Binding and uptake of surfactant protein D by freshly isolated rat alveolar type II cells

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Herbein, Jo F., J ordan Savov, and Jo Rae Wright. Binding and uptake of surfactant protein D by freshly isolated rat alveolar type II cells, Am J Physiol Lung Cell Mol Physiol 278: L830–L839, 2000.—Alveolar type II cells secrete, internalize, and recycle pulmonary surfactant, a lipid and protein complex that increases alveolar compliance and participates in pulmonary host defense. Surfactant protein (SP) D, a collagenous C-type lectin, has recently been described as a modulator of surfactant homeostasis. Mice lacking SP-D accumulate surfactant in their alveoli and type II cell lamellar bodies, organelles adapted for recycling and secretion of surfactant. The goal of current study was to characterize the interaction of SP-D with rat type II cells. Type II cells bound SP-D in a concentration-, time-, temperature-, and calcium-dependent manner. However, SP-D binding did not alter type II cell surfactant lipid uptake. Type II cells internalized SP-D into lamellar bodies and degraded a fraction of the SP-D pool. Our results also indicated that SP-D binding sites on type II cells may differ from those on alveolar macrophages. We conclude that, in vitro, type II cells bind and recycle SP-D to lamellar bodies, but SP-D may not directly modulate surfactant uptake by type II cells.

Lung; lamellar bodies; surfactant lipids; alveolar macrophages; surfactant recycling

SURFACTANT PROTEIN (SP) D is a large, oligomeric, calcium-dependent lectin and a component of pulmonary surfactant, a lipid, protein, and carbohydrate complex that lines the alveolar epithelium. Studies indicate a role for SP-D in the regulation of the two primary functions of pulmonary surfactant: reduction of surface tension at the alveolar air-liquid interface (3, 21) and modulation of the innate immune response of the lung (reviewed in Refs. 7, 41). SP-D has been localized to alveolar macrophages, alveolar type II epithelial cells, and nonciliated airway epithelial cells (Clara cells). Macrophages endocytose and degrade SP-D (12, 39), whereas type II cells and Clara cells have been shown to synthesize and secrete SP-D (6, 26, 39, 40).

Type II cells occupy 5–10% of the alveolar surface area where they are dispersed among gas-exchanging type I epithelial cells (27). Type II cells are primarily responsible for the production of pulmonary surfactant and are involved in the regulation of the surfactant pool. They package newly synthesized surfactant into unique secretory organelles called lamellar bodies. To efficiently coordinate the rapid turnover of surfactant necessary for proper respiratory function, type II cells recycle surfactant products such as phospholipids and SP-A, SP-B, and SP-C from the alveolar space and incorporate them directly into their secretory pathway (1, 4, 33, 45). Surfactant internalization, reutilization, and secretion is a regulated process. SP-A increases lipid uptake by type II cells and macrophages (42, 43) and enhances type II cell recycling of dipalmitoylphosphatidylcholine (DPPC) (42). Additionally, SP-A inhibits phospholipid secretion by type II cells in vitro (10, 31).

The role of SP-D in the regulation of surfactant lipid metabolism has yet to be elucidated. A fraction of SP-D recovered from bronchoalveolar lavage fluid coisolates with lipids (25), and SP-D binds and aggregates phosphatidylinositol (PI)-containing liposomes (28, 29). SP-D has also been shown to partially reverse SP-A inhibition of stimulated type II cell phospholipid secretion in vitro (24). Most recently, studies (3, 21) with mice made deficient in SP-D by homologous recombination have an overaccumulation of surfactant lipid in the alveolar space and in the lamellar bodies of type II cells. This is in contrast to SP-A-null mice (20), which show little alteration of surfactant homeostasis (18) despite numerous descriptions of SP-A regulation of lipid metabolism in type II cells and macrophages in vitro (37).

The goal of this study was to describe the characteristics of SP-D association with alveolar type II cells freshly isolated from rat lungs. Furthermore, the effect of this association on SP-D and surfactant lipid internalization was examined. A preliminary report including a portion of these data has appeared in abstract form (16).

