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Cell Biology

Alveolar type II cells express a high-affinity receptor for pulmonary surfactant protein A

(lipoproteins/lung/cell surface/epithelial cells)

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ABSTRACT **Primary cultures of rat alveolar type II cells bind radiolabeled pulmonary surfactant protein A (SP-A) with high affinity. The binding of ^{125}I-labeled SP-A is time- and temperature-dependent and is not accompanied by significant degradation. The binding process is saturable at low concentrations of SP-A (5 μg/ml), and unlabeled SP-A readily competes with labeled SP-A for cellular binding sites. Subsequent to binding, two pools of cell-associated ^{125}I-labeled SP-A can be identified based upon sensitivity to trypsin at 0°C. It is likely that the trypsin-sensitive pool comprises ^{125}I-labeled SP-A bound to the cell surface and the trypsin-insensitive pool comprises the internalized protein. Scatchard analysis of cell surface binding of SP-A at 0.1–10 μg/ml shows positive cooperativity at concentrations between 0.1 and 1 μg/ml. Hill plots give n_H = 1.34 ± 0.08 with an apparent dissociation constant K'_d = 1.02 ± 0.32 μg/ml (which is 0.64 ± 0.19 nM if the native molecular mass of oligomeric SP-A is assumed to be 1.6 MDa). The binding of SP-A to type II cells shows an absolute requirement for Ca^{2+}. The putative receptor for SP-A is unaffected by treatment of type II cells with a variety of proteases and N-Glycanase (EC 3.5.1.52). Alveolar macrophages also exhibit high-affinity binding of SP-A, but rat lung fibroblasts and the alveolar epithelial cell line L2 exhibit only nonspecific binding.**

Alveolar type II cells produce, store, and secrete pulmonary surfactant, a heterogeneous complex of lipid and protein that stabilizes alveoli at the end of expiration. Pulmonary surfactant apoprotein A (SP-A) is the major protein component of the surfactant complex (1). The protein is polymorphic (26–38 kDa) when analyzed by one-dimensional electrophoresis under reducing and denaturing conditions. This polymorphism is a consequence of posttranslational modifications, principally variable glycosylation (2–4). SP-A has been purified to homogeneity from human, rat, and canine sources and their cDNAs have been isolated and sequenced (5–7).

Several functions have been proposed for SP-A. Results from physical studies (8) demonstrate that the protein can interact with the extremely hydrophobic proteins associated with the surfactant complex (8–10) to promote the rapid formation of stable surface films of phospholipid that are required for the reduction of surface tension at the alveolar air/water interface. SP-A has been shown to inhibit secretion of surfactant phospholipid by primary cultures of rat alveolar type II cells (11–12). The protein also facilitates the uptake of liposomal phospholipids by type II cells (13) and may be involved in the recycling of surfactant components (14–16) from the alveolar space back to the type II cell.

The interactions of SP-A with isolated type II cells suggest the presence of a specific receptor. The purpose of this study was to characterize the binding of ^{125}I-labeled SP-A (^{125}I-SP-A) to isolated type II cells. The results demonstrate the presence of a high-affinity SP-A receptor on alveolar type II cells.

MATERIALS AND METHODS

Cells. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum. Alveolar type II cells were isolated from adult male Sprague–Dawley rats by tissue dissociation with elastase and purification in metrizamide density gradients (17). The cells were cultured and their surfactant lipid secretion was measured as described (18). Release of lactate dehydrogenase into the medium (19) was measured to determine nonspecific secretion. Alveolar macrophages were isolated by lavaging lungs with isotonic saline and centrifuging the recovered solution to sediment the cells. The macrophages were cultured at 0.5 × 10^6 cells per 35-mm dish. Binding of ^{125}I-SP-A to alveolar type II cells and macrophages was assayed after cultivation of the freshly isolated cells for 20 hr. ^{125}I-SP-A binding to L2 cells and adult rat lung fibroblasts was assayed in confluent dishes of these cells.

