Phospholipid Binding and Biophysical Activity of Pulmonary Surfactant-associated Protein (SAP)-35 and Its Non-collagenous COOH-terminal Domains*

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Surfactant-associated protein of $M_r = 35,000$, SAP-35, is the major glycoprotein present in mammalian pulmonary surfactants. In this study, canine SAP-35 and several of its COOH-terminal peptides were purified and characterized by amino acid composition and NH$_2$-terminal sequencing analysis. These proteins were then studied in terms of their specific lipid-binding characteristics and surface activity when combined with a synthetic phospholipid mixture, SM, chosen as an approximation of lung surfactant phospholipids. Purified, delipidated SAP-35 bound SM strongly. In contrast, SAP-21 (a non-collagenous fragment generated by collagenase digestion) bound phospholipid weakly; SAP-18 (an acidic COOH-terminal fragment comprising residues Gly-118 to Phe-231) did not bind phospholipid, demonstrating the importance of hydrophobic amino acid residues Cly-81 to Phe-231 to Val-117 and the NH$_2$-terminal collagenous domain in interaction of the SAP-35 with phospholipids.

In surface activity experiments, purified SAP-35 enhanced the adsorption of SM phospholipids in terms of both rate and overall surface tension lowering. However, the adsorption facility of the SM-SAP-35 mixture did not approach that of either whole surfactant or the surfactant extract preparations, calf lung surfactant extract or surfactant-TA, used in exogenous surfactant replacement therapy for the neonatal respiratory distress syndrome. In addition, the dynamic surface activity of the SM-SAP-35 mixture was well below that of natural surfactant or surfactant extracts. This was also true of mixtures of SM phospholipids combined with the SAP-18 and SAP-21 fragments of SAP-35.

Surfactant-associated glycoprotein of $M_r = 30,000$–40,000 (herein denoted surfactant-associated protein SAP-35$^*$) is the major pulmonary surfactant-associated protein and has been detected in numerous mammalian surfactants (1–10). However, the existence of other surfactant apoproteins has also been reported in a variety of studies (1–10), and it is now clear that smaller surfactant-associated proteins of $M_r = 6,000$, which are clearly not fragments of SAP-35, are also prominent in mammalian pulmonary surfactant extracts (11). The various roles played in lung surfactant activity by SAP-35 and other surfactant-associated proteins are currently a subject of much debate and interest. For example, a number of studies have demonstrated that lipid-rich extracts of natural lung surfactant (such as calf lung surfactant extract, CLSE or calf lung lipid (12-16) and surfactant-TA (17–19)) have equivalent biophysical or physiological activity to whole surfactant. However, recent evidence suggests that such preparations lack SAP-35 and are enriched in other surfactant-associated proteins which may confer surface activity to the mixtures (11, 20–23).

SAP-35, first described by King and Clements (1), is now known to be synthesized primarily by pulmonary Type II epithelial cells, and in the rat and dog, is derived from a primary translation product of $M_r = 26,000$ which is highly glycosylated by the addition of asparagine-linked complex carbohydrate (9, 24). The protein has been referred to as apolipoprotein A, glycoprotein A, pulmonary surfactant apolipoprotein, and SAP-35 by various investigators. The entire amino acid sequence of this protein has been derived from cDNA sequence and has been recently reported for both canine and human SAP-35 (25, 26). Sulphydryl-dependent dimerization of the various glycosylated and unglycosylated forms present in canine and rat surfactant occur in the N-terminal region of the molecule and the sulphydryl-dependent interchain cross-linking is lost by treatment of the molecule with bacterial collagenase selective for the Gly-X-Y amino acid repeats in the collagen-like region of the molecule (8, 27). In the presence of calcium, SAP-35 binds to phospholipids forming vesicles and aggregates with some of the features of natural surfactant (28, 29).

In the present study, we have carried out specific studies of the binding of SAP-35, and several well-defined segments of SAP-35, with a synthetic phospholipid mixture containing an acyl chain distribution and headgroup constituents (zwitterionic, anionic) related to lung surfactant phospholipids. These lipid-binding studies are then complemented with direct determinations of the surface activity in vitro of the lipid-apoprotein recombinants in terms of their ability to adsorb to the air-water interface and to lower surface tension under dynamic compression in an oscillating bubble at physiologically relevant temperature, humidity, and cycling rate. In addition to addressing potential contributions of SAP-35 to
Characterization of Pulmonary SAP-35 COOH-terminal Domains

