Purification and Biochemical Characterization of CP4 (SP-D), a Collagenous Surfactant-Associated Protein†

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ABSTRACT: CP4 is a collagenous glycoprotein (43 kDa, reduced) synthesized by rat type II pulmonary epithelial cells in primary culture (Persson et al., 1988). In order to better characterize this protein, CP4 was isolated from rat bronchoalveolar lavage and EDTA extracts of lung surfactant by adsorption to barium sulfate and elution with sodium citrate followed by reverse-phase HPLC. Amino acid analysis of purified CP4 demonstrated 4-hydroxyproline (Hyp), hydroxylysine (Hyl), and acid-labile components coeluting with Hyl glycosides. In addition, gas-phase amino-terminal microsequencing of two CP4 CNBr peptides demonstrated nonoverlapping collagenous sequences comprised of nine and six Gly-X-Y triplets, containing a total of four residues of Hyp and two of Hyl. There was <50% sequence homology of these peptides with the cDNA-derived sequence of the collagenous domain of rat SP-A. Two-dimensional IEF/SDS–PAGE resolved the protein into a charge train of basic isoforms (p*I* approximately 6–8), similar to those of newly synthesized CP4 and the class D surfactant proteins (Phelps & Taeusch, 1985). Gel filtration of nondenatured CP4 on 4% agarose showed a high apparent molecular mass complex comprised of disulfide-bonded trimers of the 43-kDa subunits. Antibodies to purified lavage CP4 showed specific binding to newly synthesized and surfactant-associated CP4. We propose that CP4 be designated "surfactant protein D" (SP-D) in accordance with an accepted nomenclature for surfactant-associated proteins (Possmayer, 1988).

Pulmonary surfactant is required for normal gas exchange and may also play important roles in epithelial permeability and host defense. This material, which is synthesized by type II epithelial cells, is comprised of specific phospholipids and several surfactant-associated proteins. There is evidence that these proteins play important roles in modulating surface activity and in regulating the secretion and metabolism of pulmonary surfactant (van Golde et al., 1988; Possmayer, 1988). The most abundant and best characterized of these surfactant-associated proteins is a collagenous glycoprotein designated SP-A (32–38 kDa, reduced). Surfactant also contains two genetically different noncollagenous, hydrophobic proteins designated SP-B and SP-C (Glasser et al., 1988; Hawgood et al., 1987; Jacobs et al., 1987; Warr et al., 1987). In addition, surfactant contains uncharacterized basic proteins of 40–45 kDa (Ng et al., 1983; Phelps & Taeusch, 1985), referred to as class D surfactant proteins (Phelps & Taeusch, 1985).

We have recently identified a second collagenous surfactant-associated glycoprotein (43 kDa, reduced) that is similar in size and charge to the class D surfactant proteins (Crouch et al., 1987; Persson et al., 1988). This protein previously designated CP4, is synthesized and secreted by freshly isolated rat type II cells and can be extracted from rat surfactant with EDTA or EGTA (Persson et al., 1988). In this paper we describe the purification and further biochemical characterization of lung CP4 and present evidence that the protein is identical with the pneumocyte-derived protein. We also show that CP4 is compositionally and immunologically different from SP-A and the major surfactant-associated hydrophobic proteins.

Materials and Methods

Bronchoalveolar Lavage and Surfactant Isolation. Rat lungs were lavaged in situ with a total of 50 mL of buffered saline (125 mM NaCl, 2.5 mM Na_2HPO_4, 5 mM KCl, 17 mM HEPES, pH 7.4, containing 0.75 mg/L gentamycin) at a hydrostatic pressure of 20 cm. The lavage was centrifuged at 400*g* for 10 min, and the surfactant pellet was collected from the supernatant by centrifugation at 48000*g* for 30 min at 4 °C. For most experiments, contaminating albumin and other soluble proteins were extracted from the surfactant pellet by washing with 0.15 M NaCl–50 mM Tris-HCl, pH 7.5 (TBS), containing 2 mM $CaCl_2$. CP4 and a fraction of the SP-A were solubilized from the pellet with 10 mM EDTA in TBS as previously described (Persson et al., 1988).