Purification of SP-A. Surfactant was isolated from 200-g rats given intratracheal instillation of 40 mg of silica in phosphate-buffered isotonic saline 4 weeks before lung lavage (20). The surfactant was purified as described by Hawgood *et al.* (21) and delipidated by extraction with 1-butanol (98 ml of butanol per 2 ml of pellet). The butanol-insoluble protein precipitate was isolated by centrifugation at 2000 × *g* for 30 min. Residual solvent was removed by evaporation under a stream of nitrogen. The protein was suspended in 5 mM Tris/HCl buffer (pH 7.4) and dialyzed against the same buffer at 4°C for 36–48 hr. The macromolecular material was centrifuged at 150,000 × g_{av} for 1 hr and the supernatant was applied to an affinity column of mannose-Sepharose 6B (22). The SP-A bound to the affinity matrix in the presence of Ca^{2+} and was eluted with 2 mM EDTA. Further purification and removal of EDTA were accomplished by gel filtration using Bio-Gel A-5m (Bio-Rad). Electrophoretic analysis of the purified SP-A was performed as described (18). Complete details of this purification will be published elsewhere. A similar purification scheme was described by Haagsman *et al.* (23).

Preparation of ^{125}I-SP-A. ^{125}I-SP-A was prepared by the method of Innerarity *et al.* (24) based on the Bolton and Hunter (25) iodination procedure. Yields of ^{125}I-SP-A ranged from 300 to 470 μCi (1 μCi = 37 kBq). Final specific activities varied between 153 and 470 cpm per ng of protein. In all preparations, more than 96% of the radioactivity was precipitated by treatment with 10% (wt/vol) trichloroacetic acid. At the highest specific activities utilized only 0.41% of monomeric SP-A molecules were modified by iodination, and this low level of modification yielded preparations that

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Abbreviations: SP-A, surfactant protein A; ^{125}I-SP-A, ^{125}I-labeled SP-A.

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exhibited full biological activity as defined by the ability to inhibit phospholipid secretion by primary cultures of alveolar type II cells.

^{125}I-SP-A Binding to Cells. The methods used in our binding studies were adapted from those described for binding of low density lipoprotein (LDL) to its receptor (26). Alveolar type II cells were cultured for 20 hr after isolation. The medium was removed and each monolayer was washed with 10 ml of DMEM containing 1 mg of bovine serum albumin per ml. Next, 1.6 ml of DMEM containing 10% fetal bovine serum and the indicated amount of ^{125}I-SP-A was added. After incubation at 37°C each dish was placed on ice and the medium was removed. The cell monolayers were washed rapidly with 3 ml of ice-cold buffer B, containing 50 mM Tris buffer (pH 7.4), 0.15 M NaCl, and 2 mg of bovine serum albumin per ml. Each monolayer was then incubated on ice for 10 min with 3 ml of ice-cold buffer B. The medium was removed and this washing step was repeated. Next, the monolayer was rapidly washed with 3 ml of ice-cold buffer A, containing 50 mM Tris buffer (pH 7.4) and 0.15 M NaCl. The cells were removed from the dishes by dissolution in 2 ml of 0.1 M NaOH and the amount of ^{125}I-SP-A bound to the cells was determined with a γ counter. To study the effect of Ca^{2+} on ^{125}I-SP-A binding to type II cells at 37°C, 50 mM Tris buffer (pH 7.4) containing 0.15 NaCl, 0.1 mM EDTA, and 5 mg of bovine serum albumin per ml was used during the incubation of ^{125}I-SP-A with the cell monolayer. The formation of acid-soluble materials from ^{125}I-SP-A was determined by the method of Goldstein and Brown (27).

Protease Pretreatment of Cells. Cell preparations were pretreated with proteases to remove putative receptor molecules from the cell surface. The adherent cells were treated with the proteases in serum-free DMEM at 37°C for 45 min or 20 min (trypsin). The proteases and their respective concentrations were as follows: Pronase, 6 μg/ml; trypsin, 12 μg/ml; staphylococcal protease, 5 μg/ml; papain, 50 μg/ml; chymotrypsin, 6 μg/ml; and pepsin, 6 μg/ml. In addition to proteases, cultures of type II cells were also treated with 1.0 unit of N-Glycanase [0.625 unit/ml; peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase, EC 3.5.1.52; from Genzyme, Boston].

