by Alveolar Type II Cells—Alveolar type II cells were isolated from adult male Sprague-Dawley rats by tissue dissociation with elastase and purification on metrizamide gradients (36). Uptake of liposomes by freshly isolated type II cells was performed by the method based on that described by Wright et al. (18). Type II cells (106 cells) were incubated with 0.5 ml of Dulbecco's modified Eagle's medium containing 10 mM HEPES, pH 7.4, radiolabeled phospholipid liposomes (100 μg/ml), SP-A (5 μg/ml), and monoclonal antibodies (50 μg/ml) at 37 °C for 1 h. After incubation, cells and media were separated by centrifugation at 160 X g for 5 min at 4 °C. The medium was removed and the cells were gently resuspended in 1 ml of ice-cold phosphate-buffered saline containing 1 mg/ml bovine serum albumin. The washings were done three times. Before the final centrifugation, the cell suspension was transferred to a fresh tube. The final cell pellet was analyzed for radioactivity.

Other Methods—Protein contents were estimated by the bicinchoninic protein assay kit (BCA) (Pierce) using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (37).

RESULTS

Construction of Mutant Proteins and Proteins Expressed in Insect Cells—Group III C-type lectins such as MBP-A, SP-A, and SP-D are isolated from four different structural components which are: 1) an NH2-terminal domain involved in interchain disulfide formation; 2) a collagenous domain; 3) a neck domain; and 4) a carbohydrate recognition domain (CRD) (4, 32, 38, 39). For simplicity these domains are denoted A1-A4 in SP-A, M1-M4 in MBP-A, and D1-D4 in SP-D. All proteins expressed in baculovirus-infected insect cells lack proline hydroxylation and hence are denoted SP-Ahyp (29). In this study we constructed three deletion mutants of SP-A, a chimeric molecule of SP-A/MBP-A and three chimeric molecules of SP-A/SP-D as follows: 1) mutants in which Ala81 to Gly103, Leu110 to Leu112, or Cys206 to Cys218 of SP-A are deleted (SP-Ahyp-Leu112-Leu112 (205 amino acids), SP-Ahyp-Leu112-Leu112 (220 amino acids), or SP-Ahyp-Leu112-Leu112 (213 amino acids), respectively); 2) a chimera A1A2M3A4 which is composed of Asn1 to Pro109 of SP-A, Arg110 to Lys206 of MBP-A, and Gly113 to Phe208 of SP-A (230 amino acids); or 3) chimeras A1A2M3D4 (233 amino acids), A1A2A3D4 (229 amino acids), and D1D2A3A4 (351 amino acids) as described in the accompanying paper (43). The recombinant proteins used in this study are schematically represented in Fig. 1.

Analysis of native SP-A by SDS-PAGE reveals the protein migrates as a triplet at 26, 32, and 38 kDa (Fig. 2, lane a). In contrast, SP-A produced by the baculovirus expression system migrates as a set of bands at 25–32 kDa on SDS-PAGE under reducing conditions (SP-Ahyp) (Fig. 2, lane b). The difference in molecular weight between native and recombinant protein is related to the glycosylation pattern by insect cells and the lack of hydroxylation of proline residues as described previously (29). SP-Ahyp-M1A2-M3A4 and SP-Ahyp-D1D2-D3D4, and the A1A2M3A4 chimera used for the monoclonal antibody binding assay were also analyzed by electrophoresis (Fig. 2, lanes c, d, and e, respectively). These mutant proteins also migrated as broad bands as observed in the SP-Ahyp. The protein bands evident after Coomassie Blue staining of the electrophoretic gels are identical to those reactive with polyclonal antibody upon immunoblot analysis indicating that the purified proteins are isoforms of SP-A. The electrophoretic profiles also demonstrate the purity of the protein preparations and the minimal degree of proteolysis. Electrophoretic characterization of chimeras of SP-A and SP-D also employed in this study is described in the accompanying paper (43).

