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CP4: A Pneumocyte-Derived Collagenous Surfactant-Associated Protein. Evidence for Heterogeneity of Collagenous Surfactant Proteins†

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ABSTRACT: Type II pneumocytes secrete pulmonary surfactant and are known to synthesize SP-35, a collagenous surfactant-associated protein. Freshly isolated type II cells also synthesize other bacterial collagenase-sensitive and hydroxyproline-containing proteins, including a glycoprotein designated CP4. CP4 was isolated from rat pneumocyte culture medium by immune precipitation with polyclonal antibodies to rat surfactant proteins or by DEAE chromatography and reverse-phase or gel permeation HPLC. CP4 did not cross-react with polyclonal antibodies to SP-35 and was completely resolved from SP-35 by SDS-PAGE (M_r 43K reduced) or isoelectric focusing. Unlike SP-35, which consists of acidic isoforms assembled as disulfide-bonded dimers and multimers, CP4 was secreted as basic isoforms assembled as disulfide-bonded trimers. Differences in primary structure were demonstrated by CNBr and V8 protease peptide mapping. The secretion of both proteins was inhibited by 2,2'-dipyridyl, an inhibitor of posttranslational prolyl and lysyl hydroxylation and collagen triple helix formation. CP4 was isolated from EDTA extracts of rat surfactant. These studies provide evidence for the heterogeneity of pneumocyte-derived collagenous surfactant-associated proteins.

Pulmonary surfactant is comprised of specific phospholipids and several surfactant-associated proteins. The most abundant and best characterized of these proteins is designated SP-35 or SP-A. SP-35 has several distinctive structural features, including an amino-terminal collagenous domain, a central amphipathic domain believed to play a role in phospholipid binding, and a glycosylated carboxy-terminal domain that may mediate calcium-dependent carbohydrate binding (Benson et al., 1985; Floros et al., 1986b; Rannels et al., 1987; Sano et al., 1987; Ross et al., 1986). Surfactant also contains at least two different low molecular weight, noncollagenous, hydrophobic proteins (SP-18 and SP-5) (Claypool et al., 1984; Hawgood et al., 1987). There is evidence that these pneumocyte-derived proteins play important roles in modulating surface activity and in regulating the secretion and turnover of pulmonary surfactant (Dobbs et al., 1987; Hawgood et al., 1985, 1987; King et al., 1983; Wright et al., 1987).

We have observed that rat type II cells synthesize several collagenous components in primary culture. The major proline-labeled components secreted by adherent cells after 12-24 h in culture include type IV procollagen chains (CP1 and CP2) and a low molecular weight collagenous protein (CP3) that does not cross-react with polyclonal antibodies to rat surfactant proteins (Crouch et al., 1987a). We have recently identified two additional pneumocyte-derived collagenous proteins (CP4 and CP5). These proteins are secreted by type II cells during the first day of primary culture and are immunologically related to proteins in normal rat surfactant. CP5 corresponds to SP-35. However, CP4 is immunologically and structurally different from SP-35, the previously described hydrophobic surfactant-associated proteins, and other low molecular weight collagenous proteins.

Materials and Methods

Type II Cell Isolation. Rat type II cells were isolated by limited modifications of the method of Dobbs et al. (1986). Briefly, adult male Sprague-Dawley rats were anesthetized, heparinized, tracheostomized, and sacrificed by exsanguination as previously described (Crouch et al., 1987a). The lungs were perfused via the pulmonary artery with phosphate-buffered saline (PBS) to remove blood and lavaged repeatedly via the trachea with divalent cation-free HEPES-buffered saline to obtain crude surfactant and to remove macrophages. Porcine pancreatic elastase (Elastin Products, Inc., Pacific, MO; 30 orcein units/mL) was instilled into the airway in three aliquots, each to total lung capacity, for a total of 20 min at 37 °C. The lungs were minced in the presence of 250 μg/mL DNase I (Boehringer-Mannheim), and elastase was inactivated by the

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addition of fetal calf serum (FCS, Gibco). The macerated lungs were then incubated with agitation for several minutes at 37 °C, and the dissociated cells were separated from tissue fragments by filtration through nylon mesh. Residual macrophages were removed by incubating the cells on bacteriologic plastic plates coated with rat IgG (Sigma) for 3 h at 37 °C (Dobbs et al., 1986), and the nonattached cells were collected by centrifugation. Cell yields were approximately (53 ± 13) × 10^6 cells/lung with an average purity of approximately 90% as assessed by the tannic acid polychrome stain (Mason et al., 1985). Viability by trypan blue exclusion was >98%.

Metabolic Labeling. For metabolic labeling, cells were resuspended in serum-free preincubation medium, incubated for 30 min, and then labeled for up to 16 h with L-[2,3-^{3}H]-proline [New England Nuclear (NEN)]. At the end of the labeling period, the medium was harvested into inhibitors [0.2 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM *N*-ethylmaleimide (NEM), and 100 μg/mL ε-aminocaproic acid]. The cells were collected by centrifugation and lysed in immune precipitation buffer (see below). Culture medium supernatants and cell layer homogenates were temporarily stored at −85 °C prior to further processing.

Surfactant Isolation. Rat lavage obtained during the purification of type II cells (approximately 50 mL per rat) was subjected to centrifugation at 400*g* for 10 min to remove macrophages. The surfactant pellet was collected by centrifugation at 48000*g* for 1 h and stored at −85 °C. The majority of the contaminating albumin and other soluble proteins were extracted from the surfactant pellet by repeated washing with 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5 (TBS), containing 2 mM calcium chloride. SP-35 isoforms (32–38 kDa) and dimers were the major protein components visualized following silver staining of SDS gels of the washed surfactant. For some experiments, CP4 and a fraction of the SP-35 were solubilized from the pellet with 10 mM EDTA in TBS at 4 °C, extensively dialyzed versus 0.1 M acetic acid at 4 °C, and lyophilized.

SDS–PAGE and Fluorescence Autoradiography. Proteins were examined by SDS–PAGE on discontinuous methylenebis(acrylamide) slab gels (Crouch et al., 1987a). For fluorography, gel slabs were washed and fixed in 30% methanol–10% acetic acid, permeated with Enhance (NEN), dried, and exposed at −85 °C to sensitized Kodak XRP film. The relative recovery of radioactivity in proteins resolved by SDS–PAGE was determined by laser densitometry of appropriately exposed fluorograms. Unlabeled proteins were visualized by silver staining (Morrissey, 1981). Molecular weights were estimated relative to internal, unlabeled or ^{14}C-labeled, globular protein standards (Bethesda Research Laboratories).