Determination of Internalized and Surface-Bound Pools of ^{125}I-SP-A. To differentiate between surface-bound and internalized pools of labeled SP-A, the cells were treated with ^{125}I-SP-A and washed as described above and then subjected to trypsin treatment at 0°C to remove the cell surface pool. The cells were incubated for 10 min on ice in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline containing 1 mM EDTA and 4 mg of trypsin per ml. During this time all of the cells detached from the dish. Next, the entire cell suspension was removed from the dish and placed in a centrifuge tube. The dish was washed with 1 ml of DMEM containing 10% fetal bovine serum, and this wash was added to the centrifuge tube. The cells were sedimented at 850 × *g* for 10 min and the supernatant was removed. The cell pellet was resuspended in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and centrifuged again. The resultant supernatant was added to the first supernatant and the radioactivity found in this fraction was designated as trypsin-sensitive (i.e., surface-bound). The radioactivity associated with the pellet was designated as trypsin-resistant (i.e., the internalized pool). The amount of radioactivity that was trypsin-resistant increased in a time-, temperature-, and concentration-dependent fashion.

RESULTS

Binding and Degradation of ^{125}I-SP-A by Alveolar Type II Cells. The purification procedures used for the isolation of SP-A yielded a homogeneous preparation of the protein (Fig. 1*A*). Minor amounts of the protein migrated with an apparent

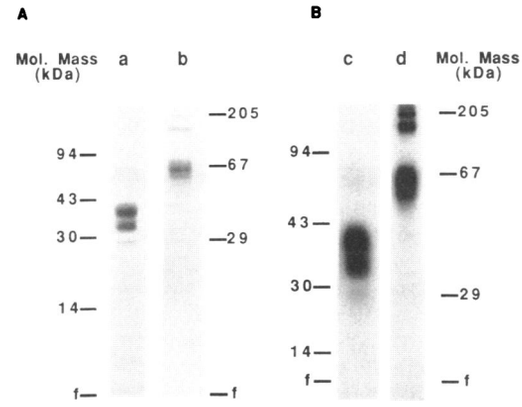

FIG. 1. Electrophoretic analysis of SP-A and ^{125}I-SP-A. (*A*) Preparations of purified SP-A were analyzed by electrophoresis in 7–22% gradient polyacrylamide gels in the presence of sodium dodecyl sulfate under reducing (lane a) and nonreducing (lane b) conditions and stained with Coomassie blue. (*B*) Preparations of ^{125}I-SP-A (10^5 cpm) were analyzed by electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulfate under reducing (lane c) and nonreducing (lane d) conditions and autoradiographed for 48 hr. Positions of protein size standards run in parallel are indicated. f, Dye front.

molecular mass of 26 kDa and the major forms had a mass of 32 and 38 kDa when analyzed by electrophoresis under reducing and denaturing conditions. The polymorphism of SP-A is due to posttranslational modifications, primarily variable glycosylation (2–4). When the protein was analyzed by electrophoresis under nonreducing conditions the major form had a molecular mass of ≈60 kDa and minor forms were observed to have a mass of 120, 180, and >200 kDa. The ^{125}I-SP-A was also examined by electrophoresis (Fig. 1*B*). The major forms of the radiolabeled protein correlated well with the major forms observed for the unlabeled protein. There is little evidence to suggest that the iodination procedure has yielded markedly altered forms of the protein.

When monolayers of alveolar type II cells were incubated with ^{125}I-SP-A (1 μg/ml) at 37°C, a time-dependent binding of radioactivity to the cells was observed (Fig. 2*A*). In contrast to binding, there was no significant increase of acid-soluble

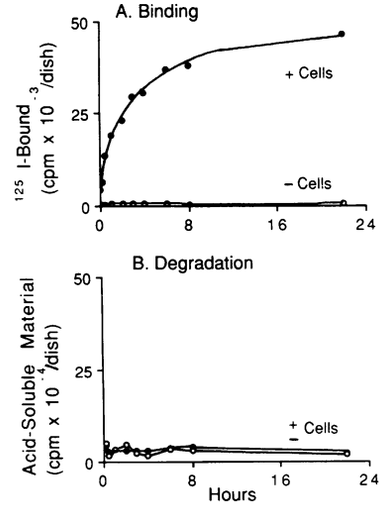

FIG. 2. Time-dependent binding and degradation of ^{125}I-SP-A by primary cultures of rat alveolar type II cells. Monolayers of type II cells were incubated for the indicated time at 37°C in 1.6 ml of DMEM containing 10% fetal bovine serum and 1 μg of ^{125}I-SP-A per ml. The cell monolayers were then washed and harvested, and the binding (*A*) and degradation (*B*) of the labeled apoprotein were determined. Data presented are from a representative one of five experiments.

materials appearing as a degradation product for at least 22 hr of incubation (Fig. 2*B*). The amount of acid-soluble radioactivity recovered from the dishes was essentially the same in the presence and absence of cells.