Barium Sulfate Extraction. Barium sulfate extraction of the 48000*g* lavage supernatant or type II cell culture medium was performed following the addition of sodium citrate to 2.5 mM. Barium sulfate powder (Fisher) was added to 2 mg/mL, and the suspension was stirred for 30 min at room temperature. The adsorbent was collected at 10000*g*, washed twice with TBS containing 2.5 mM sodium citrate, and eluted with 200 mM sodium citrate, pH 7.5. For some experiments the supernatant was reextracted with barium sulfate to optimize recoveries and to monitor the efficiency of barium adsorption. Adsorption of the proteins extracted from crude surfactant with 10 mM EDTA was accomplished by diluting the EDTA concentration to 2.5 mM prior to the addition of $BaSO_4$. The adsorbent was washed and eluted as above. Aliquots of eluted protein were examined by SDS–PAGE or further purified by reverse-phase HPLC or gel filtration chromatography. For some experiments, proteins adsorbed to $BaSO_4$ were directly eluted with

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SDS sample buffer and resolved by SDS–PAGE. Relative recoveries were estimated by densitometry of silver-stained gels or appropriately exposed fluorograms.

Type II Cell Isolation and Metabolic Labeling. Rat type II cells were isolated from adult Sprague-Dawley rats as previously described (Persson et al., 1988). Briefly, cells were released from lung with porcine pancreatic elastase, and contaminating macrophages were removed by "panning" on IgG-coated plastic plates. Freshly isolated cells were incubated for up to 16 h in serum-free medium containing 20 μCi/mL L-[2,3-^{3}H]proline [New England Nuclear (NEN)] in the presence of ascorbate and β-aminopropionitrile (Crouch et al., 1987; Persson et al., 1988). 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 medium was clarified by centrifugation for 10 min at 400*g* prior to storage at −85 °C.

Reverse-Phase HPLC Chromatography. For reverse-phase chromatography, citrate extracts of $BaSO_4$ pellets were applied in <500-μL aliquots to a C4 reverse-phase column (Bio-Rad, RP-304, 4.8 mm × 300 mm) equilibrated with 30% (v/v) acetonitrile–0.1% TFA. In some experiments, lyophilized proteins were denatured and solubilized by warming to 50 °C for 10 min in 0.1% TFA prior to injection. 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, and 0.5-mL fractions were collected. Radioactive proteins were quantified by liquid scintillation spectrometry. Fractions were dried with a Speed-Vac (Savant), or volatile components were removed by gassing with N_2 prior to lyophilization. The recovery of lavage CP4 was quantified by amino acid analysis and normalized to total lavage protein as estimated by a dye binding assay (Bradford, 1976) using bovine serum albumin as standard.

Gel-Filtration Chromatography. Proteins in TBS or 200 mM sodium citrate, pH 7.5, were applied to a 1.5 × 170 cm column of 4% agarose (Bio-Rad, A-15m, 200–400 mesh) and eluted at room temperature with TBS containing 10 mM EDTA at a flow rate of 6 mL/h. Eluted proteins were monitored at 278 nM and examined by SDS–PAGE. Radioactive proteins were quantified by liquid scintillation spectrometry. The column was calibrated with bovine thyroglobulin (670 kDa, Sigma), rabbit IgG (150 kDa, Sigma), and tritiated water.

SDS–PAGE and Isoelectric Focusing. Proteins were resolved by SDS–PAGE on discontinuous methylenebis(acrylamide) slab gels and silver stained, or examined by fluorography (Persson et al., 1988). Two-dimensional resolution of proteins was performed with the Anderson Iso-Dalt system as previously described (Anderson & Anderson, 1977; Persson et al., 1988). Ampholytes were from Serva (Servalyte, pH 2–11), and the pH gradient was directly measured with a surface electrode. Molecular masses were estimated relative to internal, unlabeled or ^{14}C-labeled, globular protein standards [Bethesda Research Laboratories (BRL)].

Amino Acid Analysis. For amino acid analysis proteins were dialyzed against 0.1 M acetic acid at 4 °C, lyophilized, and hydrolyzed in constant-boiling HCl for 24 h at 108 °C. Analyses were performed on a Beckman 119C amino acid analyzer. Alkaline hydrolysis for detection of γ-carboxyglutamic acid (Gla) was performed according to Price (1983), and thermal decarboxylation of Gla was performed according to Bajaj et al. (1982). Gla standards were purchased from Sigma and quantified by amino acid analysis following acid hydrolysis of Gla to glutamic acid. Hydroxylysine glycosides were identified following alkaline hydrolysis (Butler, 1982).