Isoelectric Focusing. 2-D resolution of proteins by SDS–PAGE and equilibrium or nonequilibrium isoelectric focusing were performed with the Anderson Iso-Dalt system (Anderson & Anderson, 1977). Proteins were solubilized in 0.05 M 2-(cyclohexylamino)ethanesulfonic acid, pH 9.5, containing 2% (w/v) SDS, 1% (w/v) DTT, and 10% (v/v) glycerol. The isoelectric focusing gradient was established by using a 1:1 mixture of pH 3.5–10 ampholines (LKB) and pH 3.0–10.0 ampholines (Serva, Iso-Dalt grade). The proteins were focused at 80 V/cm over 16 h. SDS–PAGE was performed in the second dimension by using a 10% acrylamide slab gel. Molecular weights were estimated relative to internal globular standards.

Peptide Mapping. CNBr mapping of proteins in gel slices was performed as previously described (Barsh et al., 1981). Briefly, proteins were resolved by SDS–PAGE on 0.75 mm thick slab gels (first dimension). Gel strips containing individual lanes were incubated with CNBr (75 mg/mL) in 70% formic acid, equilibrated with sample buffer, horizontally positioned above a discontinuous slab gel (e.g., 5%/12.5–15%), and sealed in place with agarose ± DTT. CNBr peptides were resolved by SDS–PAGE in the second dimension.

Variations of this technique were also used to identify collagenase-resistant domains and V8 protease cleavage products. For bacterial collagenase digestion, each first dimension gel strip was incubated for 10 min at 37 °C with 25 mM calcium acetate and 25 mM Tris–acetate, pH 7.2, containing 0.1% Triton X-100, washed twice for 5 min in buffer without detergent, and transferred to a small siliconized glass tube with 200 μL of digestion buffer containing 800–1200 units of purified bacterial collagenase (Advance Biofactures), 0.2 mM PMSF, and 2 mM NEM. The samples were incubated for 2 h at 37 °C with agitation and equilibrated with sample buffer prior to second dimension SDS–PAGE. For V8 protease mapping, each first dimension gel strip was incubated 2 × 5 min with urea-free SDS–PAGE sample buffer, positioned above a second dimension slab gel, covered with 0.5 mL of urea-free buffer containing 10 μg/mL V8 protease (Sigma), and overlaid with urea-free running buffer. SDS–PAGE was performed in the second dimension for 16 h at a constant current of 4 mA, followed by 2–3 h at 25 mA.

Interchain Disulfide Bonds. Patterns of interchain disulfide bonding were also examined by 2-D SDS–PAGE (Crouch et al., 1987c). Briefly, unreduced proteins in first dimension gel strips were incubated for 10 min at 37 °C in sample buffer containing 50 mM DTT. The gel was positioned above the second dimension slab gel and sealed in place with agarose containing 50 mM DTT.

Glycosidase Digestions. Immune complexes were washed with the appropriate enzyme buffer, resuspended in buffer containing enzyme, and incubated for 8–16 h at 37 °C with agitation. Digestions were terminated by the addition of concentrated SDS–PAGE buffer and boiling. Endoglycosidase F digestions (NEN, 1 unit) were performed in 50 μL of 0.1 M sodium phosphate, 50 mM EDTA, 0.1% (w/v) SDS, 1% (v/v) NP-40, 2 mM PMSF, pH 6.1, and ±1% β-mercaptoethanol. Neuraminidase digestions (Sigma type V, 0.5 unit) were performed in 50 μL of 0.1 M sodium acetate and 0.1% (v/v) NP-40, pH 5.5, containing 0.2 mM PMSF and 2 mM EDTA. For each digestion parallel samples were also incubated in the absence of enzyme. The digested proteins were mixed with concentrated sample buffer and resolved by SDS–PAGE.

Radioactive Hydroxyproline Assay. The ratio of radioactive hydroxyproline (Hpr) to proline (Pro) in CP4 and SP-35 was determined by ion-exchange HPLC (Stimler, 1984). Column fractions were dialyzed versus 0.1 M acetic acid, lyophilized, and subjected to acid hydrolysis in 6 N HCl for 24 h at 108 °C. The hydrolysates were redissolved in 0.05 mL of 0.01 M calcium acetate, pH 5.5, clarified by centrifugation in a microfuge, and chromatographed on an HPX-87C ion-exchange column (7.8 mm × 300 mm, Bio-Rad) equilibrated with the above buffer at 87 °C. Hpr and Pro were resolved by isocratic elution at a flow rate of 1 mL/min. One-milliliter fractions were collected and quantified by liquid scintillation spectrometry. The recovery of applied radioactivity was >90%. For some experiments, proteins were immune precipitated, resolved by SDS–PAGE, and visualized by fluorography. The desired peptide band was excised from the gel, rehydrated with water, washed with acetic acid/methanol/water (1:3:6 v/v/v)

overnight, washed extensively with water, lyophilized, and pulverized with a small pestle. Radiolabeled protein was extracted by digestion with 20 μg of proteinase K (Boehringer-Mannheim) in 0.5 mL of 0.05 M ammonium bicarbonate, pH 8.0, for at least 16 h at 37 °C (Sage et al., 1980). The gel residue was removed by centrifugation, and soluble peptides in the supernatant were lyophilized, acid hydrolyzed, and analyzed for radioactive Hpr and Pro as above. Proteinase K solubilized >80% of the total radioactivity in the excised protein bands.

DEAE Chromatography. Type II cell culture medium supernatants were dialyzed versus 50 mM Tris–acetate, 0.1 M NaCl, and 1% (v/v) Triton X-100, pH 8.0, and chromatographed on DEAE-cellulose (1.0 × 4.0 cm; Whatman, DE-52) equilibrated with the same buffer at 21 °C, as described by Sueshi and Benson (1981) for SP-35. Total recovery of [^{3}H]Pro was approximately 70%, and approximately 11% of the recovered counts bound to the column. Bound proteins were eluted with a linear gradient of 0.1–0.5 M NaCl over a total volume of 400 mL at a flow rate of 25 mL/h. Conductivities were determined at 21 °C. Proteins in pooled fractions were concentrated and delipidated according to the method of Wessel and Flugge (1984).