Surfactants for Binding or Surface Activity Studies—A number of synthetic phospholipids and other surfactants were used in this study in addition to the surfactant-associated protein SAP-35 and its various molecular segments described below. Synthetic phospholipids were purchased from Sigma and were used without further purification after verification of content by thin layer chromatography using the system of Touchstone et al. (30). For binding and surface activity studies of synthetic phospholipids and surfactant-associated proteins, a specific phospholipid mixture was used 65% dipalmitoyl phosphatidylcholine, 20% egg phosphatidylcholine, 7.5% egg phosphatidylglycerol, and 7.5% soyphosphatidylglynositol. This synthetic phospholipid mixture (SM) was chosen because its fatty acyl chain composition is relatively reflective of that in lung surfactant phospholipids, and the major zwitterionic and anionic headgroups are also closely relevant for natural surfactant.

For experiments involving surfactants representing highly active surfactants, both whole lung surfactant (LS) and organic solvent extracts of lung surfactant (CLSE and surfactant-TA) were used. Natural surfactant was obtained by bronchoalveolar lavage of intact calf lungs followed by centrifugation. For binding and surface activity studies of synthetic phospholipids and surfactant-associated proteins, a specific phospholipid mixture was used 65% dipalmitoyl phosphatidylcholine, 20% egg phosphatidylcholine, 7.5% egg phosphatidylglycerol, and 7.5% soyphosphatidylglycositol. This synthetic phospholipid mixture (SM) was chosen because its fatty acyl chain composition is relatively reflective of that in lung surfactant phospholipids, and the major zwitterionic and anionic headgroups are also closely relevant for natural surfactant.

For experiments involving surfactants representing highly active surfactants, both whole lung surfactant (LS) and organic solvent extracts of lung surfactant (CLSE and surfactant-TA) were used. Natural surfactant was obtained by bronchoalveolar lavage of intact calf lungs followed by centrifugation, as detailed by Notter et al. (13). CLSE was obtained from this whole calf LS by chloroform/methanol extraction following the method of Bligh and Dyer (31). The composition of natural LS and CLSE obtained by these methods has previously been reported (13–16, 32). Surfactant-TA, which is an organic solvent extract of minced bovine lungs supplemented with dipalmitoyl phosphatidylcholine and palmitic acid, was provided by Abbott with a composition similar to that reported by Vidyasagar et al. (19). Both CLSE and surfactant-TA have been shown to be biophysically and physiologically active lung surfactants (13–16, 18, 19), and have been used successfully to treat premature infants with the neonatal respiratory distress syndrome (17, 33, 34). Delipidation and Initial Purification of SAP-35—Mongrel dogs were sacrificed by pentobarbital KCl injection. The lungs were rapidly removed and placed on ice. The trachea was cannulated and the lung infused with 0.9% NaCl, 1 mM phenylmethylsulfonyl fluoride. Surfactant from lavage of intact dog lung was collected and placed on ice. Samples containing visible blood were not utilized. Lung lavage was then centrifuged twice at 1000 × g for 5 min at 4°C to remove cellular debris. Supernatant was dialyzed against three 400-ml changes of 0.1 M sodium phosphate, pH 7.8, 0.5% Nonidet P-40. Endoglycosidase F from *Clostridium histolyticum* (Advanced BioTechnologies Corp., Lynbrook, NY) was added (50–100 units/ml) and digestion carried out at 37°C for 16–20 h. The resulting SAP-21 was isolated from the supernatant by precipitation by dialysis against 50% saturation with H2O using an Amicon filter or by HPLC gel filtration using a Du Pont GF-250 column (27).