Incubation of various concentrations of ^{125}I-SP-A with type II cells at 37°C revealed a biphasic binding pattern. At concentrations of ^{125}I-SP-A below 5 μg/ml, binding increased abruptly with increasing amounts of SP-A; this component of the binding curve is designated as high-affinity binding (Fig. 3). At levels of SP-A above 5 μg/ml, binding continued to increase but the slope of the line relating binding and SP-A concentration was considerably less, suggesting that at these higher SP-A levels a second process was taking place, representing low-affinity or nonspecific binding. The data relating total binding of SP-A to type II cells as a function of ^{125}I-SP-A concentration were replotted by the "slope peeling" method of Goldstein and Brown (27) to subtract the contribution of the low-affinity processes. By this analysis, specific binding appeared to reach saturation below 5 μg/ml. The ability of excess unlabeled SP-A to compete with ^{125}I-SP-A for the high-affinity sites was also examined (Fig. 4). The specific binding of ^{125}I-SP-A to type II cells observed between 0.1 and 2.0 μg of labeled protein per ml was essentially eliminated by the addition of unlabeled SP-A at 100 μg/ml. This result is consistent with receptor binding that is specific for the SP-A molecule.

Determination of Binding Constants. The binding of SP-A to alveolar type II cells was further analyzed by the method of Scatchard (28). In these experiments the total specific binding was operationally subdivided into two separate pools: a trypsin-sensitive and a trypsin-insensitive pool. The trypsin-sensitive pool of bound SP-A is that which remains accessible to protease attack at the cell surface. The trypsin-insensitive pool of SP-A is that which becomes refractory to protease attack, presumably due to internalization. The amount of ^{125}I-SP-A bound and internalized was found to be dependent upon the extracellular concentration of ^{125}I-SP-A (Fig. 5*A*). The internalization process is also time- and temperature-dependent (data not shown). The results from three separate experiments were essentially the same as that given for the data of Fig. 5. Between 0.1 and 1.0 μg/ml of ^{125}I-SP-A, the Scatchard plot of cell surface ^{125}I-SP-A yields a curve with positive slope indicative of positive cooperativity in binding. The observed positive cooperativity was not a consequence of failure to attain equilibrium binding at low levels of ^{125}I-SP-A, since incubation of labeled ligand with the cells for 22 hr gave the same result (data not shown). When these same data were analyzed by using Hill plots, the coefficient n_H was 1.34 ± 0.08 (mean ± SD, n = 3) and the apparent dissociation constant K'_d was 1.02 ± 0.32 μg/ml. Analysis of purified SP-A by gel filtration has shown that it exists as an oligomer consisting of ≈45 monomers and exhibiting a molecular mass

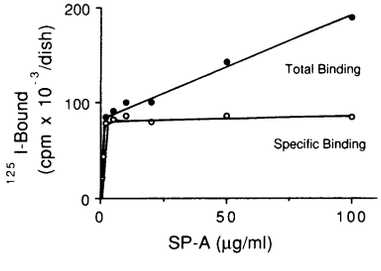

FIG. 3. Concentration-dependent binding of ^{125}I-SP-A to primary cultures of rat alveolar type II cells. Type II cell monolayers were incubated for 5 hr as for Fig. 2 but with various concentrations of ^{125}I-SP-A. The cell monolayers were washed and harvested, and the total (●) and specific (○) binding of the labeled apoprotein was determined. Data presented are from a representative one of four experiments.