METHODS

Materials. Animals were purchased from Charles River (Raleigh, NC) and Taconic Farms (Germantown, NY). Enzymes for type II cell isolation were from Worthington Biochemicals (Freehold, NJ). Dulbecco’s phosphate-buffered saline (PBS), DMEM, fetal bovine serum (FBS), and trypsin were obtained from Life Technologies (Gaithersburg, MD). Tran-[35S]-label, [3H]proline, and CytoScint were purchased from ICN Biochemicals (Costa Mesa, CA). Lipids for synthetic liposome preparation were obtained from Avanti Polar Lipids (Birmingham, AL). L-[3H]DPPC was from New England Nuclear (Boston, MA). Proteinase K and Pefabloc were purchased from Boehringer Mannheim (Indianapolis, IN). Bicinchoninic acid protein detection reagents and sulfo-NHS-biotin were obtained from Pierce Chemical (Rockford, IL). [3H]lpopolysaccharide

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[**H**]LPS from Escherichia coli K 12 LDC 25 (Rb chemotype) was purchased from List Biological Laboratories (Campbell, CA). PI-specific phospholipase C (PI-PLC), low-endotoxin bovine serum albumin (BSA), and all other chemical reagents were obtained from Sigma (St. Louis, MO).

Isolation of wild-type rat SP-D. Wild-type rat SP-D was isolated from the lung lavage fluid of silica-treated rats as previously described (12). Briefly, SP-D was purified by maltose affinity from serum-free medium incubated with Chinese hamster ovary cells expressing a full-length rat SP-D cDNA clone. Radiolabeled SP-D was generated by inclusion of 20 µCi/ml of [3H]proline with HB CHO serum-free medium. Preparations were tested for endotoxin contamination and functionality as outlined in Endotoxin and functionality tests of SP-D preparations.

Production of recombinant rat SP-D. Recombinant rat SP-D was expressed, purified, and characterized as previously described (12). Briefly, SP-D was purified by maltose affinity from serum-free medium incubated with Chinese hamster ovary cells expressing a full-length rat SP-D cDNA clone. Radiolabeled SP-D was generated by inclusion of 20 µCi/ml of [3H]proline with HB CHO serum-free medium (Irvine Scientific, Santa Ana, CA) or 20 µCi/ml of [35S]cysteine to toxin contamination and functionality as outlined in PI-PLC treatment of cells. Cells (5 x 10^6/ml) resuspended in PBS with 0.1% BSA were incubated at 37°C for 1 h with 2.5 U/ml of PI-PLC. The cells were washed twice by centrifugation with ice-cold PBS containing 0.9 mM CaCl_2, 0.5 mM MgCl_2, and 0.1% BSA before being transferred to a new tube for binding (see Binding and uptake assays). Protease control cells were treated identically to trypsin- or proteinase K-treated cells except that they were never exposed to enzyme. Trypsin was from a tissue culture grade solution (0.25%; Life Technologies), and proteinase K tested free of nuclease activity (Boehringer Mannheim).

PI-PLC treatment of cells. Cells (5 x 10^6/ml) resuspended in PBS with 0.1% BSA were incubated at 37°C for 1 h with 2.5 U/ml of PI-PLC. The cells were washed twice by centrifugation with ice-cold PBS containing 0.9 mM CaCl_2, 0.5 mM MgCl_2, and 0.1% BSA before being transferred to a new tube for binding (see Binding and uptake assays). Cells for control treatments were incubated identically to PI-PLC-treated cells except that they were never exposed to enzyme. PI-PLC activity was at least 3,000 U/mg, with spikingomyelinase (<40 U/mg) and PLC (2 U/mg) as measurable contaminants (Sigma).

Binding and uptake assays. Unless otherwise indicated, the cells were resuspended at a final concentration of 5 x 10^6 cells/ml in binding buffer (PBS containing 0.9 mM CaCl_2, 0.5 mM MgCl_2, and 0.1% BSA) and then divided into aliquots of 500 µl into 1.5-ml microfuge tubes precoated with 1% BSA in PBS (overnight at 4°C). After incubation with gentle rotation (6-10 rpm) at the specified temperature, the cells were washed by centrifugation (200 g for 7 min) with PBS containing CaCl_2 and MgCl_2 (unless otherwise indicated). After the first wash, the cells were transferred to new PBS-coated tubes and washed twice more before lysis (50 mM sodium phosphate buffer (pH 7.2), 150 mM NaCl, 2 mM EDTA, and 0.5% Nonidet P-40). All washes were done at 4°C. Associated label was detected by mixture of a portion of the lysed sample with 4 ml of CytoSint scintillation cocktail and analysis in a Packard 4000 series liquid scintillation counter. Samples were analyzed for protein content with bicinchoninic acid protein detection reagents, and the radioactive signal was normalized to cellular protein (in mg/ml) as calculated from a BSA standard curve.