Endoglycosidase F Treatment of SAP-18—In order to better estimate the polypeptide molecular weight of SAP-18, the peptide was digested as described above, using (0.2 mg/ml) endoglycosidase F (Diagnostic Products Corp.) and sodium deoxycholate, EDTA, and 0.1% Nonidet P-40. Endoglycosidase F from New England Nuclear was added at a final concentration of 2 units/ml and the sample incubated at 37°C. HPLC Separation of Tryptic Fragments—In order to compare their polypeptide structural similarities, purified SAP-35 and SAP-18 were reduced and carboxymethylated and digested with trypsin-TPCK (Cooper Biomedical, Malvern, PA). Tryptic fragments were separated using an Ultrapore analytical C5 column (4.6 mm × 75 mm, Beckman Instruments) as previously described (27). Details of the elution procedure are provided in the legend to Fig. 3 later under "Results." Enzyme-linked Immunosorbent Assay (ELISA) of Canine SAP-35—Monospecific antisera were prepared against canine SAP-35 in both goats and rabbits by standard immunization protocols using Freund's incomplete adjuvant. Resultant antiserum was fractionated with ammonium sulfate to obtain immunoglobulin for use in a capture ELISA assay. ELISA assays followed the general procedure described by Katyal and Singh (42). Plastic plates were coated with goat anti-SAP-35 (1:100) in 0.9% NaCl, 50 mM Tris-HCl, pH 7.4, overnight at 4°C. Unknown samples were added in the same buffer containing 0.5% Nonidet P-40 and incubated for 2 h at 37°C. Rabbit anti-SAP-35, 1:200 followed by specific goat anti-rabbit IgG conjugated to horseradish peroxidase (Behring Diagnostics). Color development was assessed by absorbance after addition of 0.15% hydrogen peroxide, 0.01 M o-phenylenediamine as the substrate. Both antiserum were reactive against glycosylated and non-glycosylated SAP-35 forms,
detecting only SAP-35 in immunoblots of whole canine surfactant, and were unreactive to canine serum proteins. Both antisera detected only the $M_t = 26,000$ translation products after in vitro translation of adult canine lung poly(A)$^+$ mRNA (9). Standard curves were constructed with purified SAP-35. Protein was assessed by the method of Lowry et al. (40) using bovine serum albumin as the standard.

**Immunoblot Assay**—Surfactant proteins were separated by one- or two-dimensional SDS-gel electrophoresis and transferred electrophoretically to nitrocellulose. The nitrocellulose sheet was treated with rabbit anti-SAP-35 antisera followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. The blocking buffer described by Johnson et al. (44) was included in the incubation to reduce nonspecific background. Color was developed as described by Towbin et al. (45) except that 4-chloro-naphthol was used as a substrate.

**Amido Acid Composition Analysis**—Details of amino acid analytical procedures for surfactant-associated proteins are given by Ross et al. (27). Purified SAP-35 samples and the various peptides above were hydrolyzed in 300 μl of 5.7 N HCl, 0.01% phenol, and 0.1% β-mercaptoethanol at 110 °C under vacuum. Free amino acids were resolved using a Beckman 6300 amino acid analyzer. Automated Edman degradation was performed using an Applied-Biosystems model 470A protein sequencer.

**Phospholipid Binding Studies**—The binding of SAP-35, and specific molecular segments of SAP-35, to phospholipids was assessed using both the ELISA assay and silver-stain analysis. The phospholipids studied were the SM described earlier. Phospholipid vesicles were prepared by sonication at 45 °C of SM phospholipids, dried to a thin film under nitrogen, into a buffer composed of 20 mM Tris-HCl, pH 7.4, 0.1 M NaCl, and 5 mM CaCl$_2$. Purified SAP-35 or its SAP-21 and SAP-18 fragments were then added to the phospholipids. The dispersion was incubated for 2 h at 37 °C with rotation, and bound protein was then separated from soluble protein by centrifugation at 10,000 × g for 15 min. Recovery of $[^{14}C]$phosphatidylcholine, added to assess the recovery of lipid in the presence of each peptide, was always greater than 99% in the 10,000 × g pellet. SAP-35 binding was assessed using two-antibody ELISA and SDS-PAGE analysis of the resultant lipid pellet and aqueous fractions. Phospholipid binding of SAP-18 and SAP-21 was analyzed directly by immunoblot analysis after SDS-PAGE, since the capture ELISA did not detect the COOH-terminal fragments.

**Interfacial Biophysical Methods**—Biophysical activity of lipid-protein mixtures was evaluated by measurements of adsorption facility in the absence of diffusion resistance (12-14) and dynamic surface tension-lowering ability on an oscillating bubble apparatus (46). The physical activity of surfactants on the oscillating bubble apparatus has been found to correlate well with their physiologic efficacy in lungs (18). Details of oscillating-bubble methods applied to pulmonary surfactant and phospholipid-containing systems have been given previously (14, 16, 32).

In the biophysical experiments here, bubble measurements were made on the same lipid-apoprotein dispersions studied above for adsorption facility. As standards representing highly active surfactants for comparative purposes, additional adsorption and oscillating-bubble experiments also investigated the surface activity of natural LS, and the organic solvent surfactant extracts CLSE and surfactant-TA. For these experiments, CLSE was dispersed by vortexing as above for the lipid-apoprotein mixtures. Surfactant-TA was dispersed by sonication (Heat Systems sonicator, model W-220F, microtip, 35-watts), and natural surfactant was simply resuspended in the standard calcium-supplemented saline solution used in all interfacial biophysical experiments. For all biophysical experiments, phospholipid concentration was defined by phosphate determinations using the method of Chen et al. (48).

