Pulmonary Surfactant Protein A (SP-A) Specifically Binds Dipalmitoylphosphatidylcholine*

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Phospholipids are the major components of pulmonary surfactant. Dipalmitoylphosphatidylcholine is believed to be especially essential for the surfactant function of reducing the surface tension at the air-liquid interface. Surfactant protein A (SP-A) with a reduced denatured molecular mass of 26–38 kDa, characterized by a collagen-like structure and N-linked glycosylation, interacts strongly with a mixture of surfactant-like phospholipids. In the present study the direct binding of SP-A to phospholipids on a thin layer chromatogram was visualized using 125I-SP-A as a probe, so that the phospholipid specificities of SP-A binding and the structural requirements of SP-A and phospholipids for the binding could be examined. Although 125I-SP-A bound phosphatidylcholine and sphingomyeline, it was especially strong in binding dipalmitoylphosphatidylcholine, but failed to bind phosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine. Labeled SP-A also exhibited strong binding to distearoylphosphatidylcholine, but weak binding to dimyristoyl-, 1-palmitoyl-2-linoleoyl-, and dilinoleoylphosphatidylcholine. Unlabeled SP-A readily competed with labeled SP-A for phospholipid binding. SP-A strongly bound dipalmitoylglycerol produced by phospholipase C treatment of dipalmitoylphosphatidylcholine, but not palmitic acid. This protein also failed to bind lysophosphatidylcholine produced by phospholipase A2 treatment of dipalmitoylphosphatidylcholine. 125I-SP-A shows almost no binding to dipalmitoylphosphatidylglycerol and dipalmitoylphosphatidylethanolamine. The addition of 10 mM EGTA into the binding buffer reduced much of the 125I-SP-A binding to phospholipids. Excess deglycosylated SP-A competed with labeled SP-A for binding to dipalmitoylphosphatidylcholine, but the excess collagenase-resistant fragment of SP-A failed. From these data we conclude that 1) SP-A specifically and strongly binds dipalmitoylphosphatidylcholine, 2) SP-A binds the nonpolar group of phospholipids, 3) the second positioned palmitate is involved in dipalmitoylphosphatidylcholine binding, and 4) the specificities of polar groups of dipalmitoylglycerophospholipids also appear to be important for SP-A binding, 5) the phospholipid binding activity of SP-A is dependent upon calcium ions and the integrity of the collagenous domain of SP-A, but not on the olosaccaride moeity of SP-A. SP-A may play an important role in the regulation of recycling and intra- and extracellular movement of dipalmitoylphosphatidylcholine.

Alveolar type II cells produce and secrete a complex mixture of lipids and proteins called pulmonary surfactant, which functions to keep the alveoli from collapsing at the end of expiration (1, 2). The major protein component of surfactant, surfactant protein A (SP-A), is a glycoprotein with a reduced denatured molecular mass of 26–38 kDa in the rat (3). The cDNAs of the human, rat, and dog SP-A molecules have been isolated. This protein possesses the striking feature of collagen-like sequences. SP-A has been identified as a potent negative regulator of surfactant phospholipid secretion by primary cultures of alveolar type II cells (4, 5). Recent studies (6, 7) have demonstrated the presence of a high affinity receptor for SP-A expressed on alveolar type II cells.

Phospholipids are the major components of pulmonary surfactant, making up 80–90% of its weight. Two major classes of surfactant phospholipids are phosphatidylcholine and phosphatidylglycerol, which constitute 70–80% and 5–10% of phospholipids, respectively. Disaturated species, especially with fatty acids being palmitic acid (2), form about 60% of the phosphatidylcholine. Dipalmitoylphosphatidylcholine is believed to be essential for the surfactant function of reducing surface tension at the air-liquid interface. SP-A interacts with surfactant-like lipids, such as dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol (8–11), causes phospholipid aggregation (12), and promotes the rapid formation of stable surface films of phospholipid cooperating with the hydrophobic surfactant proteins (13). SP-A has also been demonstrated to enhance the uptake of phospholipid liposome by alveolar type II cells (14). However, whether SP-A binds all phospholipids, which classes of lipids it binds, and how it binds are not fully understood. In the present study the direct binding of SP-A to phospholipids was visualized using 125I-SP-A as a probe, and the structural requirements of SP-A and phospholipids for the binding was examined. This work provides direct evidence that SP-A specifically binds dipalmitoylphosphatidylcholine.

