The Major Glycolipid Recognized by SP-D in Surfactant Is Phosphatidylinositol†

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ABSTRACT: Surfactant protein D (SP-D), a multimeric calcium-dependent lectin isolated from pulmonary alveolar lavage, has been previously shown to interact reversibly with crude surfactant [Persson et al. (1990) *J. Biol. Chem. 265*, 5755–5760]. In this study, SP-D is shown to interact reversibly with a preparation of organelles enriched in lamellar bodies, in a manner inhibited by calcium-chelating agents and by competing saccharides. An interaction with an endogenous glycoprotein could not be identified by electrophoresis of surfactant or lamellar body-associated proteins followed by electrotransfer of the separated proteins to nitrocellulose and then probing with radioiodinated SP-D via lectin overlay. Separation of the surfactant or lamellar body lipids on two-dimensional thin-layer chromatography (2D-TLC) followed by probing with radioiodinated SP-D via lectin overlay demonstrated binding to a single lipid. This interaction was dependent on the presence of calcium and was inhibited by competing saccharides. By assaying column fractions for the ability to bind radioiodinated SP-D after TLC, the glycolipid was purified to homogeneity and identified as phosphatidylinositol (PI). Identification was confirmed by mass spectrometry. We further demonstrate the ability of radiolabeled SP-D to bind to PI presented in a lipid bilayer through separation of free SP-D from liposome-bound SP-D on density gradients of Percoll. The interaction of SP-D with PI is dependent on calcium and inhibited by competing saccharides. SP-D binds with similar efficiency to liposomes with mole fractions of PI ranging from 2.5% to 30%, thereby demonstrating the lectin's ability to recognize mole fractions of PI available in surfactant. SP-D was shown to agglutinate unilamellar liposomes in a manner dependent on the presence of PI in the liposomes. The agglutination was dependent on the presence of calcium and was inhibitable with competing saccharides. These findings are consistent with a probable physiological interaction between SP-D and phosphatidylinositol in the lipid bilayer.

Pulmonary surfactant is a morphologically heterogeneous mixture of proteins and lipids obtained on differential centrifugation of the lavage fluid of lungs. The proteins associated with surfactant include two small hydrophobic proteins, SP-B and SP-C, as well as the extensively described major surfactant protein SP-A (Possmayer, 1988). A quantitatively minor protein, SP-D, is also associated with surfactant (Persson et al., 1990), and like the other surfactant-associated proteins, SP-D is markedly increased in silica-induced alveolar lipoproteinosis (Crouch et al., 1991). SP-A and SP-D are immunologically distinct (Persson et al., 1988) and differ in their amino acid composition, isoelectric points, and assembly (Persson et al., 1989). Like the major surfactant protein SP-A, SP-D has a C-terminal domain that shares sequence homology with a family of calcium-dependent lectins (Rust et al., 1991; Shimizu et al. 1992). SP-D demonstrates calcium-dependent saccharide specificity that favors terminal glucosyl residues as the α-anomer (Persson et al., 1990) and overlaps the saccharide specificity reported for SP-A (Haagsman et al., 1987). Both proteins are synthesized and secreted by type II alveolar epithelial cells and have been histochemically localized to both type II alveolar epithelial cells and nonciliated bronchiolar epithelial cells (Crouch et al., 1992).

Upon differential centrifugation, a significant proportion of SP-D in alveolar lavage fluid is not associated with the high-speed pellet, termed crude surfactant (Persson et al., 1990), an observation independently confirmed (Kuroki et al., 1991). The SP-D associated with the high-speed pellet can be quantitatively dissociated by calcium chelators or competing saccharides in concentrations dependent on their relative affinities for SP-D, suggesting that the association of SP-D with surfactant is reversible and dependent on binding to a surfactant-associated glycoprotein or glycolipid.

The sugar specificity of SP-D for terminal glucose in an α-anomeric configuration leads to speculation on the nature of the surfactant-associated ligands. The unidentified ligands might include collagens with glucosyl(α1–4)galactosylhydroxylysine (Butler, 1982). Neutral and acidic glucoglycerolipids with (α1–6)-linked terminal glucose residues isolated from rabbit lung lavage (Slomiany et al., 1979) and human salivary secretions (Slomiany et al., 1978) might also function as ligands for the association of SP-D with the lipid bilayers in surfactant. Glucosylceramide, even though the β-anomer, might also function as a surfactant-associated ligand. The purpose of the present studies was to identify the major ligand for SP-D in surfactant.

We have previously demonstrated the specific interaction between SP-D and a ligand present in surfactant and now demonstrate reversible interaction with an organellar subfraction from rat lung enriched in lamellar bodies. The major glycolipid recognized by SP-D was purified from lamellar bodies and shown to be phosphatidylinositol. The major glycolipid recognized by SP-D in surfactant isolated from rat, guinea pig, and cow was also shown to be phosphatidylinositol. SP-D binds to phosphatidylinositol when the lipid is incorporated into liposomes, and the lectin–liposome interaction can be inhibited either by calcium chelators or by competing saccharides. Moreover, SP-D can agglutinate unilamellar

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liposomes containing this phospholipid, further confirming multivalency of the lectin.