**RESULTS**

**Purification and Characterization of SAP-35 and an Acidic Peptide SAP-18**—Purification of canine SAP-35 from ethanol/ether delipidated canine surfactant was accomplished primarily using preparative isoelectric focusing. Silver-stain analysis of the fractions from the isoelectric-focusing column are shown in Fig. 1A. Acidic protein migrating with $M_t = 32,000-38,000$ (pI 4.5-5.0) is the major form of the SAP-35 molecule, although smaller amounts of unglycosylated polypeptide are noted at $M_t = 26,000$. Further purification of these SAP-35 forms was achieved by Cibacron Cl-6B chromatography as previously reported (27). SAP-35 utilized in experiments described in the present work, therefore, consists of a

![Fig. 1. A, SDS-PAGE silver-stain analysis of preparative IEF separation of canine surfactant. Organic solvent-insoluble surfactant proteins were fractionated by the preparative IEF procedure (see "Materials and Methods"). Fifty-microliter aliquots of selected fractions (ranging from pH 3.4 to 6.7) were subjected to SDS-PAGE and silver stained. The left lane represents starting material. SAP-35 forms have $M_t = 38,000, 32,000$ and 26,000. The $M_t = 18,000$ band is designated SAP-18 and is significantly more acidic. B, immunoblot of preparative IEF separation of canine surfactant proteins. Surfactant proteins were separated by preparative IEF and then SDS-PAGE as in Fig. 1A. The proteins were transferred to nitrocellulose and identified using monospecific rabbit anti-canine SAP-35 as described under "Materials and Methods." SAP-35 forms are detected in the pH range of 4.5-5.2. SAP-18 has an isoelectric point estimated to be 3.8.
mixture of glycosylated and unglycosylated proteins which copurify (27). A second acidic protein migrating with pI 3.5–3.8, $M_r$ approximately 18,000, was also detected and is displayed in Fig. 1A. The most acidic surfactant-associated protein fractions (pI < 3.8) provided an essentially homogeneous preparation of this polypeptide (designated SAP-18) for further analysis.

Immuno blot analysis of the various SAP-35 forms isolated in a second set of isoelectric-focusing fractions is shown in Fig. 1B. Monospecific rabbit anti-dog SAP-35 antiserum re-acted primarily with the $M_r$ = 26,000, 32,000, and 38,000 forms of the protein. However, the acidic peptide of $M_r$ = 18,000 (SAP-18) was also immunoreactive, consistent with it being a fragment of SAP-35,000. Treatment of this acidic peptide with endoglycosidase F demonstrated the presence of asparagine-linked carbohydrate and a polypeptide molecular weight of approximately 14,000 (Fig. 2).

Comparison of the structural features of SAP-35 and SAP-18 was achieved by analysis of tryptic fragments contained within each polypeptide. The SAP-18 elution pattern (Fig. 3) shares identical tryptic peaks with the SAP-35 profile previously reported by our laboratory (27) again suggesting that the SAP-18 polypeptide is a subset of the SAP-35 structure. Several tryptic SAP-35 peaks, which are absent from the SAP-18 profile, contain collagen-like amino acid sequences with high glycine content (27). The resistance of SAP-18 to bacterial collagenase (data not shown) also indicates that the SAP-18 peptide represents a distinct non-collagenous domain of the SAP-35 molecule. The amino acid composition of SAP-35 and the acidic fragment (SAP-18) are listed in Table I. The SAP-18 composition has a lower percentage of glycine residues than does SAP-35, consistent with it being a non-collagenous peptide. Amino-terminal sequencing of SAP-18 yielded overlapping sequences (Val)–Gly–Arg–Lys–Val–Phe–Ser–Ser–Asn–Gly–Gln–Ser–Ile corresponding to residues Val-117 or Gly-118 to Ile-129 in the canine SAP-357000 sequence reported by Benson et al. (25). The sequence beginning with

![Fig. 2. Endoglycosidase F digestion of canine SAP-18.](image) SAP-18 peptide was incubated at 0.2 mg/ml in 50 µl of sodium phosphate, pH 6.1, 0.1% Nonidet P-40, 10 mM disodium EDTA and incubated in the absence (a) or presence (b) of 2 units/ml endoglycosidase F, 37 °C, 16 h. The bands shown represent 10 µg of the peptide run on an SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with rabbit anti-canine SAP-35 serum.
Val-117 accounted for 35–40% of the amino acid yield in each cycle.