Gas-Phase Microsequencing. Lyophilized HPLC-purified CP4 was cleaved with cyanogen bromide in 70% (v/v) formic acid (Crouch & Bornstein, 1978). The peptides were rechromatographed under the same conditions on the reverse-phase HPLC. The fractions in the first major peak were pooled, and peptides were resolved by SDS–PAGE on 15% acrylamide gel, transferred to Immobilon membranes (Millipore), excised, and sequenced according to Matsudaira (1987). Transfers were performed for 2 h at 4 °C under a constant current of 0.5 A in a minitransfer apparatus (Hoefer). Molecular weights were estimated relative to internal prestained globular standards (BRL).

Immunochemistry. Antisera to rat CP4 were prepared in rabbits by subcutaneous injection of 10 μg of HPLC-purified CP4 in complete Freund's adjuvant (Sigma). Rabbits were boosted with biweekly subcutaneous injections of 10 μg of CP4 in incomplete adjuvant. The serum was adsorbed by passage over rat serum–Sepharose (Sigma). The adsorbed sera showed no cross reaction with rat serum proteins by ELISA and did not cross react with rat SP-A or the hydrophobic proteins by immunoblot. Indirect immune precipitation assays demonstrated specific precipitation of CP4 from metabolically labeled type II cell medium (see below) and cell lysates. Immune precipitations and immunoblotting were performed as previously described (Persson et al., 1988).

Immunocytochemistry. Freshly isolated type II cells were collected by cytocentrifugation in a Shandon Cytospin, fixed for 10 min at 4 °C in absolute ethanol, air-dried, rehydrated in phosphate-buffered saline, and incubated for 30 min at room temperature with adsorbed anti-CP4 (1:200) or control immunoglobulin. Immune complexes were visualized by an indirect immunoperoxidase technique using biotinylated affinity-purified goat anti-rabbit IgG and streptavidin–peroxidase (BRL) with 3,3′-diaminobenzidine as substrate.

Results

Adsorption of CP4 with Barium Sulfate. Rat surfactant and the high-speed (48000*g*) supernatant of rat bronchoalveolar lavage were found to contain collagenous proteins identical in electrophoretic mobility with newly synthesized pneumocyte CP4. Adsorption of the lavage supernatant with barium sulfate in the presence of 2.5 mM citrate gave an approximately 40-fold purification of the protein (Figure 1). In addition, CP4 was quantitatively and relatively selectively eluted from the barium sulfate with 200 mM sodium citrate, pH 7.5 (Figure 1). Citrate elution gave some additional purification and was used to concentrate CP4 prior to chromatography. Adsorption to barium and quantitative elution with citrate were also observed for pneumocyte-derived CP4 (Figure 2) and the surfactant-associated protein (not shown). In the latter case, it was necessary to dilute the EDTA in the surfactant extract to 2.5 mM prior to the addition of barium sulfate. CP4 from all three sources was degraded by purified bacterial collagenase (not shown).

Chromatographic Purification of CP4. Final purification of lavage and surfactant-associated CP4 was achieved by reverse-phase high-pressure liquid chromatography (HPLC). Lung CP4 eluted early in the acetonitrile gradient at the expected position of pneumocyte CP4 (Figure 3A) and was completely resolved from rat albumin or rat SP-A. Following HPLC, the barium-adsorbed and citrate-eluted proteins were pure by silver staining of SDS gels (Figure 1, lane 4). Lavage CP4 was obtained in quantities of approximately 1 μg of CP4/mg of total lavage protein.