Reverse-Phase and Gel Permeation HPLC Chromatography. For reverse-phase chromatography, lyophilized or dried alcohol-precipitated proteins were redissolved in 0.1% (v/v) trifluoroacetic acid (TFA), heated for 15 min at 50 °C, and clarified by centrifugation with a microfuge. Samples were applied in up to 500-μL aliquots to be a C4 reverse-phase column (Bio-Rad, RP-304, 4.8 mm × 300 mm) equilibrated with 30% (v/v) acetonitrile–0.1% TFA. Bound components were eluted with a linear gradient of 30–70% acetonitrile in 0.1% TFA at a flow rate of 0.5 mL/min. Absorbance was monitored at 214 nm, 0.5-mL fractions were collected, and radioactivity was quantified by liquid scintillation spectrometry. Fractions were dried with a Speed-Vac (Savant), or briefly gassed with N_2 prior to lyophilization, and examined by SDS–PAGE. Total recovery was >80% of applied radioactivity.

For gel permeation chromatography, proteins were dissolved in 6 M guanidine hydrochloride (BRL) and 50 mM Tris-HCl, pH 7.5, by heating for 15 min at 50 °C in the presence or absence of 10 mM DTT. Samples were clarified by microcentrifugation and applied in 50-μL aliquots to a Protein-Pak 300 SW column (Waters, 7.5 mm × 300 mm) equilibrated at room temperature with 2 M guanidine hydrochloride and 50 mM Tris-HCl, pH 7.5. Proteins were resolved by isocratic elution at a flow rate of 0.5 mL/min. Absorbance was monitored at 214 nm, 0.5-mL fractions were collected, and the radioactivity in each fraction was quantified as above. Pooled fractions were dialyzed at 4 °C versus 0.1 M acetic acid in the presence of rat serum albumin (Sigma) and lyophilized prior to further analysis. Relative molecular weight was estimated by using lysozyme, ovalbumin, and rat serum albumin as standards (Sigma).

Immunochemistry. Antisera to surfactant proteins were prepared in rabbits by subcutaneous injection of a 1:1 emulsion of crude rat surfactant in complete Freund's adjuvant. Rabbits were boosted with biweekly subcutaneous injections in incomplete adjuvant. Each injection consisted of surfactant derived from four rats and contained approximately 40 mg of protein as estimated by the Bradford assay (1976) using albumin as standard. The serum was adsorbed by passage over rat serum Sepharose (Sigma). The titer against crude surfactant was determined by ELISA (Rennard et al., 1982). The

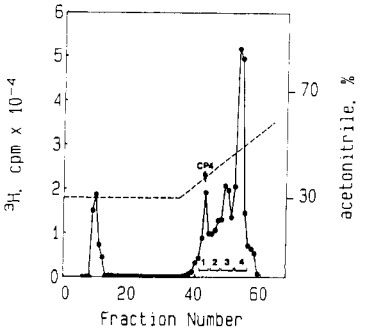

FIGURE 1: Reverse-phase HPLC of radiolabeled type II cell culture medium. Freshly isolated type II cells were labeled for 20 h with [^{3}H]proline. The medium was centrifuged at 400*g* for 10 min, harvested into inhibitors, dialyzed versus acetic acid at 4 °C in the presence of pepstatin, and lyophilized. Proteins were dissolved in 0.1% trifluoroacetic acid, resolved by reverse-phase HPLC, and quantified by liquid scintillation spectrometry as described under Materials and Methods.

adsorbed sera showed no cross-reaction with rat serum proteins or rat serum albumin (Sigma) by immunoblot or ELISA (not shown). For immune precipitation, medium supernatants were diluted 5-fold with buffer A [50 mM Tris-HCl, 150 mM NaCl, 0.5% (w/v) deoxycholate, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 2.5 mM EDTA, 0.1 mM PMSF, pH 7.5]. Excess antiserum was added, and samples were incubated for 18 h at 4 °C with agitation. The optimal amounts of medium and antiserum were determined by titration. The resultant immune complexes were adsorbed with a 2-fold excess of IgG-Sorb (The Enzyme Center), washed three times with buffer A, and washed three more times with buffer A without Triton X-100 prior to SDS–PAGE or enzymic digestion. Immunoblotting was performed by the method of Towbin et al. (1979).

RESULTS

Synthesis and Secretion of Collagenous Proteins. Three low molecular weight bacterial collagenase-sensitive proteins were secreted by rat type II cells and were resolved by reverse-phase HPLC (Figures 1 and 2). These correspond to a non-disulfide-bonded component previously designated CP3 (26 kDa) (Crouch et al., 1987a) and disulfide-bonded components designated CP4 (43 kDa) and CP5 (34–37 kDa) (Crouch et al., 1987b).

Characterization of the 35-kDa Protein (CP5). Several lines of evidence indicate that CP5 is identical with the major surfactant protein SP-35. CP5 comigrated on SDS–PAGE with SP-35 isolated from rat surfactant, both under reducing and nonreducing conditions, and cross-reacted in indirect immunoprecipitation assays with polyclonal antibodies to rat surfactant proteins (Figure 3, lane 1) and with polyclonal antibodies specific for SP-35 kindly provided by Dr. J. Whitsett (University of Cincinnati, Cincinnati, OH) (Figure 4, lane 1). CP5 was also selectively precipitated with polyclonal antibodies to human SP-35 isolated from alveolar proteinosis fluid (not shown). CP5, isolated from culture medium, migrated as several discrete acidic isoforms in the Anderson Iso-Dalt system (see below). The major group consisted of a charge train of at least five isoforms (p*I* = 4–5) ranging from 34 to 37 kDa as previously reported for rat SP-35 (Whitsett et al., 1985b; Floros et al., 1986a). In addition, the protein showed increased mobility following incubation with neuraminidase, consistent with the presence of sialylated oligosaccharides (Figure 3) (Whitsett et al., 1985a; Phelps et al., 1986). The protein was also sensitive to endoglycosidase F (not shown). Digestion of the reduced and denatured protein with highly purified bacterial collagenase (Advance Biofactures, form III)