MATERIALS AND METHODS

Purification of SP-A—Surfactant was isolated from rats given intratracheal instillation of 10 mg of silica in saline 4 weeks prior to lung lavage (15). The surfactant was purified as described by Hawgood et al. (12). SP-A was purified by the method described previously (6). Briefly, surfactant was delipidated with 1-butanol and the organic solvent soluble lipids were separated from the protein when precipitated by centrifugation at 2,000 × g for 30 min at room temperature. After the residual butanol was evaporated under a gentle stream of

*This research was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Japan and a Grant from the Akiyama Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: SP-A, surfactant-associated protein A; CRF, collagenase-resistant fragment of SP-A; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; TLC, thin layer chromatography; ANSA, 8-anilino-1-naphthalenesulfonic acid.
nittogen, the protein was suspended in 5 mM Tris buffer (pH 7.4) and dialyzed against the same buffer. The suspension was then centrifuged at 150,000 x g for 1 h at 4 °C and the supernatant was applied to a mannose-Sepharose 6B (16) column. The SP-A bound content was estimated by the method of Lowry et al. (17) using bovine alveolar proteinosis in the same fashion as the rat SP-A. The protein content was estimated by the method of Lowry et al. (17) using bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (18).

Iodination of Rat SP-A—Rat SP-A was iodinated by the method of Bolton and Hunter (19) using the Bolton-Hunter reagent (Amer sham Corp.) as described previously (6). The specific activity of the 125I-Sp-A used ranged between 140 and 432 cpm/ng. In all experiments more than 90% of the radioactivity was precipitated by treatment with 10% (w/v) trichloroacetic acid.

Preparation of Modified SP-A—Deglycosylated rat SP-A was prepared based on the method described previously (3). Briefly, the rat SP-A (0.5 mg of 1.78 mg/ml) in 5 mM Tris buffer (pH 7.4) containing 1 mM EDTA was incubated with N-glycanase (Genzyme Corp.) at a concentration of 1 mg/mg protein for 1 h at 37°C. The reaction mixture was then applied to a Bio-Gel A5m (Bio-Rad) column (1.5 x 59 cm) to separate free oligosaccharide from the deglycosylated form of the protein. The deglycosylated SP-A was eluted with 5 mM Tris buffer (pH 7.4) containing 1 mM EDTA.

The noncollagenous carboxyl-terminal domain of SP-A was prepared as described below. Human SP-A (0.5-1.0 mg) was suspended with 50 mM Tris buffer (pH 7.4) containing 2 mM CaCl2. Collagenase (Advance Biofactures Corp.) was added (500 units/mg protein) to the SP-A preparation and incubated at 37 °C for 24 h. The reaction mixture was applied to a Bio-Gel A5m (Bio-Rad) column (1.5 x 59 cm) to separate free oligosaccharide from the deglycosylated form of the protein. The deglycosylated SP-A was eluted with 5 mM Tris buffer (pH 7.4) containing 1 mM EDTA.

RESULTS

Electrophoretic Analysis of Iodinated and Modified SP-A—Purified rat SP-A and 125I-SP-A were analyzed by electrophoresis (Fig. 1B, lanes c and d, and Fig. 1A, lanes a and b, respectively). The major forms of iodinated protein correlated well with the major forms of unmodified protein. Rat deglycosylated SP-A is shown in Fig. 1B, lane e. The majority of the oligosaccharide was removed. Human SP-A and human CRF were also electrophoresed as shown in Fig. 1B, lanes f and g. Collagenase treatment of human SP-A created an apparent 20-kDa peptide.