MATERIALS AND METHODS

Isolation of Surfactant and Purification of SP-D. SP-D was purified in enhanced quantities from the broncho–alveolar lavage of rat lungs after induction of lipoproteinosis with silica (Crouch et al., 1991). To induce lipoproteinosis, 250-g male Sprague-Dawley rats were anesthetized with an intramuscular injection of 1.0 mL/kg xylazine/ketamine/acepromazine (20 mg/mL:100 mg/mL:10 mg/mL), the trachea were cannulated using a modified pediatric laryngoscope, and 200 mg/kg of a 100 mg/mL suspension of 5-μm silica prepared according to Dethloff et al. (1986) was instilled intratracheally. Two weeks later, the animals were anesthetized with 200 mg/kg intraperitoneal pentobarbital and sacrificed by exsanguination, and the lungs were lavaged in situ with 5 mM HEPES, 150 mM NaCl, pH 7.4. The lavage fluid was centrifuged at 150*g* for 10 min to remove cells and debris, and the crude surfactant pellet was separated from the supernatant by centrifugation at 48000*g* for 30 min at 4 °C and stored at −20 °C. The supernatant was supplemented with $CaCl_2$ to 2 mM and passed over maltosyl-Sepharose (Pharmacia) prepared according to Fornstedt and Porath (1975). The column was washed with 5 column volumes of 25 mM HEPES, 150 mM NaCl, pH 7.4 (HBS) containing 2 mM $CaCl_2$ (HBS-Ca), and SP-D was eluted with HBS containing 10 mM EDTA (HBS-EDTA). Fractions containing SP-D were further purified by gel filtration over A15M (Bio-Rad) equilibrated with 50 mM Tris, 150 mM NaCl, 10 mM EDTA, pH 7.4 (TBS-EDTA). Purity of the lectin was verified by SDS–polyacrylamide gel electrophoresis as previously described (Persson et al., 1990).

Radioiodination of SP-D. Purified SP-D was rechromatographed on maltosyl-Sepharose to concentrate the protein and to exchange TBS-EDTA for HBS-EDTA. Iodination was performed with HPLC-purified Bolton–Hunter reagent (Bolton & Hunter, 1973) (Amersham) as previously described (Persson et al., 1990) yielding specific activities of (3–6) × 10^6 cpm/μg SP-D. The radioiodinated lectin was stored at 4 °C in the presence of 0.1% Triton X-100 in HBS-EDTA. Immediately before use in experiments, the radioiodinated lectin was readsorbed to a 0.3-mL maltosyl-Sepharose column and washed with 5 column volumes of HBS containing 2 mM $CaCl_2$, 0.5% bovine serum albumin (BSA). The lectin was eluted with HBS containing 10 mM EDTA, 0.5% BSA, and the affinity-purified lectin was used immediately in subsequent experiments.

Solid-Phase Carbohydrate-Binding Assays. SP-D was modified by periodate oxidation and biotinylated with biotin-LC-hydrazide (Pierce) per O'Shannessy et al. (1987). The biotinylated lectin (Bt-SP-D) was repurified on maltosyl-Sepharose as above. Maltosyl-BSA (10 μg/mL) synthesized by reductive amination (Schwartz & Gray, 1977) was adsorbed to polystyrene microtiter plates for 1 h at 37 °C, and all subsequent steps were performed at room temperature. The wells were blocked with 1% BSA, washed with TBS, 2 mM $CaCl_2$, 0.1% Tween 20, and incubated for 1 h with Bt-SP-D in the presence of varying concentrations of competing inhibitors. Wells were then rapidly washed with two 100-μL aliquots of wash buffer, complexes were stabilized with 0.125% glutaraldehyde in phosphate-buffered saline for 15 min, and excess glutaraldehyde was quenched with 1 M ethanolamine, pH 8.0. After washing, bound Bt-SP-D was detected by sequential incubation with streptavidin–horseradish peroxidase conjugate (Pierce) and a commercial peroxidase substrate (ABTS; Kirkegaard and Perry, Gaithersburg, MD).

Liposome Generation. Phospholipids in chloroform/methanol (1:1 v/v) were added to a test tube in appropriate ratios, and organic solvent was removed with N_2 followed by 1 h at high vacuum. The lipids were hydrated in HBS at a concentration of 2 mg/mL and sonicated for 10 min at high power in a Heat Systems horn sonicator at room temperature to obtain multilamellar liposomes for use immediately in Percoll gradients. For measurement of agglutination by changes in light scattering at 400 nm, unilamellar liposomes were prepared by hydrating the mixed and dried lipids in HBS with intermittent vortexing for 30 min and passing the crude liposomes through two layers of Nucleopore filters of 0.1-μm pore size assembled in a device constructed according to MacDonald et al. (1991).

Percoll Gradients. Percoll (Pharmacia) was adjusted to 40% in 10 mM Tris, 150 mM NaCl, and appropriate concentrations of EDTA, calcium, and maltose. Samples of radioiodinated lectin and lamellar body fraction or multilamellar liposomes were mixed in the same buffer in a volume of 0.5 mL, prior to layering on 10 mL of 40% Percoll. The gradients were formed by centrifugation at 30000*g*, at 10 °C for 1 h, and fractionated from the bottom of the tubes on a Hoefer gradient fractionator.