**PI-PLC Treatment of Cells**.
The cells were washed, transferred to a new tube, blocked (PBS with 0.9 mM CaCl$_2$, 0.5 mM MgCl$_2$, 3% BSA, and 5% FBS) for 1 h at room temperature, and incubated with anti-CD11b antibody (0.5 µg/10$^6$ cells) in fresh blocking buffer for 1 h at room temperature. The cells were washed, adhered to microscope slides by cytospin, and mounted in a solution of 50% glycerol in PBS with 2.5% 1,4-diazabicyclo[2.2.2]octane. Localization of SP-D and anti-CD11b antibody in the type II cell preparations was evaluated with a Zeiss 410 confocal microscope with a ×100 objective.

Electron microscopy. Immunogold localization of biotinylated SP-D was used to investigate SP-D uptake by type II cells by electron microscopy. Wild-type rat SP-D was dialyzed against PBS, and sulfo-NHS-biotin was added at 1:20 (SP-D:biotin molar ratio) for 30 min at room temperature. Unbound biotin was removed by dialysis against PBS.

Biotinylated SP-D was incubated with freshly isolated type II cells with the protocol in Binding and uptake assays. After being washed, the cell pellets were fixed with 2% glutaraldehyde and 1% paraformaldehyde in 0.85 M cacodylate buffer (pH 7.4) on ice for 30 min and then centrifuged at 2,500 g for 8 min. Pellets were postfixed with 2% osmium tetroxide in 0.85 M cacodylate buffer (pH 7.4) for 1 h, stained with 2% uranyl acetate for 90 min, dehydrated through graded ethanol, and embedded and polymerized in LR White resin (Polysciences, Warrington, PA) at 50–55°C for 72 h. Ultrathin sections were cut on a Reichert-jung ultratome and transferred to Formvar-coated 200-mesh nickel grids to air-dry for 30 min. The sections were stained by immersion at room temperature in 30-µl drops on dental wax with the following sequence: 1) PBS + 1% BSA for 10 min; 2) 5% normal rabbit serum in PBS for 30 min; 3) goat anti-biotin primary antibody (Calbiochem, La jolla, CA) at 1:120 in PBS-0.5% Tween 20 for 6 h in a moisture chamber; 4) PBS wash for 10 min, repeated twice; 5) 10-nm gold-conjugated rabbit anti-goat IgG secondary antibody (Sigma) in PBS-0.5% Tween 20 for 4 h in a moisture chamber; 6) PBS wash for 10 min, repeated twice; 7) double-distilled water wash for 10 min, repeated twice; 8) air-drying overnight; and 9) staining with lead citrate for 4 min. The sections were viewed in a JEOL 1200 electron microscope.

Degradation assays. Type II cells and macrophages were incubated for various times with 1 µg/ml of [35S]SP-D at 37°C as described in Binding and uptake assays. After incubation, the cells were pelleted, and the incubation medium was collected and held on ice. The cells were washed as described in Binding and uptake assays and resuspended in fresh binding buffer. Samples were precipitated with 10% trichloroacetic acid (TCA) for 30 min on ice and centrifuged at 10,000 g for 10 min. Radioactivity associated with the pellet and supernatant was detected as described in Binding and uptake assays.

Statistical analysis. All data reported are means ± SE except for data in Fig. 6 that are means ± SD. Repetitions (n) used to calculate mean ± SE and mean ± SD are from independent experiments, not duplicates of conditions within an experiment. Comparison of experimental groups to their corresponding controls was evaluated by Student’s t-test. Significance was accepted at P < 0.05.

RESULTS

SP-D binds to freshly isolated type II cells. Binding increased over a [3H]SP-D concentration range of 0.5–10 µg/ml in a calcium-dependent manner (Fig. 1). The magnitude of binding (measured as ng [3H]SP-D/mg cellular protein) was much higher than that previously reported for alveolar macrophages under identical conditions (12). Additionally, macrophages in that study showed saturation of SP-D binding at 1 µg/ml. From these data, it was calculated that macrophages contaminating our type II cell isolates contributed <5% of the measured SP-D binding to freshly isolated type II cell suspensions.

At 2 µg/ml, [3H]SP-D binding to type II cells was time dependent (Fig. 2), with the most rapid binding occurring during the first 30 min of the time course; however, there was no evidence for an equilibrium of binding. [3H]SP-D binding to type II cells over time was temperature dependent (Fig. 3), with ~200% more label associating with the cells at 37°C than at 4°C. The level of association at 37°C exceeds that reported for macrophages by ~50% (12).