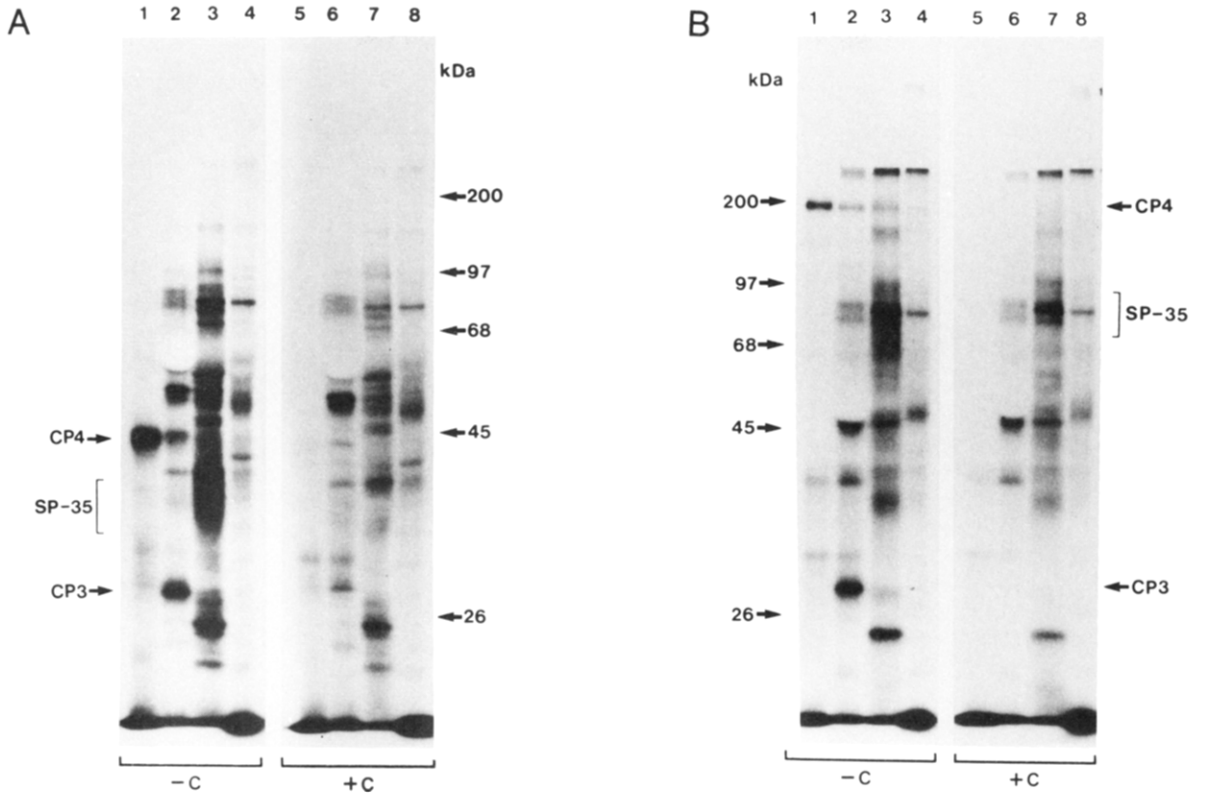

FIGURE 2: Fluorograms of proteins resolved by reverse-phase HPLC (Figure 1). Column fractions were pooled and dried. Radiolabeled proteins were resolved by SDS–PAGE on 5%/10% slab gels with (A) or without (B) prior reduction with DTT and visualized by fluorography. Half of each sample was incubated with purified bacterial collagenase (C) prior to SDS–PAGE. Lanes 1 and 5, pool 1 containing CP4; lanes 2 and 6, pool 2 containing CP4 and CP3; lanes 3 and 7, pool 3 containing SP-35; lanes 4 and 8, pool 4.

resulted in the generation of a major polydisperse collagenase-resistant fragment of 20–25 kDa, consistent with the known protein sequence and other published data (Benson et al., 1985; Ross et al., 1986). Finally, the newly synthesized protein contained radioactive Hpr with a Hpr:Pro ratio of approximately 0.39 as compared with published values of approximately 0.4 for canine SP-35 (Benson et al., 1985).

Characterization of the 43-kDa Protein (CP4). The 43-kDa component also cross-reacted in indirect immunoprecipitation assays with serum-adsorbed polyclonal antibodies to crude rat surfactant proteins (Figure 3, lane 1; Figure 4, lane 2). Identical results were obtained with two additional polyclonal antibodies to rat surfactant proteins provided by J. Floros (Harvard Medical School) and J. Whitsett. However, CP4 did not cross-react with polyclonal antibodies specific for rat SP-35 (Figure 4, lane 1) or with antibodies prepared to human SP-35 isolated from alveolar proteinosis fluid (not shown). CP4 also did not cross-react with polyclonal antibodies to type IV collagen, the major collagen synthesized by primary cultures of rat type II cells (Crouch et al., 1987a; Sage et al., 1983).

Prior to sulfhydryl reduction, CP4 migrated on SDS–PAGE as disulfide-bonded multimers consistent with trimers (i.e., between the positions of phosphorylase and myosin) (Figures 2 and 5). However, the relative molecular weight varied as a function of acrylamide concentration. Unreduced CP4 had an apparent molecular weight of 120–130K on 6% acrylamide gels, but >180K on 10–15% gels. The CP4 multimers were completely resolved from SP-35 multimers by SDS–PAGE, and no disulfide-bonded heteropolymers of SP-35 and CP4 were identified by 2-D electrophoresis (Figure 5). Minor components consistent with CP4 dimers were rarely observed.

The major secreted forms of CP4 showed a pattern of glycosylation similar to that observed for rat SP-35 (Whitsett et al., 1985a). Incubation with endoglycosidase F or neuraminidase resulted in an increase in mobility to approximately 40 kDa (Figure 3, compare lanes 1 and 2). A similar increase in mobility of immune-precipitated CP4 was observed when cells were incubated with 1 μg/mL tunicamycin (Calbiochem), an inhibitor of asparagine-linked oligosaccharide synthesis (not shown).

Unlike SP-35, CP4 migrated as several discrete basic isoforms in the Anderson Iso-Dalt system (Figure 6). The most acidic isoforms were more basic than actin and were completely resolved from the most basic and nonsialylated forms of SP-35. The isoforms showed a slight increase in apparent molecular weight with increasing p*I*, consistent with increasing glycosylation, terminal sialylation, or other modifications.