Epitope Mapping—Previous experiments from this laboratory have described the isolation and properties of two monoclonal antibodies 1D6 and 6E3 (1). These antibodies have

![Fig. 1. Schematic representation of SP-A mutants. The domain structures of the monomeric subunit of SP-A and the eight mutants examined in this study are shown. SP-Ahyp does not contain hydroxyl modification to the prolines in the collagenous domain. SP-Ahyp-Leu112-Leu112, SP-Ahyp-Leu112-Leu112, and SP-Ahyp-Leu112-Leu112 are the deletion mutants at the indicated sites. A1A2M3A4, A1A2A3D4, and D1D2A3A4 are the chimeric molecules of SP-A and mannos-binding protein A or SP-D. Domains that are black are from mannose-binding protein A, domains that are filled are from SP-D, and domains that are black are from mannose-binding protein A.](attachment:image.png)
markedly different actions when they bind SP-A. Antibody 1D6 completely blocks the action of SP-A as an inhibitor of secretion and prevents SP-A binding to its high affinity receptor (1,12). Antibody 6E3 fails to alter the action of SP-A as an inhibitor of secretion and partially blocks interaction of SP-A with its receptor (1,12). The mutant proteins and chimeras described above were analyzed for their reactivity to the antibodies 1D6 and 6E3 for the purpose of mapping the epitopes and correlating domain structure with protein function. To examine these epitopes, monoclonal antibody binding to recombinant proteins was measured by ELISA (Fig. 3). Polyclonal anti-SP-A IgG was used as a positive control and it bound to all mutant proteins used. Non-immune mouse IgG was used as a negative control and it failed to bind any of the proteins (data not shown). Both monoclonal antibodies, 1D6 and 6E3, bound to SP-A^op as well as D1D2A3A4, but neither antibody bound to A1A2D3D4, indicating these antibodies recognize epitopes located in the A3 + A4 regions (neck domain + CRD) of SP-A. Antibody 1D6 bound to SP-A^op, A1A2D3-CL^op, SP-A^op,Ala106-Leu132, and A1A2M3A4, indicating that all these proteins contain the relevant epitope. In contrast, 6E3 bound to none of the neck-deletion mutants or the chimera in which the neck of SP-A is substituted with the neck of MBP-A. Antibody 1D6 failed to bind to A1A2A3D4 but 6E3 bound to this chimera to nearly the same extent as the polyclonal antibody. These results clearly indicate that these monoclonal antibodies recognize different epitopes of SP-A and that 1D6 binds to the A4 domain and 6E3 binds to the A3 domain.

We further investigated the epitope for 1D6 by dot-blot analysis using cell lysates from S9 cells infected with the recombinant virus encoding SP-A^op,ΔCy5ΔCy204ΔCy218 as the antigen (Fig. 4). Both 6E3 and polyclonal anti-SP-A IgG bound to SP-A^op and SP-A^op,ΔCy5ΔCy204ΔCy218. In contrast, 1D6 exhibited no binding to SP-A^op,ΔCy5ΔCy204ΔCy218. Control mouse IgG did not bind any of these antigens. In addition, lysates from non-infected S9 cells failed to bind any antibodies. Analysis of cell lysates was necessary because SP-A^op,ΔCy5ΔCy204ΔCy218 was not secreted. Antibody 1D6 also recognizes reduced and alkylated SP-A that has been transferred to nitrocellulose indicating that the three-dimensional arrangement of the small disulfide loop is not required for antibody binding. Collectively, these data are consistent with the conclusion that the epitope for 1D6 is localized at Cys<sup>304</sup>, Cys<sup>318</sup> in the A4 domain (CRD) and that for 6E3 is at the A3 (neck) domain.

**Effect of Monoclonal Antibodies upon the Binding of SP-A to Lipids—**SP-A binds the lipids DPPC and GalCer. We next sought to determine what structural domains of the protein were involved in these processes and how these domains were related to the epitopes for 1D6 and 6E3. The results presented in Fig. 5 demonstrate the binding of 125I-SP-A (0.25 µg/ml) to DPPC and GalCer on TLC plates in either the absence or presence of monoclonal antibodies (20 µg/ml). Antibody 1D6 and polyclonal anti-SP-A antibody blocked the binding of SP-A to both DPPC and GalCer. In contrast, antibody 6E3 showed almost no effect on the lipid-binding property of SP-A.