In summary, composition and sequence data indicate that SAP-18 represents a non-collagenous fragment of SAP-35, starting with the glycine residue at position 118 and extending to the COOH terminus. It contains 1 asparagine-linked oligosaccharide likely at position Asn-190 and is significantly acidic in nature (pI < 3.8).

**Production and Analysis of a Collagenase-resistant Fragment SAP-21**—We have recently reported an initial characterization of the major non-collagenous domain of SAP-35 (27). This major segment, SAP-21, is a collagenase-resistant fragment of SAP-35 and incorporates the SAP-18 fragment above. Specifically, following treatment of SAP-35 with bacterial collagenase, a large resistant fragment of \( M, = 21,000, \) containing asparagine-linked carbohydrate and having an apparent polypeptide molecular weight of 16,000 is produced, herein termed SAP-21. The amino acid composition of this collagenase-resistant peptide, isolated by HPLC gel filtration using a Du Pont GF-250 column, is also listed in Table I. Collagenase digestion of the canine SAP-35 sequence reported by Benson et al. (25) should produce a non-collagenous fragment extending from glycine at amino acid residue 81 to the COOH terminus. The major NH2-terminal amino acid sequence of this collagenase-treated SAP-35 begins Gly(81)-Leu-Pro-Ala with a minor sequence beginning Gly(63)-Val-Ala-Gly-Glu, corresponding to the canine SAP-35 sequence of Benson et al. (25). The amino acid composition reported in Table I for this collagenase-resistant fragment closely matches the corresponding composition predicted by the SAP-35 sequence. It can be inferred, therefore, that the collagenase-resistant fragment, SAP-21, contains within its structure the acidic SAP-18 sequence.

**Phospholipid Binding**—As one measure of the interactions of SAP-35 in lung surfactant, specific studies were carried out on the binding of this surfactant-associated protein and its specific fragments (SAP-18 and SAP-21) with a complex mixture of synthetic phospholipids reflective of those in natural surfactant. The results of the lipid-binding studies are shown in Figs. 4 and 5. The overall protein, SAP-35, had a clear affinity for the SM dispersed by sonication into vesicles. SAP-35 was precipitated with lipid from aqueous solution by centrifugation (see “Materials and Methods”). SAP-35 alone did not pellet at 10,000 \( \times g \) in the absence of lipids. Binding of SAP-35 to synthetic phospholipid vesicles was rapid (complete at 1 min) at 37 °C and apparently nonsaturable up to 1 mol of protein/300 mol of phospholipid (Fig. 4). Results using silver staining after SDS-PAGE (Fig. 4A) or the ELISA assay (Fig. 4B) to determine binding were comparable. The SAP-21 fragment, however, bound phospholipid weakly, while the SAP-18 fragment failed to bind and was completely recovered in the supernatant of the reconstitution assay (Fig. 5). This latter finding is of particular interest since, as shown below, SAP-18 had equivalent effects to SAP-35 in enhancing the adsorption of SM phospholipids.

**Biophysical Surface Activity Results**—Adsorption results for SAP-35 and its fragments combined with SM phospholipids are shown in Table II and Fig. 6. The data indicate that mixtures of synthetic phospholipids with SAP-35 and its fragments do not exhibit adsorption facility comparable to whole LS or to the surfactant extracts CLSE and surfactant-TA. The maximum adsorption surface pressure reached by the SAP-35-phospholipid mixture in Table II was 18 dynes/cm, less than half of the 46–47 dynes/cm equilibrium spread-

Fig. 4. A, silver-stain analysis of SAP-35 binding to phospholipid. Varying amounts of SAP-35 were incubated with the SM (1 mg/ml) at 37 °C. Lipid-protein pellets were obtained by centrifugation, washed once with buffer (20 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 5 mM CaCl2) and finally resuspended into 150 \( \mu l \) of SDS-PAGE sample. Twenty-five-microliter aliquots were electrophoresed through a 13% acrylamide gel and silver stained. The lipid to protein molar ratios during the incubation ranged from 10\(^{-1}\) to 1 (lane a) to 3.3 \( \times 10^{5} \) to 1 (lane 1). B, ELISA assay of SAP-35 binding to phospholipid. SAP-35 was incubated at 37 °C with the synthetic phospholipid mixture SM (1 mg/ml). The lipids were separated from soluble protein by centrifugation. SAP-35 was assessed in both the supernatants (A) and pellets (B) by a two-antibody capture ELISA assay, described under “Materials and Methods.” As in A, the lipid to protein molar ratios ranged from 10\(^{-1}\) to 1 to 3.3 \( \times 10^{5} \) to 1.