CP4 and SP-35 showed differences in the size distribution of proline-labeled CNBr and V8 protease-derived peptides (Figure 7). Cleavage of proline-labeled CP4 with CNBr reproducibly generated at least two relatively large CNBr peptides (18 and 14 kDa, globular standards), whereas the majority of the SP-35 peptides derived from the same immunoprecipitate migrated with the dye front on 5%/12.5% slab gels. The size distribution of proline-labeled SP-35 CNBr peptides is consistent with the predicted amino acid sequence of rat SP-35 derived from cDNA sequencing (Sano et al., 1987).

The collagenase-resistant domain of CP4 was, however, similar in size to that of SP-35. Incubation of reduced CP4 with highly purified bacterial collagenase resulted in the generation of a polydispersed collagenase-resistant fragment of approximately 20 kDa that was poorly labeled with proline (not shown). In addition, some similarities in the CNBr and V8 protease maps were noted. For example, both proteins showed a major polydispersed V8-resistant peptide of 20–25 kDa.

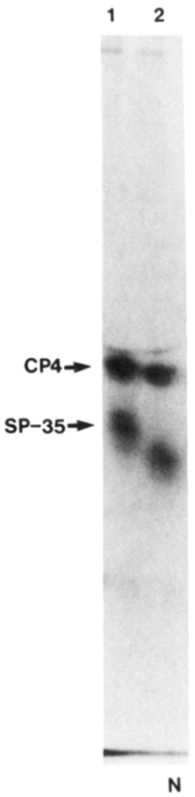

FIGURE 3: Neuraminidase sensitivity of CP4 and SP-35 (CP5). Type II cells were labeled for 20 h with [^{3}H]proline. The medium was clarified at 400g, harvested into neutral protease inhibitors, and dialyzed versus 150 mM NaCl and 50 mM Tris-HCl, pH 7.5 (TBS). Secreted [^{3}H]proline-labeled proteins that cross-reacted with polyclonal antibodies to rat surfactant proteins were precipitated with IgSorb, incubated in the presence or absence of neuraminidase (N) as described under Materials and Methods, resolved by SDS–PAGE on a 5%/12.5% slab gel, and visualized by fluorography.

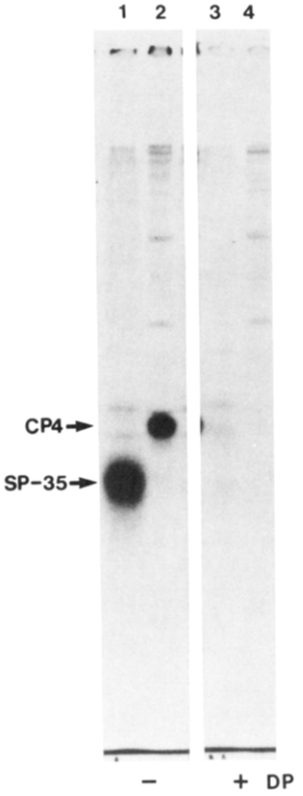

FIGURE 4: CP4 is immunologically different from SP-35. Type II cells were labeled for 20 h with [^{3}H]proline in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 0.1 mM 2,2′-dipyridyl (DP). The medium was first immunoprecipitated with antiserum specific for SP-35 (lanes 1 and 3) and then reprecipitated with antiserum against surfactant-associated proteins (lanes 2 and 4). Proteins were resolved by SDS–PAGE as above.

Differences in the primary structure of CP4 and SP-35 were also suggested by differences in the relative labeling of these proteins with amino acid precursors. Although both CP4 and SP-35 were readily labeled with proline, there was a higher relative recovery of radioactivity in SP-35 when cultures were labeled with [^{35}S]methionine (not shown). Radioactive Hpr analyses of immune precipitates of CP4 gave Hpr:Pro ratios of approximately 0.25. However, contamination with minor noncollagenous components was evident by SDS–PAGE. In order to obtain a more reliable ratio, [^{3}H]proline-labeled CP4 was immune precipitated as above, resolved from minor contaminants by SDS–PAGE in the presence of DTT, visualized by fluorography, and solubilized from the excised gel band as described under Materials and Methods. A ratio of 0.27 was obtained for the electrophoretically purified protein.

Purification of Radiolabeled CP4. A major purification was achieved by chromatography of whole type II cell culture medium on DEAE-cellulose in 1% (v/v) Triton X-100, pH 8.0, as previously described for canine SP-35 (Sueshi & Benson, 1981). Under these conditions, 90% of the recovered, radiolabeled protein did not bind to the column. However, CP4 was bound and was partially resolved from SP-35 and other components that eluted later in the salt gradient (Figures 8 and 9). Further purification of CP4 was readily achieved by reverse-phase or gel permeation HPLC (not shown) and confirmed by SDS–PAGE and fluorography as for the DEAE-purified proteins. Radioactive Hpr analyses of HPLC-purified CP4 gave a Hpr:Pro ratio identical with that obtained for immune-precipitated CP4 eluted from gel bands. Interestingly, reduced CP4 eluted only slightly later than reduced rat serum albumin (68 kDa) from the gel permeation column. SP-35 also showed an unexpectedly high apparent molecular weight and eluted between albumin (68 kDa) and ovalbumin (45 kDa) standards (not shown). The early elution of these proteins from gel permeation columns could reflect

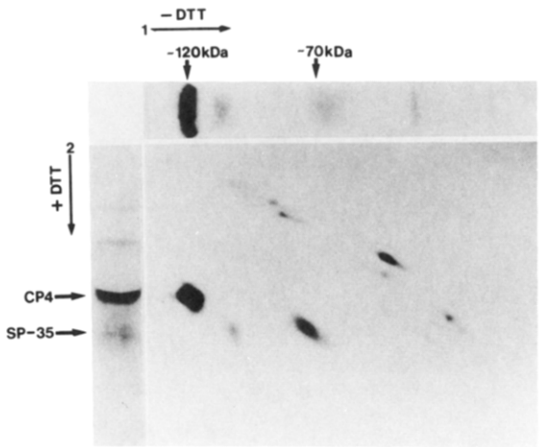

FIGURE 5: CP4 is assembled as high molecular weight disulfide-bonded multimers. [^{3}H]Proline-labeled CP4 and SP-35 were precipitated with rat antibodies to surfactant proteins and resolved by SDS–PAGE on 0.75 mm thick 5%/7.5% slab gels without prior sulfhydryl reduction (arrow 1). Individual lanes were cut from the gel, incubated in the presence of DTT to reduce interchain disulfide bonds, positioned horizontally above a 5%/12.5% slab gel, and sealed in place with agarose containing 50 mM DTT. The reduced components were resolved by SDS–PAGE in the second dimension (arrow 2). CP4 is derived from a major component that migrates between myosin (200 kDa) and phosphorylase (97 kDa), whereas SP-35 is primarily derived from a polydispersed dimeric component (approximately 70 kDa).