Thin-Layer Chromatography (TLC) and Lectin Overlay Assay. Chromatography was performed on aluminum-backed silica gel F254 plates (Whatman), with chloroform/methanol/water (60:35:8 v/v) or otherwise specified solvent systems. Glycolipids were detected with orcinol, and other lipids were identified with iodine, primulin, sulfuric acid charring, or ammonium molybdate stains. TLC plates run in parallel were subjected to lectin overlay (Magnani et al., 1982) with the following modifications. After development and air-drying, the plates were wetted in hexane and then dipped in 0.05% poly(isobutyl methacrylate) (Aldrich) with hexane for 60 s and dried. The plates were blocked with 0.5% BSA in HBS for 1 h and incubated with 0.5 × 10^6 cpm/mL radioiodinated SP-D in HBS containing 2 mM $CaCl_2$ and 0.5% BSA for 1 h. Inhibition of the lectin overlay was performed by inclusion of either 30 mM maltose or 10 mM EDTA in the incubation buffer. The plates were washed with HBS containing 2 mM $CaCl_2$ and, after drying, were submitted to autoradiography. Densitometry was performed on a Hoefer scanning densitometer coupled to a SpectraPhysics integrator.

Enriched Lamellar Body Fraction. Lavaged silicotic lungs were excised from the rat, and the large airways were removed. The lung tissue was homogenized in 145 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4, in a Waring blender, and the 500-μm mesh filtrate was submitted to differential centrifugation according to Frosolono et al. (1970). The fraction enriched in lamellar bodies was stored at −20 °C until use. Phosphate was assayed according to Bartlett (1959) as modified by Rouser et al. (1970).

Lipid Extraction and Purification of Glycolipids Recognized by SP-D. Surfactant or enriched lamellar body fraction was washed by resuspension in water and recentrifugation at 48000*g* and then lyophilized. The lyophilized material was extracted overnight with chloroform/methanol (2:1 v/v), and insoluble material was separated by centrifugation at 5000*g*. The insoluble pellet was reextracted, and supernatants were combined, dried in vacuo at 40 °C, and stored at −20 °C. DEAE-Sephadex A-25 (4.4 g) was prepared according to Ledeen and Yu (1982) to give a 1.0 × 13 cm column and washed with 100 mL of the starting solvent chloroform/methanol/water (30:60:8 v/v). After application of the lamellar body extract (860 mg) in the starting solvent, the

column was washed with 75 mL of starting solvent, followed by 50 mL of methanol, and was then eluted with a 500 mL linear gradient from 0 to 0.4 M ammonium acetate in methanol. The column fractions were assayed by phosphate determination and by TLC with radiolabeled lectin overlay. The fractions containing lectin-binding activity were pooled, evaporated in vacuo at 40 °C, and, after resuspension in water, lyophilized repeatedly to remove volatile salts. Iatrobeads (Iatron, Japan) RS-8060 (30 g) were prepared and washed according to Ledeen and Yu (1982) to give a 0.9 × 90 cm column. Pooled lectin-binding lipids from the DEAE column were redissolved in chloroform/methanol/water (65:32:3 v/v) loaded and washed with 50 mL of starting solvent, and eluted according to Bonafede et al. (1989) with the three chambers containing 250 g of solvent mixtures of chloroform/methanol/water in ratios 65:32:3, 60:37:3, and 40:57:3 (v/v).

Gas Chromatography–Mass Spectrometry (GC–MS). Two hundred micrograms of phosphatidylinositol, inositol 1-monophosphate or inositol 2-monophosphate standards or of the residue from 1 mL of the fraction containing most intense lectin binding (fraction 18 from the Iatrobeads column above) was dried under nitrogen at 55 °C and dissolved in 64 μL of chloroform/acetone/water (10:5:1 v/v) with 0.005% butylated hydroxytoluene (BHT). The samples were chilled on ice and held under nitrogen for all manipulations. Eleven microliters of trifluoroacetic acid was added to each sample, and the samples were heated for 24 h at 70 °C. Forty microliters of triethylamine was then added, and each sample was heated for 24 h at 70 °C. Retention time markers were added, and the samples were dehydrated under a nitrogen stream at 55 °C by successive additions of acetonitrile and then methylene chloride. The sample was heated for 1 h at 70 °C after the addition of 200 μL *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA). One microliter of the sample was injected into a VG Trio 2 gas chromatograph–mass spectrometer (VG Instruments, Fisons Inc., Danvers, MA) containing a 30 m × 0.32 mm DB 5 capillary column. The oven and acquisition temperatures were programmed as previously described (Shoemaker & Elliott, 1991).