Phospholipid Specificity of 125I-SP-A Binding—Each phospholipid (7 nmol) was developed on a TLC plate and stained with iodine vapor (Fig. 2A). The same amount of phospholipid was developed and 125I-SP-A (1 µg/ml) binding on the TLC plate was performed in the absence (Fig. 2, B and D) or presence (Fig. 2C) of 100 µg/ml native rat SP-A and then autoradiographed and visualized. 125I-SP-A bound phosphatidylycerine, derived from egg and rat lung, and sphingomyelin, and especially bound dipalmitolylphosphatidylcholine strongly (Fig. 2, B and D), but did not bind 7 nmol of phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, nor phosphatidylinositol. Excess unlabeled SP-A clearly reduced 125I-SP-A binding to phospholipids (Fig. 2C), suggesting that phospholipid binding of 125I-SP-A is specific for the SP-A molecule.

125I-SP-A (1 µg/ml) binding was examined on the plates which were developed with various amounts (10-500 nmol) of...
phospholipids. $^{125}$I-SP-A binding increased as the amount of phosphatidylcholine increased (Fig. 3). The result demonstrates that SP-A binding is dependent upon the amount of phosphatidylcholine. SP-A bound dipalmitoylphosphatidylcholine far more strongly than egg phosphatidylcholine. The protein, however, failed to bind in the presence of a large amount of phosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol.

Next, the question of which molecular species of phosphatidylcholine $^{125}$I-SP-A binds was tested using 10 nmol of dimyristoyl-, dipalmitoyl-, distearoyl-, 1-palmitoyl-2-linoleoyl-, and dilinoleoylphosphatidylcholine. The result is shown in Fig. 4. Labeled SP-A bound dipalmitoyl- and distearoylphosphatidylcholine strongly, but bound dimyristoyl-, 1-palmitoyl-2-linoleoyl-, and dilinoleoylphosphatidylcholine weakly (Fig. 4). The data show that $^{125}$I-SP-A binding to phosphatidylcholine is molecular species-specific. These results demonstrate that SP-A specifically and strongly binds dipalmitoylphosphatidylcholine.

**Structural Requirement of Phospholipid for SP-A Binding**—Which portion in the phospholipid molecule was required or involved in SP-A binding was examined. Dipalmitoylphosphatidylcholine treated with phospholipase C and sphingomyelinase were developed on TLC plates with a control lipid (7 nmol/lane). Palmitic acid (15 nmol/lane) was also developed and $^{125}$I-SP-A binding was performed. The data are shown in Fig. 5. SP-A bound dipalmitoylcholine very strongly, but did not bind palmitic acid in an amount almost equivalent to that of dipalmitoylphosphatidylcholine. SP-A also bound ceramide very weakly. These results indicate that SP-A binds a nonpolar group of the phospholipid molecule and that the binding site of SP-A in the phosphatidylcholine molecule is different from that in the sphingomyelin molecule.

Next, dipalmitoylphosphatidylcholine was treated with phospholipase A$_2$ and developed on a TLC plate. Phospholipase A$_2$ treatment of dipalmitoylphosphatidylcholine produced lysophosphatidylcholine (Fig. 6A). 10 nmol of phosphatidylcholine and the same amount of lysophosphatidylcholine were subjected to $^{125}$I-SP-A binding. SP-A failed to bind lysophosphatidylcholine (Fig. 6B). The result indicates that the second positioned palmitate is involved in SP-A binding to dipalmitoylphosphatidylcholine. SP-A may bind the 2-palmitate.
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**FIG. 5.** 125I-SP-A binding to dipalmitoylglycerol, palmitic acid, and ceramide. Dipalmitoylphosphatidylycerol treated with phospholipase C (DPPC/PLC, 7 nmol), sphingomyelin treated with sphingomyelinase (SM/SMase, 7 nmol), control phospholipid (DPPC and SM, 7 nmol each) and palmitic acid (15 nmol) were developed with chloroform:methanol:water (85:15:1, v/v) on TLC plates, and 125I-SP-A (1 µg/ml) binding was performed as described under “Materials and Methods.” Dotted circles indicate the apparent positions of palmitic acid and ceramide detected with ANSA under ultraviolet light.