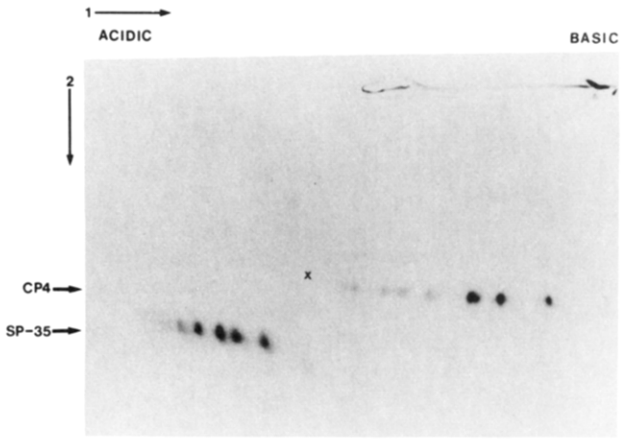

FIGURE 6: CP4 consists of basic isoforms. [^{3}H]Proline-labeled CP4 and SP-35 were precipitated from type II cell medium with antibodies to rat surfactant proteins. Precipitated components were resolved by IEF in the first dimension (arrow 1; measured pH range 3.5–8.0), followed by SDS–PAGE in the second dimension (arrow 2). The radiolabeled components were visualized by fluorography. the x indicates the position of actin (p*I* = 5.75).

the presence of a relatively extended imino acid rich (collagenous) domain.

Effects of 2,2′-Dipyridyl on the Secretion of CP4. 2,2′-Dipyridyl (DP), an iron chelator, is known to selectively inhibit the posttranslational hydroxylation of prolyl and lysyl residues in collagenous proteins and to inhibit triple helix formation and the subsequent secretion of interstitial collagens (Jimenez & Yankowski, 1978). We, therefore, examined the effects of DP on the accumulation of SP-35 and CP4 in type II culture medium. Radiolabeled secreted proteins were quantified by immunoprecipitation with polyclonal antibodies and by densitometry of the radiolabeled bands following SDS–PAGE and fluorography. DP at a concentration of 0.1 mM completely inhibited the accumulation of immunoreactive SP-35 and CP4 in the culture medium (Figure 3B) and inhibited prolyl hydroxylation of newly synthesized proteins by >90%. Under these conditions, total incorporation into nondialyzable protein was inhibited by <5%. There was also no significant difference in the intensity of minor noncollagenous bands in the immune precipitates (e.g., compare lanes 2 and 4 in Figure 4). These findings are consistent with a selective inhibition of secretion of the collagenous proteins. Intracellular forms of SP-35 and CP4 accumulating in the presence of DP showed an increased mobility on SDS–PAGE (approximately 28–30 and 40 kDa, respectively), consistent with decreased levels of posttranslational hydroxylation (not shown). Although SP-35 lacks hydroxylysine (Crawford et al., 1986), we cannot yet exclude a contribution of hydroxylysine or hydroxylysyl glycosides to the shifts in electrophoretic mobility of CP4.

Identification of CP4 in Rat Surfactant. A 43-kDa component was observed in immunoblots of rat surfactant by using polyclonal antibodies to rat surfactant proteins. Extraction of washed rat surfactant with 10 mM EDTA (or EGTA) solubilized a component that coeluted with pneumocyte CP4 from reverse-phase HPLC (not shown). The component, which was completely resolved from solubilized SP-35, was collagenase-sensitive and comigrated with CP4 on SDS–PAGE (Figure 10). Isoelectric focusing followed by immoblotting with antibodies to rat surfactant proteins revealed a family of basic isoforms that overlapped in both size and charge with those of pneumocyte-derived CP4 (not shown).

Discussion

This paper describes the initial characterization of CP4, a surfactant-associated glycoprotein secreted by primary cultures of rat type II pneumocytes. Like SP-35, CP4 is a hybrid molecule that contains both collagenous and noncollagenous domains. The presence of collagenous sequences is evidenced

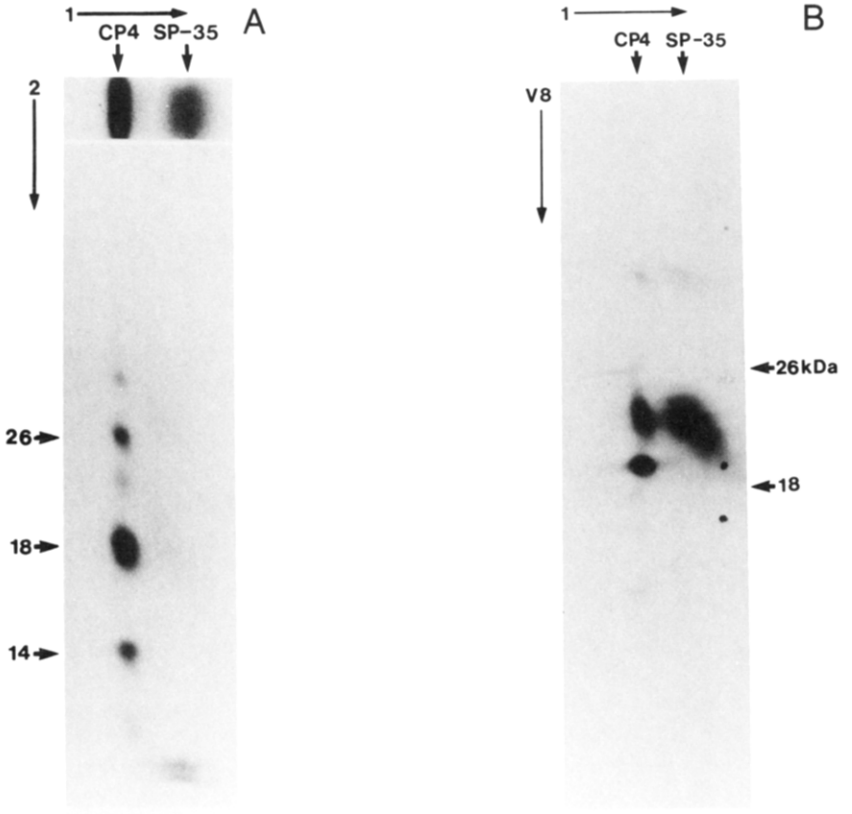

FIGURE 7: CP4 and SP-35 show different patterns of CNBr and V8 protease peptides. CP4 and SP-35 were immune precipitated as above and subjected to in situ cleavage with CNBr (A) or V8 protease (B) as described under Materials and Methods.