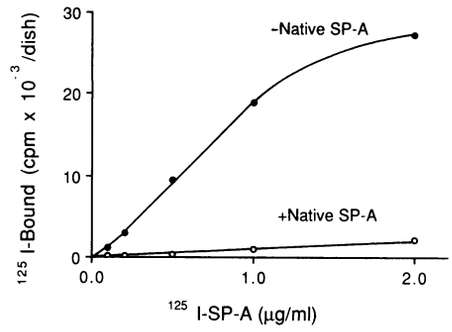

FIG. 4. Unlabeled SPA competes with ^{125}I-SP-A for binding to type II cells. Type II cell monolayers were incubated as for Fig. 3 in the absence (●) or presence (○) of an additional 100 μg of native SP-A per ml. Total binding of the labeled apoprotein was determined. Data presented are from a representative one of four experiments.

of ≈1.6 MDa (18). Thus the K'_d is 0.64 ± 0.19 nM for the oligomeric species. The maximum amount of SP-A bound to the cell surface was determined to be 360 ng per 10^6 cells. This value is equivalent to the cell surface binding of 1.35 × 10^5 SP-A oligomers per cell.

Ionic Requirements and Cell-Type Specificity. The role of specific ions in facilitating the binding of SP-A to alveolar type II cells was also examined. The specific association of ^{125}I-SP-A with type II cells displayed a near absolute requirement for Ca^{2+}. Chelation of Ca^{2+} with EDTA prevented the high-affinity binding. Substitution of Mg^{2+} for Ca^{2+} failed to support significant binding. Concentrations of Ca^{2+} as low as 0.2 mM yielded ^{125}I-SP-A binding that was 70% of that found at 2 mM Ca^{2+}.

The cell-type specificity of high-affinity ^{125}I-SP-A binding was studied by comparing the properties of alveolar type II cells, macrophages, L2 cells [a cell line derived from alveolar epithelial cells (29)], and adult rat lung fibroblasts (Fig. 6). Like alveolar type II cells, alveolar macrophages exhibited a concentration-dependent binding of SP-A indicating the presence of high-affinity receptors. In contrast, fibroblasts derived from rat lung and the L2 cell line exhibited binding properties consistent with a nonspecific process.

DISCUSSION

The data presented provide evidence that a specific, high-affinity, saturable receptor for SP-A is expressed by alveolar type II cells. The high-affinity binding is not accompanied by

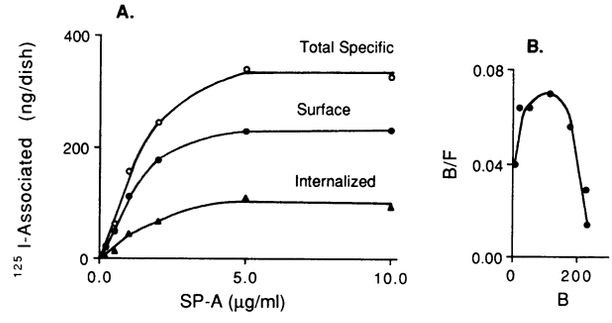

FIG. 5. Concentration-dependent binding and internalization of ^{125}I-SP-A by primary cultures of rat alveolar type II cells. Cultures were incubated for 8 hr at 37°C in 1.6 ml of DMEM containing 10% fetal bovine serum and the indicated concentrations of ^{125}I-SP-A. (*A*) Total specific binding (○), surface binding (●), and internalized (▲) labeled apoprotein were determined. (*B*) Data corresponding to the surface-bound pool in *A* were replotted by the method of Scatchard. B, bound; F, free. Data are from a representative one of three experiments.

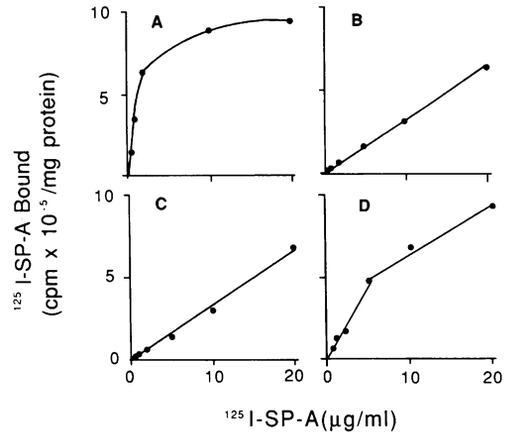

FIG. 6. Cell-type specificity of ^{125}I-SP-A binding. Monolayers of type II cells (*A*), adult rat lung fibroblasts (*B*), L2 cells (*C*), and alveolar macrophages (*D*) were incubated for 5 hr at 37°C with 1.6 ml of DMEM containing 10% fetal bovine serum and the various concentrations of ^{125}I-SP-A. Total binding of the labeled apoprotein was determined.