SP-A Binding to Dipalmitoylphosphatidylglycerol and Dipalmitoylphosphatidylethanolamine—We speculate that 125I-SP-A may bind the dipalmitoyl species of glycerophospholipids, since the protein binds dipalmitoylglycerol very strongly as shown in Fig. 5. Dipalmitoylphosphatidylglycerol and dipalmitoylphosphatidylethanolamine were tested. 125I-SP-A binding to these phospholipid is very weak (Fig. 7A), albeit there is almost no binding in this picture. But SP-A strongly bound dipalmitoylglycerol derived from dipalmitoylglycero phospholipids after treatment with phospholipase C (Fig. 7B), which is essentially the same result as shown in Fig. 5. The data demonstrate that SP-A binding to dipalmitoylglycerophospholipid is polar group specific.

**FIG. 7.** 125I-SP-A binding to dipalmitoylphosphatidylglycerol and phosphatidylethanolamine. A, dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), and dipalmitoylphosphatidylethanolamine (DPPE) were developed with chloroform:methanol:water (70:30:5, v/v) on a TLC plate (10 nmol/lane) and 125I-SP-A binding was performed as described under “Materials and Methods.” B, dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylglycerol, and dipalmitoylphosphatidylethanolamine were treated with phospholipase C and developed with chloroform:methanol:water (85:15:1, v/v) on a TLC plate (10 nmol/lane), and then 125I-SP-A binding was performed.

 Ionic and Structural Requirement of SP-A for Phosphatidylcholine Binding—The role of calcium ions, and that of the oligosaccharide moiety and the collagenous domain of SP-A for binding to dipalmitoylphosphatidylcholine were also examined. When 125I-SP-A binding to lipid was carried out in the presence of 10 mM EGTA in a binding buffer that contained 2 mM CaCl2, SP-A binding was much reduced compared with control binding (Fig. 8, lanes A and B). This result demonstrates that calcium ions are required for SP-A binding to phosphatidylcholine. The band did not disappear completely in spite of the presence of 10 mM EGTA, perhaps because the TLC plate itself contains calcium or there may exist some calcium-independent binding.

Next, 0.5 µg/ml of rat 125I-SP-A was incubated with 100 µg/ml of native or deglycosylated rat SP-A, native human SP-A,
or collagenase-resistant fragment of human SP-A (CRF) on TLC plates. The electrophoretic analysis of SP-A used for the competition experiments is shown in Fig. 1B. Deglycosylated SP-A as well as native rat SP-A competed for $^{125}$I-SP-A binding to dipalmitoylphosphatidylcholine (Fig. 8, lanes C and D). Human SP-A also much reduced the binding of labeled protein (Fig. 8, lane E). However, in the presence of excess human CRF, $^{125}$I-SP-A was capable of binding dipalmitoylphosphatidylcholine (Fig. 8, lane F), though the band visualized is thinner than in the absence of unlabeled protein. These results show that the full size of oligosaccharide moiety of SP-A is not required for phospholipid binding, that human SP-A is able to compete with rat SP-A for phospholipid binding and that the noncollagenous domain of SP-A does not appear to compete with labeled SP-A for lipid binding.

**DISCUSSION**

SP-A has been thought to contribute to the biophysical and physiological activity of surfactant (1, 2). Several studies (8–11), in which interactions of SP-A with mixtures of surfactant-like phospholipids, such as dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol, were analyzed mainly by sedimentation methods which separated associated and free constituents, suggest that SP-A binds phospholipids. However, which classes of phospholipids SP-A binds and how this protein binds lipids have not been demonstrated. Using $^{125}$I-SP-A as a probe the direct binding of SP-A to phospholipids was visualized. The results demonstrate that SP-A binds phosphatidylcholine and sphingomyelin, and especially exhibits a strong binding to dipalmitoylphosphatidylcholine. This may be important in a physiological sense (as described below) although the significance of SP-A binding to sphingomyelin is unclear. The protein also shows a strong binding to diether phosphatidylcholine, but a weak binding to dimyristoyl-, 1-palmitoyl-2-linoleoyl-, and dilinoleoylphosphatidylcholine. SP-A appears to bind saturated phosphatidylcholine with longer fatty acid acyl chains more strongly. The binding study after enzyme treatment of lipids reveals that SP-A binds the nonpolar group of dipalmitoylphosphatidylcholine and that the palmitate at the second position is involved in the binding of SP-A, but SP-A does not bind an equivalent amount of palmitic acid. Iodinated SP-A exhibits almost no binding to dipalmitoylphosphatidylglycerol nor dipalmitoylphosphatidylethanolamine. This may be due to the differences of structures and charges which are dependent on polar groups of dipalmitoylglycerophospholipids. Polar groups of glycerophospholipids also appear to be important for SP-A binding.