RESULTS

Interaction of SP-D with Surfactant and Lamellar Bodies. Previously published experiments suggested a reversible interaction between SP-D and surfactant which was inhibitable both with competing mono- and disaccharides and with calcium chelators. Moreover, an appreciable fraction (90%) of the lectin was not bound to surfactant which might suggest a weak affinity for the ligands in the surfactant, a limiting quantity of the ligands, masking of the ligands, presence of a soluble competing inhibitor, or heterogeneity in the lectin preparation. To obtain a potentially richer source of the ligands, a preparation of organelles enriched in lamellar bodies was evaluated for binding of radioiodinated SP-D. Percoll density gradients were employed to separate organelles or liposomes together with bound lectin from the nonbound lectin. Percoll was chosen to form the density gradients because the binding of SP-D to maltosyl-BSA as assessed by a solid-phase carbohydrate-binding assay was inhibited by the concentrations of sucrose, metrizamide, and glycerol typically employed in density gradients for the purification of surfactant or lamellar bodies (data not shown). Radioiodinated SP-D cocentrifuged with crude lamellar bodies in a calcium-dependent fashion (Figure 1A), and this binding was inhibited by competing saccharide, 30 mM maltose. Binding of the lectin was never quantitative, showing 56.8% ± 3.1% (n = 5) binding

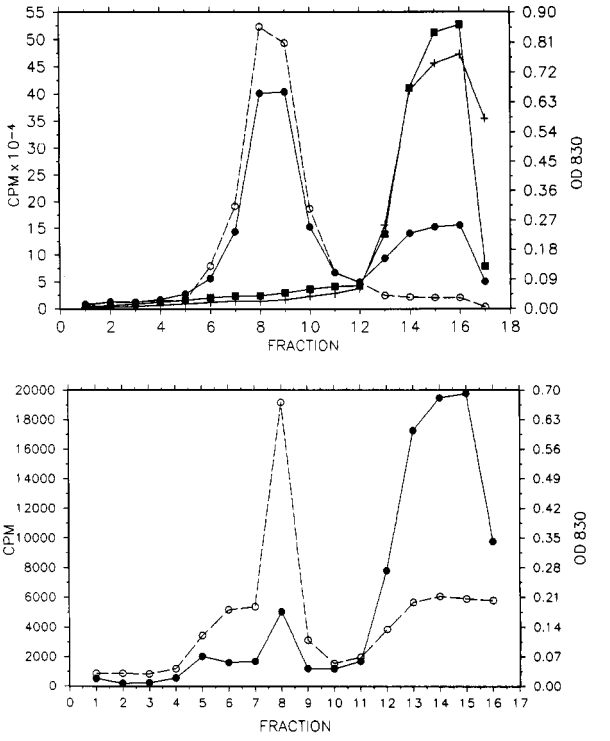

FIGURE 1: (A, top) Binding of radioiodinated SP-D to lamellar body enriched fraction. Lamellar bodies, 2 mg, were mixed with 2 × 10^6 CPM radioiodinated SP-D in TBS containing 10 mM $CaCl_2$ (solid circles), 10 mM $CaCl_2$ and 30 mM maltose (solid squares), or 10 mM EDTA (plusses) in a final volume of 0.5 mL. The sample was layered over 30% Percoll in the corresponding buffer and centrifuged as in Materials and Methods. Aliquots were assayed for radioactivity and phospholipids (open circles). (B, bottom) Binding of radioiodinated SP-D to crude surfactant. Crude surfactant, 2 mg, was mixed with 2 × 10^6 CPM radioiodinated SP-D in TBS containing 10 mM $CaCl_2$, in a final volume of 1.0 mL and layered over 30% Percoll in the corresponding buffer. Aliquots were assayed for radioactivity (solid circles) and phospholipid (open circles). Binding was abolished in the presence of 10 mM EDTA or 30 mM maltose (data not shown).

to the organelles. Similar profiles were obtained with crude rat surfactant (Figure 1B), but the proportion of SP-D bound was quantitatively less (7.0% ± 1.2% (n = 8)), suggesting that the amount, character, or accessibility of ligand was different.

Identification and Isolation of the Ligand. Binding of SP-D to proteins present in surfactant and alveolar lavage supernatant was assessed by lectin overlay (Persson et al., 1990). Preparations of whole surfactant or lamellar bodies and of proteins remaining after lipid extraction of surfactant or lamellar bodies were electrophoresed and transferred to nitrocellulose. After blocking with albumin, the nitrocellulose blots were incubated with radiolabeled SP-D, washed, dried, and autoradiographed. These methods did not identify a ligand (data not shown) even though the lectin gave a markedly positive signal with the neoglycoprotein maltosyl-BSA.

The interactions of SP-D with glycolipids present in organic solvent extracts of lamellar bodies, surfactant, and lyophilized lavage supernatant was demonstrated by separation of the lipids with 2D-TLC followed by lectin overlay. Lamellar bodies contained a lipid which interacted strongly with the lectin (Figure 2), and this interaction on the TLC plate was inhibited either by calcium chelators (10 mM EDTA) or by competing saccharides (30 mM maltose). This lipid was also identified in surfactant from several species (cow, rat, and guinea pig), and no other lipids present in these preparations were recognized by SP-D. The organic solvent extract prepared according to Slomiany et al. (1978) from the