Although the adsorption of SM lipids combined with SAP-35 did not approach that of natural surfactant, it was significantly better than that of the synthetic lipids alone for the dispersion methodology (vortexing at room temperature) used. In Table II, case 1, the adsorption of SM phospholipids alone was very low, and this was also true if ovalbumin was added as a nonspecific protein in case 2. By comparison, the adsorption data in Table II and Fig. 6 are for synthetic phospholipids at a fixed concentration (0.063 mg/ml) combined by mechanical vortexing with completely delipidated SAP-35 (and its fragments) in a final lipid/protein ratio of 99:1–98:2. It is possible that other combination methodology, or the addition of more protein or higher lipid concentrations, might improve the surface activity shown for these synthetic lipid/SAP mixtures. However, both CLSE and surfactant-TA contain equivalent amounts of protein to the SAP/lipid mixtures and are studied here at the same low lipid concentration, while exhibiting markedly greater surface activity.
led to the protein with the SM described under "Materials and Methods." The lipid/protein molar ratios in each mixture were SAP-35 (400:1); SAP-21 (1200:1); and SAP-18 (600:1). The various lanes in the figure show: Lane a and b, SAP-35, supernatant and pellet; Lane c and d, SAP-21, supernatant and pellet; Lane e and f, SAP-18, supernatant and pellet.

**TABLE II**

| Surfactant mixture | Surface pressure $\pi$ (dynes/cm) ^a |
|--------------------|----------------------------------|
|                    | 0 min  | 5 min  | 10 min | 15 min | 20 min |
| 1. SM              | <1     | 1      | 1      | 3      | 3      |
| 2. SM + ovalbumin  | 1      | 2      | 4      | 5      | 5      |
| 3. SM + SAP-35     | 9      | 13     | 14     | 15     | 18     |
| 4. SM + SAP-18     | 8      | 16     | 17     | 17     | 18     |
| 5. SM + SAP-21     | 2      | 4      | 6      | 7      | 9      |
| 6. CLSE            | 20     | 46     | 46     | 47     | 47     |
| 7. Surfactant-TA   | 31     | 43     | 44     | 44     | 44     |
| 8. Natural LS      | 17     | 45     | 46     | 47     | 47     |

^a Surface pressure $\pi$ is the amount of surface tension lowering below that of the pure subphase. The value at 0 min is that measured within 10 s after addition of a bolus of surfactant dispersion to the stirred subphase at time zero. Values given are the means of 4–10 experiments with S.E. always $\leq$ 3 dynes/cm, except for SAP-18 data which are the mean of two experiments with a deviation of $\pm$ 0.5 dynes/cm about the mean.

Delipidated proteins studied are SAP-35, SAP-21, and SAP-18 (see "Materials and Methods"). These are combined with SM composed of dipalmitoyl phosphatidylcholine/egg-phosphatidylcholine/egg-phosphatidylglycerol/soy-phosphatidylinositol 65:20:7:5:5. The surfactant extracts CLSE and surfactant-TA, along with natural LS, represent optimally active surfactants; ovalbumin is used as a non-specific protein control. Phospholipid concentration for all adsorption experiments was 0.063 mg/ml 0.15 M NaCl solution. Temperature = 35 ± 2 °C. For cases 2–5, the lipid/protein ratio was (wt/wt) 99:1–98:2. For comparison, the protein content of CLSE is 1% (12–16) while that of surfactant-TA is 1–2% (18–19). All mixtures dispersed by vortexing at room temperature except surfactant-TA (sonicated) and natural LS (resuspended).

adsorption was significantly enhanced by SAP-35 or its purified fragments SAP-18 and SAP-21. There is even a rapid rise segment present in some of the representative $\pi$–$\tau$ adsorption isotherms shown in Fig. 6. However, this enhancement of adsorption did not correlate with the phospholipid-binding studies. For example, as alluded to above, SAP-18 failed to bind phospholipid (Fig. 5), but its ability to enhance adsorption was equivalent to SAP-35 which was completely associated with phospholipid.
an oscillating bubble (see "Materials and Methods"). At concentrations as low as 0.5 mg of phospholipid/ml of subphase, physiologically active natural surfactant, or CLSE, can lower surface tension to minimum values <1 dynes/cm on dynamic cycling (32); explicit data for such surfactants are given in cases 6-8 in Table III at a dispersion concentration of 1 mg/ml. By contrast, mixtures of phospholipids and SAP-35 did not lower surface tension on the oscillating bubble below 21 dynes/cm at equivalent (or higher) concentrations, for the experimental conditions used here (20 cycles/min compression rate with a 50% area change at 37 °C).