To further characterize the effect of monoclonal antibodies, 125I-SP-A binding (1 µg/ml) to lipids coated onto microtiter wells at 1 µg/well was performed in the presence of various concentrations (1-400 µg/ml) of antibodies. When 125I-SP-A was incubated with DPPC coated onto the wells in the absence of antibodies, 16.6 ± 3.2 ng/well (mean ± S.E., n = 3) of SP-A bound to DPPC. 1D6 diminished the binding of SP-A to DPPC in a concentration-dependent manner (Fig. 6A). 1D6 completely blocked the SP-A binding to DPPC at 10 µg/ml, while inclusion of control mouse IgG at 10-400 µg/ml in the binding solution failed to eliminate 125I-SP-A binding to DPPC. 6E3 reduced the binding of SP-A to DPPC by approximately 55% at high concentrations but also failed to completely block the binding of 125I-SP-A to DPPC. Next, the effect of antibodies on the binding of SP-A to GalCer was also examined (Fig. 6B). The amount of 125I-SP-A binding to GalCer in the absence of antibodies was 18.6 ± 2.3 ng/well (mean ± S.E., n = 3). The results of antibody effects on GalCer binding were similar to that on DPPC binding. Antibody 1D6 completely blocked the binding of SP-A to GalCer but 6E3 failed to completely block SP-A binding to GalCer. These results indicate that the CRD (A4) domain is directly involved in the binding of SP-A to phospholipid and glycolipid.

**Epitope-specific Inhibition of SP-A-induced Liposome Aggregation by Monoclonal Antibodies—**In addition to binding lipid, SP-A can induce aggregation of DPPC liposomes. We next sought to determine the effects of the monoclonal antibodies 1D6 and 6E3 upon this process. SP-A and phospholipid liposomes were preincubated in the presence of monoclonal antibodies, and the turbidity caused by lipid aggregation that occurs with the addition of CaCl<sub>2</sub> was measured (Fig. 7). SP-A aggregated phospholipid vesicles in a time-dependent manner. When SP-A, liposomes, or CaCl<sub>2</sub> was deleted, negligible light scattering was observed (data not shown). Control mouse IgG showed no effect on liposome aggregation. Antibody 1D6 completely prevented the liposome aggregation effect by SP-A. The results obtained using 1D6 at 50 µg/ml were identical to those found using the antibody at 100 µg/ml (data not shown). In contrast, SP-A retained the ability to induce liposome aggregation even in the presence of 6E3, although 6E3 reduced the turbidity by approximately 35% with respect to the final extent of aggregation. Increasing the 6E3 concentration to 200 µg/ml failed to cause any further reduction in lipid aggregation (data not shown). Next, the effect of monoclonal antibodies on preformed SP-A-vesicle aggregates was investigated. SP-A was mixed with phospholipid liposomes and the aggregation was initiated by the addition of 5 mM CaCl<sub>2</sub>. Monoclonal antibodies were added after 5 min and the turbidity was further measured for 10 min (Fig. 8). Addition of 6E3 into the cuvette in which SP-A had caused liposome aggregation decreased the turbidity to a new equilibrium absorbance that was 81% of the control value. By comparison, 1D6 decreased the equilibrium absorbance to 19% of the control value. These results demonstrate that 1D6 but not 6E3 can reverse the liposome aggregation induced by SP-A.

Since the epitope for 1D6 is associated with the small disulfide loop (Cys<sup>304</sup>-Cys<sup>318</sup>), the data further indicate that this region of the CRD (A4) domain plays a critical role in aggregation of phospholipid vesicles by SP-A.
Antibody Mapping of SP-A Functional Domains

**FIG. 3.** Antibody 1D6 recognizes epitopes in the A4 domain of SP-A and antibody 6E3 recognizes epitopes in the A3 domain of SP-A. The binding of monoclonal antibodies, 1D6 and 6E3, to the purified recombinant proteins was examined by ELISA. Aliquots (50 µl, 1 µg/ml) of SP-A hyp, SP-A hyp ΔAla81-Gly103, ΔA105-112, A1A2M3A4, D1D2A3A4, A1A2D3D4, and A1A2A3D4 were coated onto the microtiter wells. The wells were then incubated with monoclonal antibody 1D6 (W), 6E3 (a), or polyclonal anti-SP-A antibody (0) as described under “Experimental Procedures.” The data presented are from the ELISA at 10 pg/ml antibodies. The relative absorbance is expressed as percent of maximal absorbance at 490 nm for each protein. Mean of maximal absorbance for each mutant from two to three experiments is: SP-A hyp, 2.0; ΔA81-103, 1.774; ΔA105-112, 1.685; A1A2M3A4, 1.694; D1D2A3A4, 0.903; A1A2D3D4, 0.546; A1A2A3D4, 0.815.