Fig. 1. Concentration-dependent binding of surfactant protein (SP) D to freshly isolated rat alveolar type II cells. Cells (5 × 10$^6$/ml) were incubated with indicated concentrations of [3H]SP-D in either calcium (0.9 mM)- or EDTA (2 mM)-containing buffer for 4 h at 4°C. Cells were washed, and associated [3H]SP-D was measured by scintillation counting and normalized to cellular protein. Values are means ± SE; n = 3 or 4 repetitions from independent experiments.

Fig. 2. Time-dependent binding of SP-D to freshly isolated rat alveolar type II cells. Cells (5 × 10$^6$/ml) were incubated with [3H]SP-D (2 µg/ml) in calcium (0.9 mM)-containing buffer for indicated times at 4°C. Cells were washed, and associated [3H]SP-D was measured by scintillation counting and normalized to cellular protein. Values are means ± SE; n = 4 repetitions from independent experiments.
To examine further the association of SP-D with the surface of type II cells, FITC-labeled SP-D binding at 4°C was assessed by FACS and confocal microscopy. FACS analysis (Table 1) showed significant calcium-dependent binding at 2 µg/ml and 2 h. Binding visualized by confocal microscopy (Fig. 4, A and B) showed localization of label on the outer margin of the type II cell plasma membrane. In the presence of 2 mM EDTA or 10 mM inositol, SP-D binding was not detectable by confocal microscopy with the same sensitivity settings employed in Fig. 4. Similarly, binding of heat-inactivated SP-D could not be detected (data not shown).

SP-D is internalized by freshly isolated type II cells. Confocal and electron microscopy were utilized for a qualitative assessment of SP-D internalization by freshly isolated rat type II cells. Confocal microscopy (Fig. 4, C-H) revealed accumulation of FITC-SP-D (green label) into intensely stained, punctate structures within type II cells after 2 h. The structures appeared vesicular in nature and were determined to be intracellular through analysis with differential interference contrast (DIC) imaging and confocal microscopy optical sectioning. Additionally, type II cells washed with EDTA-containing buffer after FITC-SP-D incubation to remove extracellularly associated SP-D have a similar staining pattern to the cells in Fig. 4, C–H (data not shown).

To investigate whether SP-D cell surface binding is a precursor to internalization, type II cells were incubated with FITC-labeled SP-D at 4°C, washed, resuspended in fresh binding buffer, and warmed to 37°C for 1 h. Confocal imaging revealed internalization of bound SP-D (Fig. 4, C–F) into type II cells in a profile very similar to that described for SP-D uptake at 37°C (Fig. 4H).

Uptake of nonspecific FITC-labeled proteins, as evaluated by confocal microscopy, was utilized to further examine the specificity of SP-D clearance by type II cells. At 2 µg/ml, FITC-RSA and FITC-HRP displayed no cell surface binding at 4°C under conditions identical to those used for SP-D. Internalization of FITC-RSA (Fig. 4, I and J) but not of FITC-HRP (data not shown) was observed at 37°C. However, uptake of FITC-RSA was not dependent on cell surface association. Type II cells incubated with FITC-RSA at 4°C, washed, resuspended in fresh binding buffer, and warmed to 37°C did not display uptake of labeled protein. Additionally, the pattern of FITC-RSA internalization at 37°C differed greatly from that of FITC-SP-D. Whereas SP-D appeared to dock with lamellar body-limiting membranes. FITC-RSA internalization at 37°C differed greatly from that of FITC-SP-D. Whereas SP-D appeared to dock with lamellar body-limiting structures within type II cells after 2 h. The structures appeared vesicular in nature and were determined to be intracellular through analysis with differential interference contrast (DIC) imaging and confocal microscope optical sectioning. Additionally, type II cells washed with EDTA-containing buffer after FITC-SP-D incubation to remove extracellularly associated SP-D have a similar staining pattern to the cells in Fig. 4, C–H (data not shown).

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A Table 1. FACS analysis of SP-D binding to freshly isolated alveolar type II cells

<table>
<thead>
<tr>
<th>Incubation Condition</th>
<th>Relative Fluorescent Units/Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells alone</td>
<td>5.6 ± 0.3*</td>
</tr>
<tr>
<td>SP-D</td>
<td>596.5 ± 41.1</td>
</tr>
<tr>
<td>SP-D + EDTA</td>
<td>39.6 ± 0.7*</td>
</tr>
<tr>
<td>SP-D + inositol</td>
<td>81.5 ± 2.3*</td>
</tr>
<tr>
<td>SP-D + maltose</td>
<td>68.5 ± 5.8*</td>
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</table>