degradation as is observed for the low density lipoprotein (LDL)-receptor system (27). The lack of degradation and the potential reutilization of SP-A may place this receptor system in the same category as the transferrin receptor (30–32). The binding of SP-A is dependent upon the extracellular Ca^{2+} concentration and can occur at 0°C (albeit to a lesser extent than at 37°C). These latter two observations are consistent with properties of a cell surface receptor. Attempts to deplete or remove the receptor with proteases (including trypsin, Pronase, chymotrypsin, pepsin, and papain) were uniformly unsuccessful. Furthermore, treatment of type II cells with N-Glycanase was also without effect upon SP-A binding. From these data it is not possible to discern whether the putative receptor is an extremely protease-resistant protein or some other class of molecule such as a glycolipid. Glycolipid receptors have been documented for cholera toxin (33, 34).

The high-affinity binding of SP-A exhibits characteristics that are consistent with other properties of the physiology of type II cells. The K'_d (0.64 nM, or 1.02 μg/ml) occurs at the same concentration considered optimal (11, 18) for SP-A-mediated inhibition of surfactant phospholipid secretion. The Ca^{2+} requirement for SP-A binding is also observed for inhibition of lipid secretion by type II cells caused by SP-A.

Although the putative receptor for SP-A is resistant to proteases, the surface-bound form of SP-A remains accessible to proteases. As the total specific binding reaction reaches equilibrium values, two pools of SP-A can be discerned. One pool of SP-A is protease labile and is most likely at the cell surface. A second pool of SP-A becomes refractory to the action of trypsin. It is very likely that the mechanism of sequestration of SP-A is internalization by the type II cells. Our observations that acquisition of protease resistance is time- and temperature-dependent (data not shown) as well as concentration-dependent are consistent with this concept.

SP-A has been shown to have a high affinity for phospholipid (35). This property of the protein is cause for significant concern with respect to the cellular binding of SP-A. The data in Fig. 6, however, demonstrate that the high-affinity binding of SP-A is a cell-type-specific process. The cell-type specificity and saturation kinetics make it unlikely that the binding reflects nonspecific affinity for phospholipid. The association of SP-A with alveolar macrophages has been noted by others (36, 37). Although the basis for the association may be explained in part by phagocytosis of surfactant, the present data demonstrate that macrophages also express a specific receptor for SP-A. The functional significance of an SP-A receptor on alveolar macrophages is unknown.

The Scatchard analysis of the concentration dependence of SP-A binding to type II cells reveals that the process exhibits positive cooperativity. The physiological relevance of this positive cooperativity is not entirely clear, but it may function to permit the accumulation of some critical level of extracellular surfactant prior to inhibition of secretion or recycling of surfactant. It is likely that phospholipid and the hydrophobic proteins SP-B and SP-C also alter surfactant secretion, synthesis, and recycling, and perhaps SP-A binding, by type II cells.

The relationship between the observed molecular mass of purified SP-A and the true physical properties of the molecule in the presence of phospholipid and the hydrophobic surfactant proteins is ambiguous. Presently there are no data available to define whether SP-A remains highly oligomeric in the presence of phospholipids and other proteins. In this study we have confined our observations to a well-defined physical form of SP-A that is known to be a potent inhibitor of surfactant lipid secretion. This simplification permits the determination of the affinity constants, the cooperative nature of binding, and the receptor number. We have assumed a stoichiometry of receptors to bound (oligomeric) SP-A of 1. The estimate for the number of receptors should not change regardless of the physical form of SP-A. However, if the self-association of SP-A changes in the presence of the other components of surfactant, then the K'_d should also change. If phospholipids do not change the intrinsic affinity of SP-A for receptor but cause SP-A to dissociate to its smallest covalent form (i.e., dimers) then the K'_d may actually be as high as 14.4 nM. Should the non-SP-A components of surfactant alter the intrinsic affinity of SP-A for receptor, then the K'_d determined in this study would be meaningful in a biochemical sense but not a physiological sense. Clearly, the present study has defined a high-affinity receptor system for SP-A. This work sets the foundation for future studies of the dynamic interactions between SP-A and the other components of the surfactant system and receptor binding activity.

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