**DISCUSSION**

SAP-35 is the major surfactant-associated protein in the mammalian alveoli. Significant evidence supports the hypothesis that specific protein components contribute to the biophysical and physiological activity of mammalian surfactants (29). Understanding the molecular mechanisms involved in surfactant protein and phospholipid interactions necessitates stringent characterization of these protein structures. In the present work, we have isolated COOH-terminal fragments of purified canine SAP-35 and measured the binding and biophysical activity of phospholipid mixtures containing SAP-35 and these specific SAP-35 fragments.

**Characteristics of SAP-35 and Its Fragments**—SAP-35 is heterogeneous with respect to both size and charge in various species (3-10, 24, 27). This is due to the presence of sialylated, asparagine-linked carbohydrate chains covalently bound to a primary translation product of $M_r = 26,000-28,000$ (8, 9, 27). Larger forms present in lung lavage arise from sulfhydryl-dependent oligomerization of SAP-35 molecules (8, 27). In the present work, an acidic polypeptide (pI < 4.0, $M_r = 16,000-18,000$), which shares antigenic sites with SAP-35 by virtue of reacting with rabbit anti-SAP-35 antisera, was also detected in canine surfactant. This acidic polypeptide, SAP-18, contains a single N-linked carbohydrate chain and an apparent molecular mass of 14,000 daltons. Amino acid composition and sequence analysis identify this peptide to be a fragment of SAP-35, extending from residues Val-117 or Gly-118 to the COOH terminus. Previous work by King et al. (2, 23, 25) demonstrated the presence of a small ($M_r = 10,000-12,000$) surfactant protein (termed surfactant apolipoprotein B) which shared immunological properties with SAP-35. It was suggested that "apolipoprotein B" might represent a metabolic product of SAP-35 (49, 50). The data presented here demonstrate that SAP-18 is a naturally occurring fragment of SAP-35, and its amino acid composition in Table I closely matches that reported earlier for apolipoprotein B (2).  

The precise location of the acidic subfragment SAP-18 was determined by comparison of the amino terminal sequence with the pulmonary surfactant apolipoprotein (SAP-35) cDNA clone sequence reported by Benson et al. (25). The overlapping sequences provided here begin either with residue Val-117 or Gly-118. These two sequences could arise from proteolytic action specific for the COOH-terminal side of the valine residues at either position Val-116 or Val-117 in the canine SAP-35 sequence. The exact origin of this fragment, as an in vivo SAP-35 metabolic product or as an artifact of our purification procedure, has not yet been established. Fig. 7 presents a model showing the linear arrangement of structural domains of SAP-35 identified by sequence analysis of the SAP-35 fragments in relation to the canine SAP-35 cDNA sequence reported by Benson et al. (25). The non-collagenous domain (Gly-81 to Phe-231) produced by bacterial collagenase treatment (SAP-21) and the acidic (Gly-118 to Phe-231) COOH-terminal fragment (SAP-18) isolated from whole surfactant are presented also. The two asparagine-linked glycosylation sites are noted, as is the cysteine residue implicated in intermolecular disulfide bonding (27) and the hydrophobic region of the SAP-35 sequence most probably involved in phospholipid binding, residues Gly-81 to Val-117.

**Discussion of Lipid Binding Studies**—King et al. (29, 51) have demonstrated that reassembly of surfactant apoprotein (apolipoprotein A or SAP-35) with synthetic phospholipid vesicles does occur. They reported that SAP-35 associated with phospholipid in a nonsaturable manner over a phospholipid/protein molar ratio range of 320:1 to 8000:1. In our studies, intact SAP-35 bound to synthetic phospholipid dispersions in aqueous solution in an apparently similar nonsaturable manner (Fig. 4). However, qualitative SDS-PAGE analysis showed that SAP-18 (Gly-118 to Phe-231), the SAP-35 COOH-terminal domain, did not associate with phospholipid (Fig. 5). This result was anticipated by the lack of the strongly hydrophobic domain in the SAP-18 primary sequence. The SAP-35 collagenase-resistant domain (SAP-21), extending from residue Gly-81 to Phe-231, associated weakly with phospholipid (Fig. 5).