Values are means ± SE; n = 3 repetitions from independent experiments. freshly isolated rat alveolar type II cells (5 × 10⁶/ml) were incubated in presence and absence of 2 µg/ml of FITC-labeled surfactant protein (SP) D for 2 h at 4°C. Binding buffer contained calcium (0.09 mM) except in samples with EDTA (2 mM). Maltose and myo-inositol were added at a final concentration of 10 mM. After incubation, type II cells were washed and fixed in freshly prepared 1% formaldehyde. Cells (10,000/sample) were analyzed by fluorescence-activated cell sorting (FACS). *Significantly different from SP-D, P < 0.001.
Freshly isolated type II cells degrade internalized SP-D to a lesser extent than alveolar macrophages. Degradation was examined as a possible fate for SP-D internalized by type II cells. TCA solubility was used as a marker for $[^{35}S]$SP-D degradation by type II cells and macrophages; radioactivity incorporated in the cell and medium fractions was analyzed as outlined in METHODS. The $[^{35}S]$SP-D stock contained 1.2% TCA-soluble counts, and $[^{35}S]$SP-D incubated with binding buffer alone for 2 h at 37°C contained 2.0% TCA-soluble counts.

For type II cells ($n = 3$) after 15 min, 1.4 ± 0.3 (SE) and 2.8 ± 0.2% of the radioactivity was TCA soluble in the cell and medium fractions, respectively. After 2 h, TCA-soluble radioactivity increased to 4.3 ± 1.6% in the cell fraction and 3.6 ± 0.2% in the medium.

For macrophages ($n = 3$) after 15 min, 3.9 ± 1.2 (SE) and 2.6 ± 0.2% of the radioactivity was TCA soluble in the cell and medium fractions, respectively. After 2 h, TCA-soluble radioactivity increased to 6.3 ± 0.9% in the cell fraction and 3.9 ± 0.3% in the medium. From these data and the percent macrophages in the type II cell preparations, it was calculated that macrophages contributed ~10–15% to the measured type II cell degradation of $[^{35}S]$SP-D.

Type II cell-conditioned medium was tested to examine the possibility of extracellular SP-D degradation by secreted or contaminating proteases. Type II cells were incubated in binding buffer for 2 h at 37°C, and the conditioned medium was removed to a new tube and incubated with SP-D for 2 h at 37°C. SP-D was not degraded in type II cell-conditioned medium, whereas type II cells from the same source degraded SP-D in a manner comparable to that reported above (data not shown).

Characterization of SP-D binding sites on freshly isolated type II cells. Protease digestion or PI-specific cleavage of cell surface moieties and competition by sugar residues were used to investigate the characteristics of SP-D binding to type II cells. Treatment with trypsin (0.05% for 30 min at 37°C) or proteinase K (100 µg/ml for 30 min at 4°C) did not affect binding of $[^{35}S]$SP-D to type II cells compared with that with
protease controls (Table 2). Conversely, binding of LPS to alveolar macrophages in the presence of 5% FBS decreased by >50% in response to trypsin and proteinase K treatment. Serum-dependent LPS binding to macrophages has previously been demonstrated to be via a protease-sensitive mechanism (19, 38).

SP-D binds type II cells in a calcium-dependent manner (Fig. 1), contains calcium-dependent lectin domains (7), and binds specific glycolipids such as PI (28, 29). Therefore, we hypothesized that carbohydrate residues could compete for SP-D binding to type II cells. Table 1 demonstrates that inositol and maltose inhibited FITC-SP-D binding to type II cells, ~85% compared with binding in the presence of calcium as evaluated by FACS. The low level of SP-D binding in the presence of competing sugars is equivalent to that of calcium-independent binding to type II cells. Additionally, inositol inhibited [3H]SP-D binding to type II cells in a similar manner (data not shown).

Studies by others demonstrated PI-PLC-sensitive binding sites for SP-D (11) and LPS (15, 35) on alveolar macrophage plasma membranes. Cleavage of PI-PLC-sensitive sites on the surface of type II cells (2.5 U/ml of PI-PLC for 1 h at 37°C) did not inhibit binding of [35S]SP-D (Table 3). This was in direct contrast to its affect on alveolar macrophages where treatment of cells with PI-PLC decreased [35S]SP-D binding ~30% and [3H]LPS binding ~40%.

Binding of SP-D to freshly isolated type II cells occurs in the presence of surfactant-like lipids. SP-D coisolates with surfactant lipids extracted from alveolar wash (25), and SP-D interacts specifically with PI (28, 29). Therefore, we speculated that lipid and SP-D interactions could influence their clearance by type II cells. At
type II cells on SP-D binding to freshly isolated rat alveolar type II cells.