The ionic and structural requirements of SP-A for phosphatidylycholine binding were studied. The results demonstrate that phospholipid binding of SP-A is dependent upon calcium ions as well as some other functions of SP-A, i.e. the aggregation of phospholipid liposomes (12), the inhibitory effect of lipid secretion by type II cells (4, 5), and receptor binding activity (6,7). The full size of oligosaccharide moiety in the SP-A molecule does not appear to be a required structural feature of SP-A binding to phosphatidylcholine. In contrast, CRF fails to compete with $^{125}$I-SP-A for binding to dipalmitoylphosphatidylcholine, but the band seen in Fig. 8, lane F, appears to be less dark than that seen in Fig. 8, lane A. These findings suggest that CRF competes with labeled SP-A incompletely. CRF, in which the amino terminus starts at Gly-75, Gly-78 or Ala-81 of SP-A and in which the carboxyl terminus of SP-A is preserved (data not shown), may bind only weakly. This is consistent with the result described by Ross et al. (11). They emphasized the importance of hydrophobic amino acid residues Leu-102 to Val-117 and the aminoterminal collagenous domain in the interaction of SP-A with phospholipids. Phospholipid binding activity of this protein appears to be dependent upon the integrity of the collagenous domain.

Several calcium-dependent phospholipid binding proteins have been isolated from lungs (23–25). In the presence of micromolar Ca$^{2+}$, these phospholipid binding proteins specifically bind acidic phospholipids, usually found at the cytoplasmic face of the membranes (26). These proteins and SP-A possess different specificities for phospholipid. Thus SP-A appears to differentiate from a family of so called calcium-dependent phospholipid (and membrane)-binding proteins.

Wright et al. (14) found that SP-A enhances the uptake of phospholipid liposomes labeled with $[^3]$Cl dipalmitoylphosphatidylcholine by alveolar type II cells and that only 86% of the radioactivity is recovered in phosphatidylcholine and 7% is recovered in phosphatidylglycerol in the absence of added SP-A, whereas more than 97% of the cell-associated radioactivity is recovered in phosphatidylcholine when SP-A is included in the incubation buffer. Wright et al. (14) suggested that calcium ionophore A may direct lipids to lamellar bodies where they are not catabolized. The results from the present study are on the lines of this hypothesis. Lamellar bodies are secreted by exocytosis, form tubular myelin (27, 28) and then can generate a surface film (29), which is believed to be enriched in dipalmitoylphosphatidylcholine, which is probably the most important factor in reducing surface tension at the air-liquid interface. SP-A may selectively bind dipalmitoylphosphatidylcholine, an essential factor of pulmonary surfactant, and lead it back to the lamellar body, preventing futile recycling and reutilization of surfactant.

In summary, the present study provides direct evidence that SP-A specifically and strongly binds dipalmitoylphosphatidylcholine. SP-A binding to phosphatidylcholine is specific for the molecular species of the fatty acid acyl chain. SP-A binds the nonpolar group of phospholipids. The second positioned palmitate is also involved in dipalmitoylphosphatidylcholine binding. The selectivities of partial groups of dipalmitoylglycerophospholipids also appear to be important for SP-A binding. The phospholipid binding activity of SP-A is dependent upon calcium ions and the integrity of the collagenous domain of SP-A, but not on the oligosaccharide moiety of SP-A.

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