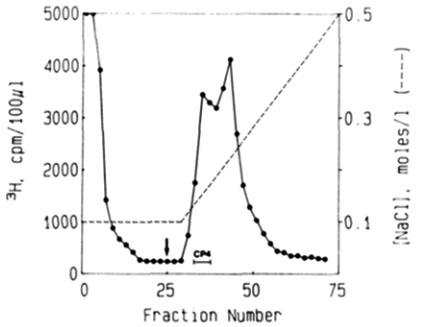

FIGURE 8: DEAE chromatography of radiolabeled medium proteins. Type II cells were radiolabeled for 20 h with [^{3}H]proline. The medium was harvested in the presence of inhibitors, dialyzed, and chromatographed on DEAE-cellulose in the presence of Triton X-100 as described under Materials and Methods.

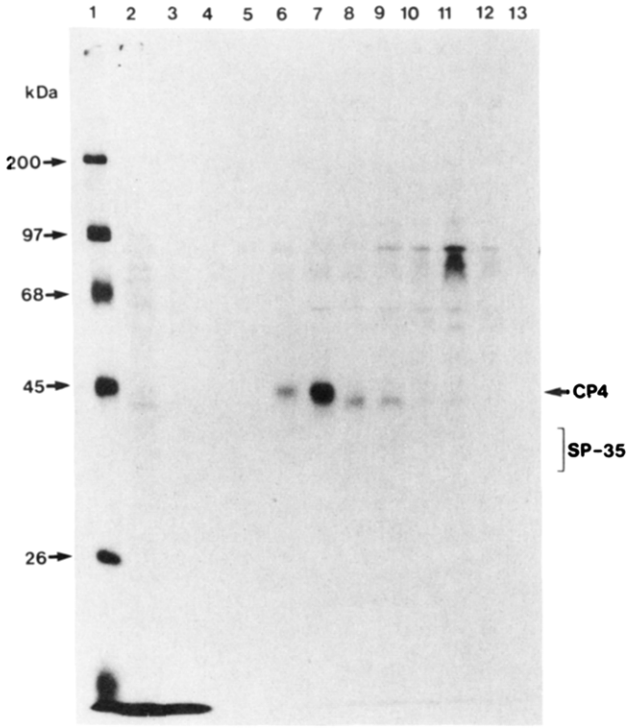

FIGURE 9: SDS–PAGE of DEAE fractions (Figure 8). DEAE fractions were processed for SDS–PAGE and fluorography as described. Lane 1, globular standards; lane 2, aliquot of sample applied to column; lane 3, nonbound proteins; lanes 4–13, equal aliquots of odd fractions 29–47. CP4 is found in fractions 33–35. SP-35 elutes in fraction 39–45 but is difficult to visualize because of its broad elution profile and marked size heterogeneity.

by the protein's sensitivity to highly purified bacterial collagenase and by the presence of hydroxyproline.

Both SP-35 and CP4 are associated with surfactant obtained from rat lungs and cross-react with antibodies to crude surfactant. In addition, CP4 and a significant fraction of the total SP-35 are extracted from surfactant in the presence of EDTA or EGTA. These observations strongly suggest that at least a fraction of CP4 interacts with other components of surfactant via divalent cation dependent mechanisms. The effects of calcium on SP-35-mediated phospholipid adsorption and phospholipid aggregation have been well described (Benson et al., 1984; Efrati et al., 1987; Hawgood et al., 1985), and an important role for calcium in the function of SP-35 is implied by the presence of γ-carboxyglutamic acid residues in the rat protein (Rannels et al., 1987).

Despite the similarities between SP-35 and CP4, the proteins are immunologically and structurally different. CP4 is not recognized by polyclonal antibodies obtained against purified

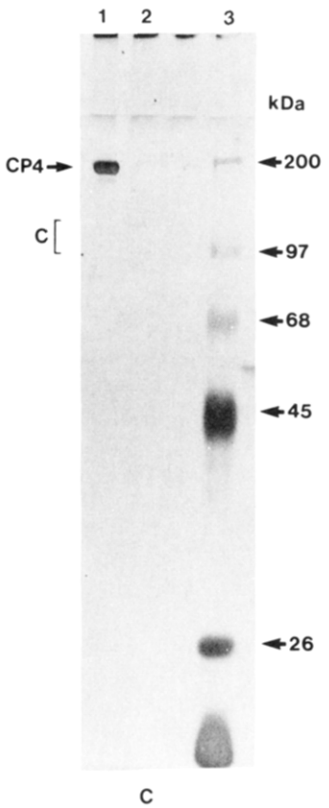

FIGURE 10: Electrophoresis of CP4 extracted from surfactant. The fractions coeluting with CP4 from the HPLC were dried, resolved by SDS–PAGE in the absence of DTT, and visualized by silver staining (lane 1). Samples were also examined following prior digestion with highly purified bacterial collagenase (lane 2). Reduced globular protein standards are at right (lane 3).

rat or human SP-35. The proteins show different behavior in a variety of chromatographic systems and have different subunit and nonreduced molecular weights by SDS–PAGE. The CP4 isoforms are more basic than SP-35. Furthermore, the proteins have different cyanogen bromide and proteolytic cleavage patterns. The observations indicate that CP4 is not a precursor to SP-35 nor a more extensively posttranslationally modified version of the SP-35 polypeptide.