### Table 2. Effect of cell surface protease treatment on SP-D binding to freshly isolated rat alveolar type II cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Binding Molecule</th>
<th>Binding, %control</th>
<th>Proteinase K</th>
<th>Trypsin</th>
</tr>
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<tbody>
<tr>
<td>Type II cells</td>
<td>SP-D</td>
<td>151.7 ± 26.4</td>
<td>160.7 ± 28.4</td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>LPS</td>
<td>32.0 ± 8.0*</td>
<td>40.7 ± 14.4*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3 repetitions from independent experiments. Type II cells (5 × 10⁶/ml) were treated for 30 min in presence and absence of either proteinase K (100 µg/ml; 4°C) or trypsin (0.05%; 37°C). Treatment was terminated with protease inhibitor, and cells were washed before incubation with [³⁵S]SP-D (1 µg/ml) for 2 h at 4°C. Type II cells were washed, and associated [³⁵S]SP-D was measured by scintillation counting and normalized to cellular protein. As a control for protease activity, alveolar macrophages (5 × 10⁶/ml) were protease treated as for type II cells and incubated with [³⁴S]lipopolysaccharide (LPS; 100 ng/ml) for 2 h at 4°C in presence of 5% fetal bovine serum (FBS). Cells were washed, and associated counts were measured by scintillation counting and normalized to cellular protein. *Significantly different from control, P ≤ 0.02.

25 µg lipid/ml, neither PG- nor PI-containing liposomes altered [³H]SP-D association with type II cells after 2 h at 37°C (data not shown).

SP-D does not enhance lipid uptake by freshly isolated type II cells. A direct influence of SP-D on lipid clearance by freshly isolated type II cells was also examined. Wild-type rat SP-D (2 µg/ml) did not alter the magnitude or clearance rate of synthetic surfactant-like liposomes (100 µg/ml) at 37°C over 2 h (Fig. 6). Longer time courses (4 h) and various SP-D concentrations (0.5–5 µg/ml) showed the same trend (data not shown). Human alveolar proteinosis SP-A (2 µg/ml), used as a positive control for increased type II cell uptake of liposomes, significantly increased uptake (data not shown).

Although synthetic surfactant-like liposomes are a reasonable model system for lipid clearance, it is possible that components key to a SP-D effect on clearance might be absent from synthetic lipid preparations. Therefore, liposomes were prepared from an organic extraction of cell-free normal rat lavage fluid. This method allowed for purification of surfactant lipids still associated with the hydrophobic SPs SP-B and SP-C (14, 36). As with the synthetic liposomes, SP-D (2 µg/ml) did not affect clearance of lavage fluid lipid liposomes at 37°C, whereas SP-A controls significantly increased uptake (data not shown).

### DISCUSSION

A direct role for SP-D in the regulation of surfactant homeostasis remains an intriguing possibility because mice deficient in SP-D have an increased alveolar surfactant pool size (3, 21). The goal of this study was to examine the association of SP-D with alveolar type II cells in hopes that an understanding of the interaction between these two components of the pulmonary surfactant system might assist in explaining a role for SP-D in surfactant homeostasis. The results reported here indicate that SP-D binds the external surface of type II cells as a precursor to internalization into degradative and recycling pathways. However, binding is apparently not a direct signal for modulation of surfactant lipid uptake by type II cells under the current experimental conditions.

SP-D binding to type II cells. SP-D binding to freshly isolated rat type II cells was concentration and time dependent; however, saturation of the label and equilibrium of binding over time were not observed. Competition for SP-D binding sites with excess, unlabeled SP-D to calculate nonspecific binding was not successful due to self-association of the protein at high concentrations in the presence of calcium. Self-association may also explain why SP-D binding failed to equilibrate over time, although low concentrations of SP-D were used for the time-course study.

The propensity for SPs to self-associate has been previously reported. Pison et al. (30) described calcium-dependent SP-A aggregation in a binding study with alveolar macrophages. Furthermore, they observed...