**FIG. 4.** The epitope for 1D6 lies within Cys204-Cys218 of SP-A. The monoclonal antibody binding assay for SP-A hyp ΔAla204-Gly218 was carried out by dot-blot analysis. The cell lysate (5 µg of total protein) from SF9 cells infected with a recombinant virus stock of SP-A hyp ΔAla204-Gly218 (SF9/SP-A hyp ΔAla204-218), or from noninfected SF9 cells (SF9/control), or the purified SP-A hyp (0.5 µg) was applied onto a nitrocellulose sheet. The nitrocellulose sheet was incubated with anti-SP-A polyclonal antibody (polyclonal), antibody 1D6 (1D6), antibody 6E3 (6E3), or control mouse IgG (control) at 5 µg/ml followed by anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase, as described under “Experimental Procedures.”

**Effect of Monoclonal Antibodies on SP-A-mediated Phospholipid Uptake by Alveolar Type II Cells**—Another measure of the interaction of SP-A with phospholipid is the ability of the protein to augment liposome uptake by freshly isolated alveolar type II cells. The basis of this uptake is poorly understood but it is thought to be related to the phospholipid recycling documented to occur within the alveolus (40, 41). When type II cells were incubated with phospholipid liposomes containing [3H] DPPC in the presence of SP-A (5 µg/ml), the protein enhanced the uptake of liposomes by approximately 5 times the levels found without SP-A (Fig. 9). We examined the effect of monoclonal antibodies on the SP-A-mediated liposome uptake by type II cells. The radioactivity sedimented in the presence of SP-A and monoclonal antibodies in the absence of cells was less than 2% of that occurring in the presence of cells. When 1D6 and 6E3 were incubated with cells and liposomes in the presence of SP-A, the protein-mediated uptake of lipid was essentially reduced to basal levels. These data implicate the neck domain of SP-A as well as the CRD as an important structural domain involved in the process of SP-A-mediated uptake of phospholipid vesicles by alveolar type II cells.

**DISCUSSION**

SP-A has been shown to inhibit secretion of surfactant lipids by primary cultures of alveolar type II cells (7, 8) and to bind to a high affinity receptor expressed on type II cells (1, 10, 11). Interactions of SP-A with phospholipids have also been reported. SP-A specifically binds to DPPC (15) and GalCer (16, 17), and causes the aggregation of phospholipid liposomes con-
Antibody Mapping of SP-A Functional Domains

**Fig. 6.** Antibody 1D6 blocks 125I-SP-A binding to lipid in a concentration-dependent manner. 125I-SP-A binding (1 ng/ml) to DPPC (panel A) and GalCer (panel B) coated onto microtiter wells (1 ng lipid/well) was performed in the presence of 1–20 ng/ml antibody 1D6 (●), 10–400 ng/ml antibody 6E3 (○), or control mouse IgG (□). 125I-SP-A was incubated at room temperature for 1 h. After incubation, the wells were washed and SP-A bound to the lipids was determined as described under “Experimental Procedures.” The data are expressed as percent of control binding in which the binding was carried out in the absence of antibody (mean ± S.E., n = 3).

**Fig. 7.** Antibody 1D6 inhibits SP-A induced liposome aggregation. Unilamellar liposomes (200 µg/ml) and SP-A (5 µg/ml) were preincubated in the absence (●) or presence of 100 µg/ml monoclonal antibody 1D6 (○), 6E3 (□), or control IgG (□). Following the initial absorbance reading at 400 nm, CaCl₂ was added to a final concentration of 5 mM at a time of 30 s and light scattering was further measured until a time of 5 min. Data presented are from a representative one of three experiments.