SP-35 and lower molecular weight hydrophobic proteins (i.e., SP-18 and SP-5) have been identified as the major protein species of surfactant. However, lesser quantities of other proteins have been identified, including proteins similar in charge and/or size to CP4. Ng et al. (1983) identified components of 43–45 kDa in rabbit lung surfactant that are more acidic than CP4. Phelps and Taeusch (1985) observed a basic charge train of roughly 45 kDa, termed "class D" proteins, in immunoblots of lavage from several species, using antibodies to proteins from human alveolar proteinosis lavage. Rannels et al. (1987) identified a 40-kDa species in rat lung surfactant that, like the major surfactant protein SP-35, yielded γ-carboxyglutamic acid on base hydrolysis. The authors suggested a role for the posttranslationally modified residue in SP-35 in calcium-dependent association with phospholipids. Crawford et al. (1986) identified a disulfide-bonded protein of similar molecular weight; however, it was reportedly not sensitive to bacterial collagenase. Postle et al. (1985) isolated a 43-kDa protein from lavage which is also different from CP4; the protein did not contain hydroxyproline and formed higher molecular weight disulfide-bonded aggregates. The cDNA-derived sequences of the precursors of the lower molecular weight surfactant-associated proteins (Glasser et al., 1987; Hawgood et al., 1987; Jacobs et al., 1987; Warr et al., 1987)

do not contain collagenous sequences. Although other cell types secrete low molecular weight collagens, these proteins are structurally different from CP4 (Pihlajaniemi et al., 1987; Sage, 1985; Majack & Bornstein, 1985; Gordon et al., 1987). The subunit molecular weights are different from C1q, acetylcholinesterase, and the core-specific lectin. Unlike the latter protein, CP4 contains asparagine-linked carbohydrate (Colley & Baenziger, 1987a).

The functional role of the collagenous domain of SP-35 is not yet known. It has been suggested that the comparatively rigid amino-terminal collagenous domains of acetylcholinesterase, C1q, and the core-specific lectin serve to anchor and spatially organize the larger, functionally important carboxy-terminal globular domains (Drickamer et al., 1986; Colley & Baenziger, 1987b). Interestingly, these proteins can also interact with lipids or membranes.

Hydroxylation of proline and acquisition of a triple helical conformation are essential for the assembly and subsequent secretion of interstitial procollagen (Jimenez & Yankowski, 1978), for the secretion of C1q (Muller et al., 1978), and for the assembly and secretion of the core-specific lectin (Colley & Baenziger, 1987b). Our observation that the secretion and normal posttranslational glycosylation of SP-35 (Crouch et al., 1987b; O'Reilly et al., 1987) and CP4 by rat type II cells are selectively inhibited by 2,2′-dipyridyl, an inhibitor of posttranslational prolyl and lysyl hydroxylation, suggests that triple helix formation is required for the secretion of the collagenous surfactant proteins. Ross et al. (1986) observed that fragments of SP-35 which lack the amino-terminal collagenous domain are poorly adsorbed to lipids and suggested that the collagenous domain is essential to maintaining the structural features of SP-35 necessary for phospholipid binding. Further studies comparing SP-35 and CP4 may provide important insights into the functional role of the collagenous domain and into the divalent cation dependence of phospholipid binding.

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Interaction of Phospholipids with the Detergent-Solubilized ADP/ATP Carrier Protein As Studied by Spin-Label Electron Spin Resonance†

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ABSTRACT: The interaction of spin-labeled phospholipids with the detergent-solubilized ADP/ATP carrier protein from the inner mitochondrial membrane has been investigated by electron spin resonance spectroscopy. The equilibrium binding of cardiolipin and phosphatidic acid was studied by titration of the protein with spin-labeled phospholipid analogues using a spectral subtraction protocol for the evaluation of the mobile and immobilized lipid portions. This analysis revealed the immobilization of two molecules of spin-labeled cardiolipin per protein dimer. Phosphatidic acid has a similar affinity for the protein surface as cardiolipin. The lipid–protein interaction was less pronounced with the neutral phospholipids and with phosphatidylglycerol. The importance of the electrostatic contribution to the phospholipid–protein interaction shows up with a strong dependence of the lipid binding on salt concentration. Cleavage by phospholipase A_2 and spin reduction by ascorbate of the spin-labeled acidic phospholipids in contact with the protein surface suggest that these lipids are located on the outer perimeter of the protein. At reduced detergent concentration, the protein aggregated upon addition of small amounts of cardiolipin but remained solubilized when more cardiolipin was added. This result is discussed with respect to the aggregation state of the protein in the mitochondrial membrane. It is also tentatively concluded that binding of spin-labeled cardiolipin does not displace the tightly bound cardiolipin of mitochondrial origin, which was detected previously by ^{31}P nuclear magnetic resonance spectroscopy [Beyer, K., & Klingenberg, M. (1985) *Biochemistry 24*, 3821].

The interaction of spin-labeled phospholipids with integral membrane proteins has been extensively studied in reconstituted membranes, aiming at an understanding of the specific lipid composition for protein function and biomembrane stability. As a general result, the electron spin resonance (ESR)[1] spectra revealed strongly and weakly immobilized components which were attributed to lipid molecules in transient contact with the protein surface and to lipids freely diffusing in the membrane. The strength of lipid–protein association was variable among different proteins and lipid classes (Devaux & Seigneuret, 1985). A strong interaction has been frequently observed between negatively charged phospholipids and membrane proteins (Marsh, 1985).

Recently, a tight binding of mitochondrial CL was found by ^{31}P NMR in the detergent-solubilized AAC protein from beef heart mitochondria (Beyer & Klingenberg, 1985). This finding prompted us to employ the spin-label method using phospholipids specifically labeled at carbon atom 12 of stearic acid in the *sn*-2 position. In detergent solution, labeling of this position affords a clear-cut discrimination between protein-associated phospholipids and lipid molecules in detergent micelles. In reconstituted membranes, the best results have been obtained with spin-labels in position 14 of the fatty acid chain (Marsh, 1985).

Solubilization of membrane proteins is usually attributed to the "soap effect" of the solubilizing detergent. The present experiments suggest that charged amino acids on the protein

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[1] Abbreviations: AAC, ADP/ATP carrier; CAT, carboxyatractyloside; CATSL, spin-labeled carboxyatractyloside; CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SA, stearic acid; SASL, 12-doxylstearic acid; CLSL, PASL, PCSL, PESL, and PGSL, phospholipids spin-labeled with 12-doxylstearic acid in the *sn*-2 position; ESR, electron spin resonance; NMR, nuclear magnetic resonance.

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