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**Fig. 6.** Effect of SP-D on lipid uptake over time by freshly isolated rat alveolar type II cells. Cells (5 × 10⁶) were incubated in calcium-containing buffer with synthetic surfactant-like lipid trace labeled with [³⁴S]dipalmitoylphosphatidylcholine. Uptake was conducted in presence and absence of SP-D (2 µg/ml) for indicated times at 37°C. Cells were washed in EDTA-containing buffer, and cell-associated lipid was measured by scintillation counting and normalized to cellular protein. CPM, counts/min. Values are means ± SD; n = 2 repetitions from independent experiments.
higher SP-A binding at 4°C than at 37°C. They hypothesized that aggregation contributed to the results; however, this could not be identified as the exact cause. The ability of SP-D to self-associate has also been examined. Crouch (7) recently reviewed the appearance of multimers in SP-D preparations from a variety of sources. SP-D in the multimer form has a higher apparent binding affinity for several ligands and is more effective at activating certain cellular functions than single SP-D molecules (dodecamers).

To test directly for SP-D association with the surface of type II cells, we analyzed binding by FACS and confocal microscopy. Both methods allowed for evaluation of SP-D binding to individual cells and addressed the concern of calcium-dependent aggregates copelleting with our cell preparations. Confocal microscopy and FACS confirmed that SP-D binds the surface of type II cells in a calcium-dependent manner.

We compared type II cell concentration- and time-dependent SP-D binding to the level of SP-D binding previously reported for macrophages (12). At 1 µg/ml of SP-D, macrophages bind ~20 ng SP-D/mg cellular protein, whereas type II cells bind ~100 ng SP-D/mg cellular protein. Because type II cells contain much less protein than macrophages, comparisons were also made on a per cell basis. With average protein contents of 75 µg protein/10⁶ type II cells and 225 µg protein/10⁶ macrophages, it was calculated that type II cells bind approximately twice as much SP-D per cell than macrophages. This also indicates that <5% of measured SP-D binding to type II cells is due to macrophage contamination of type II cell isolates.

SP-D internalization by type II cells. The internalization and reutilization of surfactant components by type II cells has been reported by numerous laboratories (32). Our results showing SP-D association with type II cells at 37°C indicates that they may also internalize and recycle SP-D. Optical sectioning of type II cells by confocal microscopy and the use of DIC imaging to establish the outer dimensions of the cells clearly demonstrate large, intracellular, vesicular structures with intense SP-D labeling. Our results also indicate that cell surface association is sufficient for SP-D internalization. SP-D bound to type II cells at 4°C was internalized after the cells were warmed to 37°C in a manner consistent with SP-D internalization at 37°C. Additionally, experiments with RSA and HRP demonstrated that SP-D is not binding via its fluorescent label nor is it internalized in a manner consistent with either nonspecific protein. Although RSA does appear to be internalized by type II cells at 37°C, it does not seem to enter the cell through the same mechanism as SP-D. These data suggest that type II cells have specific binding sites for SP-D that contribute to SP-D clearance.

Several features from our confocal studies indicate that vesicles labeled with internalized SP-D may be lamellar bodies, namely their size and their location within the cell. In contrast, the number of inclusions per cell appears low for identification as lamellar bodies. SP-D-labeled vesicles identified through confocal microscopy averaged ~30–40/cell, whereas a previous study (46) with electron microscopy reconstruction of type II cells estimates 150 lamellar bodies/type II cell. This discrepancy could not be accounted for empirically. One possibility is that internalized SP-D is targeted to a specific population of lamellar bodies. In an examination of the cellular localization of SP-D in rat lungs, Voorhout et al. (39) reported only minor SP-D antigenicity in type II cell lamellar bodies. Interestingly, a small subpopulation (10%) was noted to have heavier labeling. It was their conclusion, however, that SP-D did not colocalize with SP-A in lamellar bodies, and they suggested that SP-D may be secreted through a different pathway.

Electron microscopy confirmed our confocal results showing internalization of SP-D by type II cells. Although type II cells showed light staining throughout the cell, labeling was noticeably heavier in structures resembling lamellar bodies and small vesicles. Labeling of internalized SP-D in small vesicles may suggest a route of SP-D uptake by type II cells that includes deposition into lamellar bodies. Kuhn (23) previously reported that type II cells contain endogenous biotin stores. In control samples, which were type II cells incubated with nonbiotinylated SP-D, we observed no gold label over lamellar bodies and only diffuse background staining elsewhere in the cell. The low level of background staining is probably a consequence of the fixation and embedding methods employed in the current study, which were shown by Kuhn to greatly diminish detection of endogenous biotin.