Table I: Amino Acid Composition (Residues/1000)

residue	CP4[a]	SP-A[b]	MBP-C[c]	MBP-A[c]
Cys	14.3	21.9	28.7	32.5
Hyp	31.8			
Asx	77.3	109.6	114.8	81.3
Thr	35.5	48.2	57.4	56.9
Ser	65.6	35.1	73.8	77.2
Glx	141.8	105.2	98.4	105.7
Pro	48.2			
Gly	211.7	171.1	118.9	117.9
Ala	112.8	52.6	69.7	60.1
Val	26.4	52.6	77.9	65.0
Met	8.3	30.7	20.5	20.3
Ile	19.1	35.1	20.5	32.5
Leu	60.1	65.8	90.2	85.4
Tyr	6.0	43.9	12.3	8.1
Phe	20.4	21.9	36.9	28.5
Hyl	20.5	0.0		
His	4.3	13.2	4.1	16.3
Lys	49.1	39.5		
Arg	47.0	43.9	53.3	32.5
Trp	ND	8.8	12.3	8.1
Pro + Hyp	80.0	100.9	61.5	52.8
Lys + Hyl	69.5	39.5	49.2	85.4

[a] Average for two determinations using approximately 10 μg of protein per analysis. The data are not corrected for incomplete release of valine and isoleucine or hydrolytic losses. Methionine is calculated as the sum of methionine and methionine sulfoxide. [b] Calculated from the deduced amino acid composition of rat SP-A (Sano et al., 1987). Hyp identified by amino acid analysis in SP-A from several species. Hyl is absent. [c] Calculated from deduced amino acid compositions of rat mannose-binding proteins, MBP-C and MBP-A (Drickamer & McCreary, 1987). Both proteins contain Hyp (Drickamer & McCreary, 1987), and rat CSL contains Hyl and Hyl glycosides (Colley & Baenziger, 1987; Oka et al., 1987).

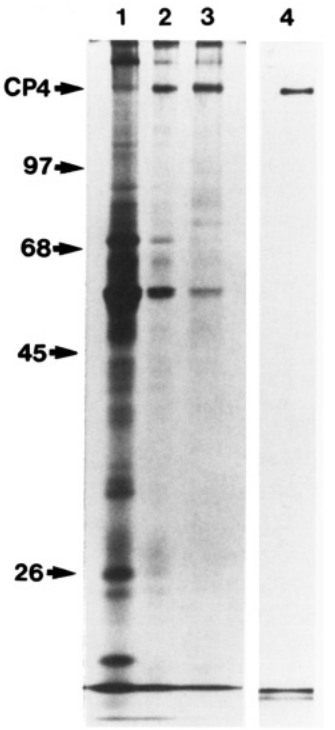

FIGURE 1: SDS–PAGE showing purification of lavage CP4. Proteins were resolved by SDS–PAGE on 5%/10% slab gels without prior reduction with DTT and visualized by silver staining. (Lane 1) High-speed supernatant of rat lavage following 6-fold concentration on an Amicon YM-10 membrane; (lane 2) proteins bound to $BaSO_4$ and eluted with SDS–PAGE buffer; (lane 3) proteins eluted from $BaSO_4$ with 0.2 N citrate buffer; (lane 4) HPLC-purified CP4 (see Figure 3A). The positions of unreduced pneumocyte CP4 and reduced globular protein standards are indicated at left.

Purification of CP4 from EDTA extracts of rat surfactant was also achieved by gel-filtration chromatography on 4% agarose in the presence of 10 mM EDTA (Figure 4). Proteins in each fraction were resolved by SDS–PAGE and visualized by silver staining (Figure 5). SP-A (Figure 5, lane 3) eluted in a position consistent with its reported aggregate size of 1.6×10^6 kDa (Kuroki et al., 1988). Surfactant-associated CP4 coeluted with pneumocyte CP4 between the void volume and SP-A (Figure 5, lane 2). Barium-adsorbed lavage CP4 also coeluted with pneumocyte CP4. The CP4 complexes were comprised exclusively of disulfide-bonded trimers of the 43-kDa subunits (e.g., Figure 5, lane 2).

Amino Acid Analysis. Acid hydrolysates of HPLC-purified lung CP4 contained both 4-hydroxyproline (Hyp) and hydroxylysine (Hyl) (Table I). The Hyp was 30–40% of the total proline (proline and Hyp), similar to the ratio of radioactive Hyp to proline in newly synthesized CP4 (Persson et al., 1988). Following alkaline hydrolysis, there was a 75% decrease in the recovery of hydroxylysine with the appearance of two peaks eluting in the positions of authentic glucosylgalactosylhydroxylysine and galactosylhydroxylysine. No γ-carboxyglutamic acid (Gla) was detected in alkaline hydrolysates under conditions that would have allowed detection of <1 mol of Gla/mol of CP4. There was also no detectable difference in the recovery of glutamic acid following acid hydrolysis or in alkaline hydrolysates following thermal decarboxylation of the lyophilized protein.