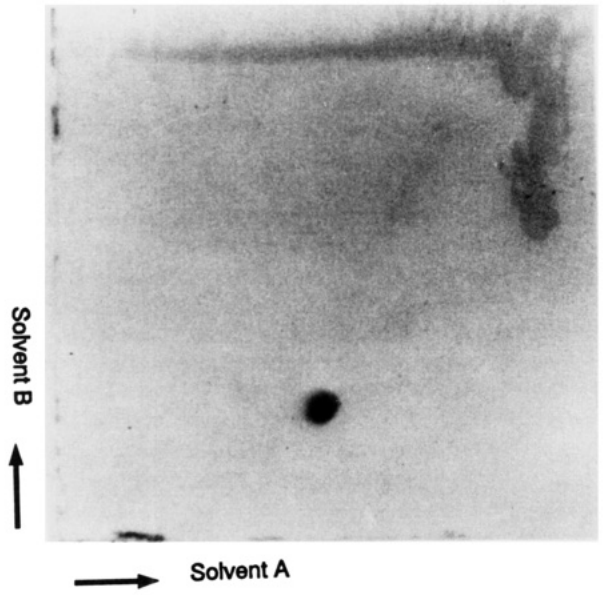

FIGURE 2: SP-D overlay of lipids from lamellar body enriched extract. An aliquot of the extract was applied and developed with solvent A (chloroform/methanol/0.5% $CaCl_2$; 60:35:0.8 v/v), dried, and followed in the second dimension by solvent B (chloroform/methanol/water/ammonium hydroxide; 70:30:3:2 v/v). The plate was blocked and probed by lectin overlay as in Materials and Methods.

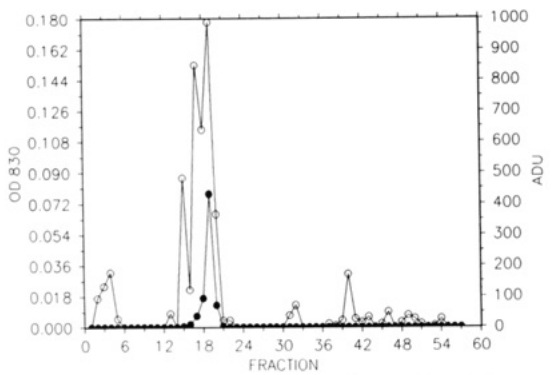

FIGURE 3: Assay of DEAE column of lamellar body extract. Phosphate determination was with optical density at 830 nm (open circles). Lectin blot assay was with arbitrary density units (ADU) (solid circles), in which only one component was detected in the developed lanes, and binding to that component was inhibited by 30 mM maltose or by the addition of 10 mM EDTA.

supernatant of rat lung lavage did not reveal other SP-D binding glycolipids on 2D-TLC and lectin overlay. The lectin binding intensity was greatest in the chromatogram of the lamellar body fraction derived from the lungs of silica-treated rats; therefore, this material was employed as the starting material for isolation of the glycolipid recognized by SP-D.

Enriched lamellar bodies were lyophilized and extracted with organic solvents, and the extract was directly applied to a DEAE-Sephadex column (Figure 3). As described by Slomiany et al. (1978), the extracts were not submitted to phase partitioning to minimize losses of water-soluble glycolipids. Employing lectin overlay of TLC for each fraction, no lectin binding was observed to the nonbound or "neutral" fraction, and the elution position of the lectin-binding compound in the salt gradient was consistent with a lipid containing a net single negative charge (Ledeen & Yu, 1982). The pool of lectin-binding lipids from the DEAE column was then applied to an Iatrobead macroporous silica column (Figure 4), and the lectin-binding lipid eluted earlier in the gradient than rat brain gangliosides purified by the same methods (Bonafede et al., 1989). The phosphate determination

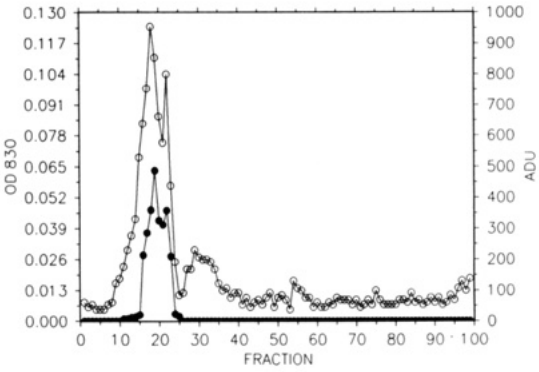

FIGURE 4: Assay of Iatrobeads column of lectin-binding lipids from DEAE column pool. Lectin blot assay (solid circles), in which only one component was detected in the developed lanes, and phosphate assay (open circles) are represented. Binding to the lipid was inhibited by 30 mM maltose or by 10 mM EDTA.

appeared to parallel the densitometrically determined binding of lectin to the TLC-separated lipids, and the ammonium molybdate stain demonstrated that a single phosphate containing compound co-chromatographed with the lectin-binding compound. No other substances were identified on TLC with visualization with iodine, primulin, orcinol, or sulfuric acid charring. Therefore, several phospholipids were compared to the isolated compound in their chromatographic properties and their ability to bind SP-D. The lectin did not bind to phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, or phosphatidic acid, but it exhibited strong binding to phosphatidylinositol. The isolated compound co-chromatographed on TLC with commercially prepared phosphatidylinositol from both soybeans and bovine liver in the following four separate solvent systems: chloroform/methanol/acetic acid/acetone/water (50:10:10:20:5 v/v), chloroform/ethanol/triethylamine/water (30:34:35:8 v/v), chloroform/methanol/0.5% calcium chloride (60:35:8 v/v), and chloroform/methanol/ammonium hydroxide/water (70:30:2:3 v/v). Phosphatidylinositol from soybeans or bovine liver co-chromatographed with the SP-D binding compound in the 2D solvent system initially employed to screen for glycolipid ligands for the lectin and gave an autoradiographic pattern identical to Figure 2 on radiolabeled lectin overlay. The isolated compound did not co-elute with glucosylceramide, with lactosylceramide, or with rat brain gangliosides prepared according to Bonafede et al. (1989).