**Amino Acid Residues**

**Fig. 7. Structural model of SAP-35 monomer and SAP-35 fragments.** Identified domains of SAP-35 and its fragments are shown schematically. The position of COOH-terminal SAP-21 and SAP-18 fragments is represented in relation to the amino sequence reported by Benson et al. (25), as noted in the text. Identification of disulfide cross-links at Cys-9 was previously reported (27).
tripholipids. The polar and nonpolar faces of a SAP-35 amphipathic helix could associate with phospholipid head groups and fatty acyl chains, respectively. The adjacent hydrophobic region Leu-102 to Val-117 might then traverse the lipid bilayer, thereby anchoring SAP-35 in the membrane. Contiguous nonpolar amino acid sequences have been demonstrated to anchor immunoglobulin heavy chains (55) and the vesicular stomatitis virus G protein (56) in cellular membranes. Furthermore, basic amino acids have been reported to mark the boundary of transmembrane sequences (56–57). Canine SAP-35 contains residues Lys-119 and Arg-120 immediately following the hydrophobic Leu-102 to Val-117 domain. If the hydrophobic region of SAP-35 (through Val-117) is buried within the lipid bilayer, then the generation of the COOH-terminal Gly-118 to Phe-231 fragment may occur through proteolysis of the molecule at the point where it extends beyond the lipid surface. In any case, the finding that SAP-21 (which in contrast to SAP-18 contains the putative NH2-terminal region of SAP-35.

Discussion of Interfacial Biophysical Results—The biophysical activity of SAP-35 and its COOH-terminal fragments was assessed in adsorption studies and in studies using an oscillating bubble. King and MacBeth (51) have reported weak biophysical effects of purified SAP-35 on lipids by monitoring SAP-35 association with dipalmitoyl phosphatidylcholine vesicles. Hawgood et al. (28) have also shown that SAP-35 reduces surface tension in adsorption experiments when added to lung surfactant lipid extracts, but interpretations are complicated because such lipid extracts contain other distinct apoprotein components (11, 22, 23). The present experiments demonstrate relatively low adsorption facility (compared to natural lung surfactant) for mixtures of synthetic phospholipids with SAP-35 and its fragments. Moreover, the results show that SAP-35, its collagenase-resistant COOH-terminal fragment (SAP-21), and the Gly-118 to Phe-231 fragment (SAP-18) induce comparable adsorption when added to a synthetic phospholipid mixture, even though only SAP-35 binds avidly to phospholipid. SAP-18, which binds lipid to a negligible extent, showed equivalent adsorption facility to SAP-35 when combined with SM phospholipids. In addition, the magnitude of the observed adsorption, while significantly greater than that of the nonspecific protein ovalbumin plus lipid, was not as large as found for natural surfactant or the surfactant extract preparations CLSE and surfactant-TA (12–18). Significant, these latter surfactant extracts lack SAP-35 or its fragments and contain only smaller hydrophobic protein(s), including SAP-6 and its oligomers as recently reported from our laboratory (11, 23). Finally, in oscillating bubble studies of dynamic surface activity, none of the mixtures of SM phospholipid plus SAP-35 and its fragments studied here exhibited dynamic surface tension-lowering ability approaching that of natural LS or surfactant extracts.

In summary, the biophysical data of the present study do not support the concept that SAP-35, combined with phospholipids, can provide the same optimal surface properties of whole LS or surfactant extracts used in exogenous surfactant replacement therapy for respiratory distress syndrome. This does not completely rule out the concept that SAP-35 or specific structural regions of the molecule may be useful in conferring surface active properties to phospholipids. In addition, combinations of SAP-35 with other distinct surfactant apoproteins such as SAP-6 (11, 23) may also prove to provide useful biophysical activity in mixtures with lipids. Nonetheless, it is significant that the dynamic surface activity of the phospholipid-SAP-35 mixtures studied here was much lower than that of CLSE and surfactant-TA, neither of which contain SAP-35. Moreover, the effects of SAP-35 on phospholipid adsorption, though reproducible, were apparently independent of the hydrophobic or lipid-binding domain of SAP-35. It remains possible that our isolation procedures have altered SAP-35 to give a falsely low assessment of its effects on lipid surface activity. However, as purified here, SAP-35 is completely soluble, binds phospholipids, confers specific calcium-dependent aggregation to SM phospholipids and inhibits phospholipid secretion from Type II cells. In addition, since SAP-35 is isolated in the absence of detergents, the observed phospholipid binding and biophysical activity should be specific for the SAP-35 structures studied.

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