The relative concentrations of glycine and imino acids were considerably lower than those for the pepsin-resistant domains of the major interstitial collagens (approximately one-third glycine and one-fifth imino acids), indicating the presence of both collagenous and noncollagenous sequences. There were also significant compositional differences between CP4 and the deduced compositions of previously described low molecular mass collagenous proteins, including rat SP-A and mannose-binding proteins (MBP-A and MBP-C) (Drickamer & McCreary, 1987; Persson et al., 1988; Sano et al., 1987). The composition was also different from that of the 43-kDa protein isolated from surfactant by Postle et al. (1985) and from the compositions of the noncollagenous precursors of human SP-B and SP-C deduced from published cDNA sequence data (Glasser et al., 1987; Hawgood et al., 1987; Jacobs et al., 1987; Warr et al., 1987).

Amino Acid Sequences. The amino terminus of the intact protein was resistant to Edman degradation. However, protein sequence was obtained for the NH_2-termini of two cyanogen bromide peptides (Figure 3B). Both peptides were collagenous with repeating of Gly-X-Y triplets containing hydroxylysine and/or hydroxyproline (Table II). The hydroxylated residues were exclusively identified in the Y position, as found in other collagenous proteins. Comparison of the sequences with the deduced amino acid sequence of rat SP-A (Sano et al., 1987) gave a maximum of 46% homology for the 14-kDa fragment and 42% homology for the 18-kDa fragment (Pustell sequence analysis software, IBI, Inc.), despite the presence of glycine in every third position. Similarly, there was less than 53% homology with the collagenous domains of the rat liver mannose-binding proteins (Drickamer et al., 1986).

Table II: Collagenous CNBr Fragments of Rat CP4

14 kDa	Gly-Ser-Ala-Gly-Ala-Lys-Gly-Pro-Ala-Gly-Pro-Cys-Gly-Glu-Arg-Gly-Ala-Hyp-Gly-Glu-Gln-Gly-Ala-Hyp-Gly-Asn-Ala-Gly-
18 kDa	?-Leu-Ser-Gly-Leu-Hyp-Gly-Pro-Arg-Gly-Pro-Val-Gly-Pro-Hyl-Gly-Glu-Glu-?-Ser-Ala-Gly-Glu-Hyp-?-Pro-Hyl-Gly-

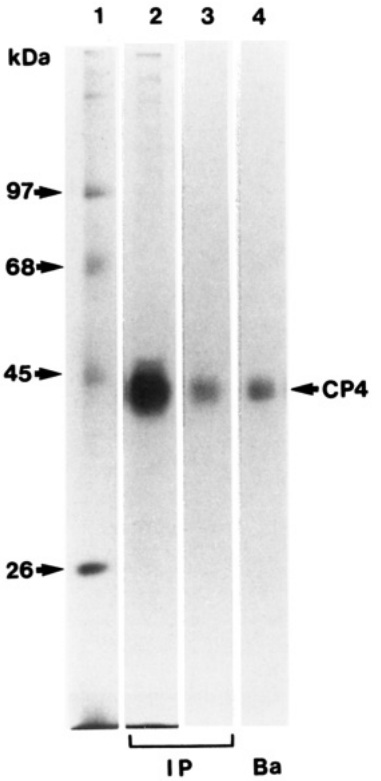

FIGURE 2: Immune precipitation and barium adsorption of pneumocyte-derived CP4. Freshly isolated rat type II cells were labeled overnight with [^{3}H]proline. Secreted CP4 was immune precipitated with antibodies to HPLC-purified lavage CP4 or adsorbed with $BaSO_4$ as described under Materials and Methods. The immune precipitated or adsorbed and eluted proteins were dissolved in sample buffer, reduced with DTT, resolved by SDS–PAGE, and visualized by fluorography. (Lane 1) Radiolabeled globular protein standards; (lane 2) overexposed fluorogram of immune precipitate of culture medium; (lane 3) immune precipitate of culture medium; (lane 4) proteins eluted with 200 mM citrate from barium sulfate extract of pneumocyte culture medium.