Our results suggest that although type II cells degrade a portion of internalized SP-D, degradation by type II cells is less than SP-D degradation by macrophages. These data do not distinguish between type II cell degradation of SP-D by acid hydrolases present in lamellar bodies (17) or incorporation of SP-D into lysosomal pathways. They do suggest that SP-D degradation is via a type II cell-associated process because SP-D is not degraded in type II cell-conditioned medium. The magnitude of degradation by macrophages in this study was less than that observed in a previous report from this laboratory (12). This is possibly due to the fact that previous experiments utilized SP-D labeled with [¹³C]proline, whereas the current study used SP-D labeled with [¹⁵N]lysine and [¹⁵N]methionine.

SP-D binding sites on type II cells. The inhibition of SP-D binding to type II cells by EDTA, inositol, and maltose suggests that the binding may be mediated through the lectin domain of SP-D. However, we cannot exclude the possibility that bound sugar residues could alter SP-D confirmation to a degree where it would block type II cell association. A previous report (11) has shown that inositol inhibits SP-D binding to alveolar macrophages and SP-D binding is inhibited by PI-PLC treatment of the cells but not by protease treatment (11). This suggests that some SP-D binding sites on macrophages may be PI or another glycolipid. We hypothesized that type II cells would display a similar binding pattern. Indeed, SP-D binding to type II cells was not inhibited by cell surface protease treatment. In
fact, protease treatment appeared to increase SP-D binding, although the increase was not significant. It is possible that enzymatic digestion of the cell surface exposes SP-D binding epitopes. The discovery that SP-D binding was not decreased by PI-PLC treatment was surprising. These data suggest that the alveolar type II cell and alveolar macrophage cell surface SP-D binding sites may differ.

Effect of surfactant lipids on SP-D clearance by type II cells. Because SP-D is known to coalesce with and bind selected surfactant lipids, we examined the effect of the presence of lipids on type II cell internalization of SP-D. Our finding that surfactant-like liposomes containing either PG or PI did not affect SP-D uptake by type II cells is similar to previously reported effects of surfactant-like lipids on alveolar macrophage uptake and degradation of SP-D (12).

Influence of SP-D on lipid clearance by type II cells. Our finding that SP-D does not increase surfactant lipid uptake by type II cells is consistent with another study (34) reporting that SP-D does not influence uptake of surfactant-like liposomes by isolated type II cells at SP-D concentrations up to 20 µg/ml. We hypothesized that the lipid composition of liposomes prepared from synthetic lipids might lack minor lipid, protein, or other compounds that mediate SP-D regulation of type II cell surfactant lipid clearance. Therefore, we prepared an organic extract of normal rat pulmonary lavage fluid to obtain a more complete profile of lipids and hydrophobic compounds resident in normal lavage fluid. Again, SP-D showed no effect on the rate or magnitude of clearance. Interestingly, the SP-A positive control for lipid uptake with the lavage fluid-extracted lipids showed a dramatically higher increase in lipid clearance over cells with liposomes alone compared with samples with synthetic surfactant-like lipids (data not shown). Finally, it is important to note that we only analyzed the uptake of [3H]DPPC; SP-D may have a regulatory role in the clearance of other surfactant lipids.

Consideration should also be given to discrepancies observed between in vivo and in vitro models of lipid clearance. SP-A has been shown to enhance surfactant lipid clearance by type II cells (42) and macrophages (43) in vitro; however, surfactant homeostasis in the SP-A-null mouse appears normal (20). Similarly, no reports to date have described a role for SP-D in the regulation of surfactant lipid turnover in vitro yet the SP-D-null mouse displays significant disruption of surfactant lipid processing (3, 21). Although these data might indicate potential problems with current in vitro models, they may also suggest avenues for additional investigation. The SP-A-null mouse phenotype could be explained through a compensatory mechanism for surfactant metabolism, or perhaps the requirement for SP-A-induced clearance is required only in times of stress or disease. Additionally, SP-D may require a cofactor for surfactant regulation, or it may not act directly; instead, SP-D could induce the production of regulatory molecules that influence surfactant turnover.

Summary. Regulation of pulmonary surfactant homeostasis is critical for normal respiratory function. A prominent role for SP-D in the maintenance of the alveolar surfactant pool has been most eloquently described through the production of SP-D-null mice (3, 21), which accumulate surfactant in the alveoli and type II cell lamellar bodies. We interpret the current study to suggest that SP-D binds to type II cells and can be internalized into their recycling pathway. However, SP-D does not directly mediate type II cell clearance of surfactant lipids in vitro. A direct role for SP-D in the regulation of the alveolar surfactant pool by type II cells cannot be dismissed; however, future studies should examine the possibility of SP-D as an indirect regulatory molecule in the modulation of type II cell lipid metabolism.

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REFERENCES