To further confirm the identity of the isolated glycolipid, the material from the peak fraction (fraction 18) of the Iatrobeads column was subjected to gas chromatography–mass spectral analysis and was shown to contain inositol 1-phosphate and trace quantities of inositol and phosphate. There were no other detectable sugars. Also present were glycerol and fatty acids with 32% palmitic acid, 27% stearic acid, 16% linoleic acid, 7% oleic acid, 15% arachidonic acid, and 3% eicosapentaenoic acid.

Interaction of SP-D with Phosphatidylinositol. To examine the specific interaction of SP-D with the polar headgroup of phosphatidylinositol, we measured the ability of several water-soluble compounds to inhibit the interaction of SP-D with maltosyl-BSA in the solid-phase lectin-binding assay (Figure 5). D-*myo*-Inositol appeared to inhibit as well as maltose, and the addition of the glycerophosphoryl group to D-*myo*-inositol moderately diminished the inhibitory potency of the cyclitol. Glycerophosphocholine did not show any inhibitory effects up to a 0.1 M concentration.

To establish the ability of SP-D to bind to phosphatidylinositol in a membrane milieu, multilamellar liposomes,

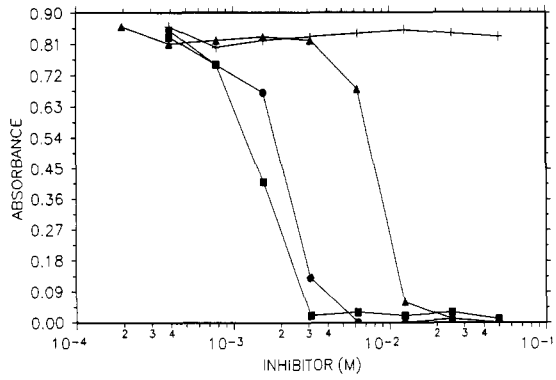

FIGURE 5: Solid phase lectin binding assay. Inhibition was performed with the indicated concentrations of *myo*-inositol (solid squares), maltose (solid circles), L-α-glycerophospho-D-*myo*-inositol (solid triangles), and L-α-glycerophosphocholine (plusses). The plate was developed as in Methods and Materials.

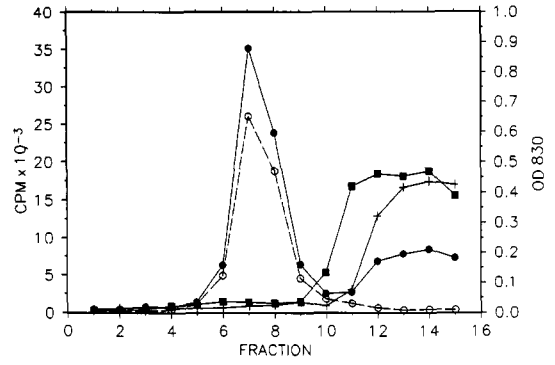

FIGURE 6: Binding of radioiodinated SP-D to liposomes on Percoll gradients. Radioiodinated lectin, immediately following affinity purification on maltosyl-Sepharose, was mixed with 2 mg of liposomes composed of phosphatidylcholine/phosphatidylinositol (90:10 w/w) (solid circles); phosphatidylcholine/phosphatidylglycerol (90:10 w/w) (solid squares); or 100% phosphatidylcholine (plusses) in the presence of 4 mM $CaCl_2$ in HBS. The mixture was applied to a 40% Percoll gradient and processed as in Materials and Methods. The phospholipid assay for phosphatidylcholine/phosphatidylinositol (90:10) is displayed (open circles).

prepared by sonication, were subjected to Percoll gradients after mixing with radiolabeled SP-D (Figure 6). Liposomes containing phosphatidylinositol exhibited binding of the lectin, but in the absence of phosphatidylinositol the lectin did not comigrate with the phospholipids. Binding of the lectin to the sedimenting phosphatidylinositol-containing liposomes could be eliminated by the addition of 30 mM maltose or 10 mM EDTA to the gradients. SP-D bound to liposomes with 65% ± 2.6% efficiency over a range of 2.5–30% phosphatidylinositol (data not shown).

Light-scattering studies (Figure 7) demonstrated reversible agglutination of unilamellar liposomes, prepared by extrusion through uniform pore membranes. In the absence of phosphatidylinositol, or SP-D, agglutination did not take place. Agglutination was dependent on the presence of calcium and was rapidly reversed by a calcium chelator (EDTA) or by competing sugars such as maltose.