This protein can also enhance the uptake of phospholipid liposomes by type II cells. We previously reported the preparation of several monoclonal antibodies recognizing peptide epitopes on SP-A (1). Monoclonal antibody 1D6 blocked the inhibitory effect of SP-A upon surfactant lipid secretion by type II cells. In contrast, monoclonal antibody 6E3 failed to alter the inhibitory effect of SP-A on lipid secretion. Our previous studies indicated that monoclonal antibodies to SP-A, which exhibited different effects on functions of the protein, ultimately should prove useful for structurally mapping these domains. The purpose of this study was to determine the relationship of SP-A structure and biological functions by mapping epitopes for monoclonal antibodies using recombinant proteins expressed in the baculovirus system.

We examined the effects of monoclonal antibodies on the functions of SP-A in lipid binding, phospholipid vesicle aggregation, and liposome uptake by type II cells. Antibody 1D6 completely blocked all of the SP-A functions examined in this study. Antibody 6E3 failed to abrogate the SP-A activities of lipid binding and phospholipid vesicle aggregation. Interestingly, 6E3 blocked the effect of SP-A on lipid uptake by type II cells to the same extent as antibody 1D6. This observation is in marked contrast to the disparate effects of the two antibodies upon SP-A-mediated inhibition of surfactant secretion (1D6 prevents SP-A from inhibiting secretion and 6E3 has no effect). These results imply that the interaction of SP-A with the cell surface receptor involved in regulating secretion is different from the interaction with components of the type II cell surface involved in lipid uptake. Such a result suggests that type II cells may have multiple receptors for SP-A. Previous work with the antibody 6E3 (12) and recombinant forms of SP-A (29) have also been consistent with the idea that there are multiple receptors for SP-A.

The data presented in this report also demonstrate that the A4 domain (CRD) is essential for the inhibitory activity of SP-A on lipid secretion and the stimulatory activity of SP-A upon lipid uptake by type II cells. Furthermore, the results suggest that the A3 (neck) domain also plays an important role in the process of SP-A-mediated uptake of phospholipids. Taken together, these observations suggest that some interaction be-
Fig. 8. Antibody 1D6 reverses SP-A induced lipid aggregation. After liposome aggregation was induced in the presence of SP-A (5 μg/ml), monoclonal antibodies (50 μg/ml) were added at a time of 5 min and the turbidity was further measured for 10 min as described under "Experimental Procedures." ○, antibody 1D6; □, antibody 6E3; ■, control. Data presented are from a representative one of three experiments.

Fig. 9. Antibodies 1D6 and 6E3 blocks SP-A-dependent lipid uptake by alveolar type II cells. Alveolar type II cells (10^6 cells) were incubated with 100 μg/ml phospholipid liposomes containing [3H]DPPC in the absence or presence of SP-A (5 μg/ml) and monoclonal antibodies (50 μg/ml) at 37°C for 1 h. After incubation, cells were washed and the radioactivity associated with the cells was measured as described under "Experimental Procedures." Control incubations without type II cells were also performed as indicated. Values are mean ± S.E. (n = 3). Asterisks indicate *p < 0.005 when compared to SP-A + control IgG.

between domains A3 and A4 is likely to be required for the lipid uptake phenomenon.

We have shown in the accompanying paper (44) that SP-A^Glu156Arg197 mutant, in which Glu156 and Arg197 of SP-A have been converted to Glu and Arg, respectively, fails to inhibit lipid secretion and to compete with SP-A for cell surface binding although it retains the ability to bind DPPC. These data are consistent with the conclusion that Glu156 and Arg197 are essential for the inhibitory effect of SP-A on lipid secretion. Although antibody 1D6 failed to bind to SP-A^Glu156Arg197 (data not shown), indicating that the epitope for antibody 1D6 is localized to the loop region (residues 204–218) but probably not to the adjacent Glu156Arg197-containing region of the protein. Our preferred interpretation of these results is that antibody binding to the epitope contained in the Cys204-Cys218 region sterically prevents receptor access to critical residues Glu156 and Arg197. Since 1D6 recognizes reduced and alkylated SP-A after transfer from denaturing electro-
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