DISCUSSION

The specificity of SP-D for sugars had been described in a solid-phase lectin-binding assay, and the lectin demonstrated a preference for glucose in the terminal position, with a slight advantage given to the α-anomer (Persson et al., 1990). Among mature mammalian glycoproteins, this ligand is present on some collagens as glucosyl(α1–4)galactosylhydroxylysine. Indeed these posttranslational modifications are present in

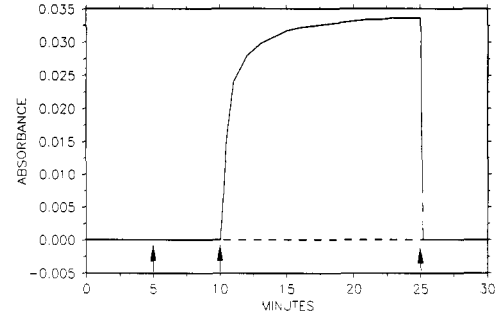

FIGURE 7: Agglutination of liposomes by SP-D. Optical density at 400 nm was employed to monitor light scattering at 21 °C. Unilamellar liposomes (phosphatidylcholine/phosphatidylinositol; 90:10 w/w; 100 μg) (solid line) were diluted into 1 mL of HBS and observed. At 5 min, unlabeled SP-D (5 μg) was added and no agglutination was observed. At 10 min, the addition of $CaCl_2$ to 5 mM resulted in rapid agglutination which approached completion within 15 min. At 25 min, addition of maltose to 30 mM or EDTA to 10 mM resulted in rapid reversal of the agglutination. No agglutination was observed with liposomes without phosphatidylinositol (phosphatidylcholine/phosphatidylglycerol; 90:10 w/w) (dashed line).

SP-D (Persson et al., 1989) and may contribute to the observed self-aggregation of the protein, an aggregation which is dependent on calcium and inhibited with competing sugars (unpublished results). While the formation of higher order aggregates of SP-D may account for part of the association of SP-D with the surfactant obtained by differential centrifugation, the association between SP-D and itself or the other proteins of surfactant was not readily apparent on lectin blots of denatured, electrophoresed, and transferred surfactant-associated proteins. This does not preclude the presence of a glycoprotein which requires a specific three-dimensional structure or other associations for successful interaction with the lectin.

Because of the lectin's preference for the glucosyl residue, and the description of glucoglycerolipids, a directed effort was made to identify the molecular basis for the interactions between SP-D and the lipids of surfactant. Presupposing a process in which a glycolipid-containing subfraction of surfactant might turnover more rapidly than other subfractions, lamellar bodies were isolated to obtain an enriched source of the glycolipid. The lectin recognized a single component in the extracts from a fraction enriched in lamellar bodies, as well as in surfactant obtained from rat, guinea pig, and cow. The lipid was purified from the enriched lamellar bodies and was shown to comigrate with phosphatidylinositol in multiple solvent systems. GC–MS confirmed the identity of the lipid, and the fatty acid composition is comparable to that shown for phosphatidylinositol in rabbit lung (Hayashi et al., 1990). We were unable to identify other glycolipids in lamellar bodies, surfactant, or lyophilized alveolar lavage supernatant as ligands for SP-D. These data support the assertions of Narasimhan et al. (1982) that the alkylacylglucoglycerolipids described by Slomiany et al. (1978) are due to contaminating bacteria as the glucoglycerolipids would be predicted to function as excellent ligands for SP-D. Glucosylceramide, when used in the TLC lectin overlay assay system, can be readily recognized by radioiodinated SP-D (data not shown), but the glycosphingolipid was not recognized in extracts from enriched lamellar bodies, surfactant, or alveolar lavage supernatant. In the lavage of patients with adult respiratory distress syndrome, the only glycolipids identified were lactosylceramide and paragloboside (Rauvala & Hallman, 1984), but since both possess terminal galactose residues they exhibit low affinity for SP-D. The assays of multivalent lectin binding

to glycolipids adsorbed to surfaces may be dependent on a threshold concentration of lipid and may not detect minor glycolipid species (Tang et al., 1985). These studies therefore do not establish the absence of the glucoglycerolipids or glucosylceramide but instead suggest that they are not the major component recognized by SP-D.

Comparison of the stereochemistry of methyl α-glucose with D-*myo*-inositol 1-monophosphate (Parthasarathy & Eisenberg, 1986) shows that the 2-, 3-, and 4-hydroxyls on the glucose are arrayed in a similar fashion to the 6-, 5-, and 4-hydroxyls, respectively, on the *myo*-inositol 1-monophosphate. The I_{50}s of both *myo*- and *scyllo*-inositol compare favorably to maltose (respectively 2.5, 2.5, and 3.0 mM), suggesting that the presence of three adjacent hydroxyl groups in equatorial positions may form the major determinant for binding. The effects of structural similarities between glucose and inositol have been observed in other biological systems such as the inositol transporter in the gut (Scalera et al., 1991). Glycerophosphoinositol appears to be moderately less effective as an inhibitor of lectin binding, and glycerophosphocholine is completely ineffective as an inhibitor. Therefore, it is plausible that phosphatidylinositol would function as a ligand for the lectin.

While the lectin may bind freely to phosphatidylinositol on a TLC plate coated with poly(isobutyl methacrylate), the specificity of interaction of a lectin with glycolipids can be dependent on the surface to which the glycolipids are adsorbed (Childs et al., 1992; Solomon et al., 1991; Yiu & Lingwood, 1992). The binding of proteins to ligands attached to liposomes has been shown to be dependent on the length of the extension between the lipid bilayer and the ligand in multiple examples including streptavidin (Blankenburg et al., 1989) and *Ricinus communis* agglutinin (Hoekstra & Duzgunes, 1986). These observations necessitate demonstration of interaction between SP-D and the inositol headgroup when the ligand is embedded in a lipid milieu. The assay used to show lectin binding to the enriched lamellar body preparation shows binding of the same efficiency as to liposomes composed of 10% phosphatidylinositol in phosphatidylcholine. The ability to interact with phosphatidylinositol in the liposomes required the presence of calcium and was inhibited by maltose. The lectin, which is postulated to be multivalent on the basis of previous studies, displays the ability to agglutinate unilamellar liposomes in a manner dependent on the presence of phosphatidylinositol, confirming the accessibility of the headgroup of phosphatidylinositol to the lectin. Weis et al. (1991) characterized the sugar-binding site of another member of these calcium-dependent lectins, the mannose-binding protein, as probably residing in a shallow depression on the surface of the protein, suggesting that the sugar-binding site on SP-D may not reside in a cleft but might be sufficiently close to the surface of the protein to allow interaction with a ligand which protrudes only slightly from the lipid bilayer. Phosphatidylinositol, embedded in the lipid bilayer, has been demonstrated to function as a ligand for concanavalin A (Wassef et al., 1985) as well as being accessible to anti-phosphatidylinositol antibodies (Loirand et al., 1992). The strength of interaction of SP-D with the lipids depends on the multiple interactions between the multimeric protein and the liposome as the monovalent interaction is probably too weak as reflected in the I_{50} of glycerophosphoinositol. Attempts to quantify the dissociation constant of SP-D and liposomes containing phosphatidylinositol have been complicated by the ability of the protein to agglutinate the liposomes and to form self-aggregates under similar conditions.

In contrast with SP-D, SP-A appears to have multiple modes by which it could aggregate liposomes. Delipidated SP-A appears to bind specifically to phosphatidylcholine and sphingomyelin when these lipids are adsorbed to a surface (Kuroki & Akino, 1991) in a manner strongly dependent on the nonpolar region and partially dependent on calcium. The association of SP-A with dipalmitoylphosphatidylcholine has been shown to be enhanced by the presence of phosphatidylglycerol and calcium (King & Macbeth, 1981). More recently, SP-A has been shown to recognize several ceramides containing terminal galactosyl residues (Childs et al., 1992). Moreover, SP-A has been shown to be dependent on the presence of its own N-linked sugars to allow the aggregation of liposomes (Haagsman et al., 1991), suggesting that the self-aggregation of SP-A, and perhaps the formation of tubular myelin, is at least in part due to the recognition of its own N-linked glycosides.

While data have accumulated to support involvement of SP-A (Manz-Keinke et al., 1991; Tenner et al., 1989; van Iwaarden et al., 1990, 1991) and SP-D (Kuan et al., 1992) in host defenses, there also exists evidence to support a role for SP-A in the structure and function of surfactant (Cockshutt et al., 1990). The present demonstration of the interaction between SP-D and phosphatidylinositol, freely reversible with competing saccharides and dependent on the presence of calcium, leads to speculation on a function other than host defense. These observations suggest that SP-D is capable of binding to the monolayer's aqueous face, cross-linking monolayer to bilayer, bilayer to bilayer, and perhaps bilayer to cell surface, and therefore SP-D may play a role in the structure and distribution of surfactant in the hypophase. SP-A and SP-D appear to recognize different subsets of lipids and may play roles in the linking or segregation of lipid subpopulations.

While the contribution of dipalmitoylphosphatidylcholine to the surface tension lowering properties of pulmonary surfactant has been well documented, the contribution of the lipids in smaller concentrations is also important. The proportion of phosphatidylglycerol in surfactant varies between species, and phosphatidylinositol becomes the second most abundant lipid in those species with small amounts of phosphatidylglycerol (Egberts et al., 1987; Hallman & Gluck, 1976; Lau & Keough, 1981; Shelley et al., 1984). In the presence of excess dietary inositol, phosphatidylinositol replaces phosphatidylglycerol in surfactant (Beppu et al., 1983; Hallman et al., 1985), and the isolated surfactant is fully functional in vitro. Fagan and Keough (1988) have shown that a model of pulmonary surfactant consisting entirely of dipalmitoylphosphatidylcholine and unsaturated phosphatidylinositol reached minimum surface tensions approaching 0 mN/m when spread as a monolayer on a surface tension balance. Taken together, the results suggest that phosphatidylinositol may substitute for phosphatidylglycerol without affecting the surface properties of surfactant. Maturation of the fetal lung can be accelerated in part by exogenous inositol; however, human lung maturity appears best measured by the amounts of phosphatidylglycerol and not dependent on absolute amounts of phosphatidylinositol (Hallman et al., 1990). Whether phosphatidylinositol plays a role distinct from phosphatidylglycerol and distinct from its involvement in the generation of the second messengers, diacylglycerol and inositolpolyphosphates, remains an open question. The role which the interaction of SP-D and phosphatidylinositol plays is similarly unanswered. Therefore, studies are in progress to examine the variables influencing the association of SP-D

with phosphatidylinositol and the distribution and metabolism of SP-D in normal and diseased states.

ADDED IN PROOF

Ogasawara et al. (1992) have recently described similar findings employing solid-phase assays of SP-D binding to adsorbed lipids.

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Registry No. Calcium, 7440-70-2; maltose, 69-79-4.