Immunochemistry. The immunologic relationships of pneumocyte-derived, lavage, and surfactant-associated CP4 were substantitated by immunoblot and immune precipitation assays using polyclonal antisera against HPLC-purified lavage CP4. Immunoblotting of reduced surfactant and lavage proteins demonstrated intense staining of a broad band that comigrated with authentic pneumocyte CP4 (Figure 6). Overdeveloped blots showed a small fraction of disulfide-bonded immunoreactive components migrating slightly slower and faster than the major CP4 band (Figure 6, lanes 4–6). These disulfide-bonded components are collagenase sensitive and copurify with CP4 (not shown). Immunoblotting of unreduced samples demonstrated selective staining of components with the mobility of unreduced pneumocyte-derived CP4 (Figure 6, lanes 7–9). These antibodies also reacted with newly synthesized CP4 in indirect immune precipitation assays of metabolically labeled type II cell medium and cell lysates (Figure 2). Freshly isolated, alcohol-fixed, and permeabilized type II cells showed specific cytoplasmic staining with the polyclonal anti-CP4 (not shown). There was no detectable cross reaction with type IV procollagen, SP-A, or the low molecular mass hydrophobic peptides in immunoblots.

Isoelectric focusing of the proteins extracted from rat surfactant in the presence of EDTA revealed isoforms of SP-A and small amounts of SP-A dimers in the acidic region. There were also comparatively basic, 43-kDa isoforms (p*I* of 5–6) similar in charge to the most acidic isoforms of pneumocyte-derived CP4 (Figure 7) (Persson et al., 1988). Immunoblotting of parallel gels with anti-CP4 showed selective staining of the 43-kDa components, including more basic isoforms of CP4. The latter components developed more slowly than the acidic isoforms and were below the limits of detection by silver staining. Similar profiles of CP4 isoforms were obtained for HPLC-purified CP4 isolated from lavage supernatant (not shown).

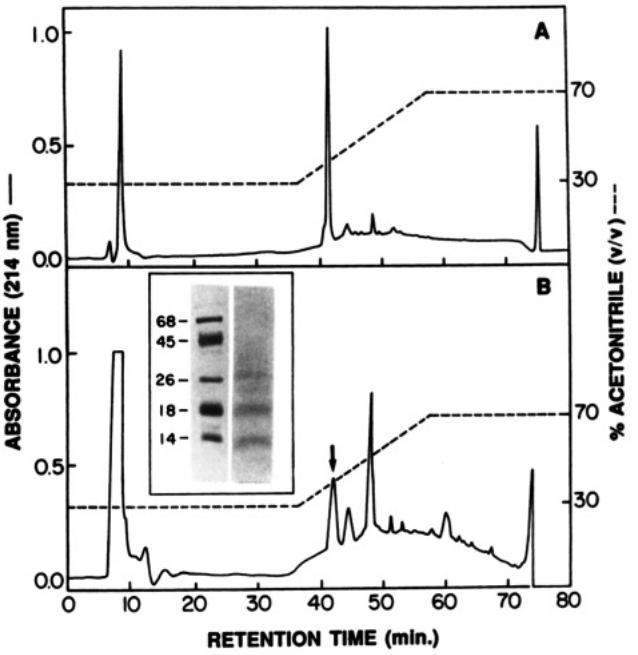

FIGURE 3: Isolation of CP4 and CP4-derived CNBr peptides. Barium-adsorbed and citrate-eluted proteins isolated from the high-speed supernatant of rat lavage were dialyzed against 0.1 N acetic acid and lyophilized. Proteins were redissolved in 0.1% trifluoroacetic acid, resolved by reverse-phase HPLC, and quantified by absorbance at 214 nm as described under Materials and Methods (panel A). The purified CP4, eluting at 41 min (see Figure 1, lane 4), was fragmented with CNBr, lyophilized, and reapplied to the same chromatographic system (panel B). The peak at 42 min (arrow) was pooled and lyophilized. Peptides in this peak were reduced with DTT, resolved by SDS–PAGE on a 15% acrylamide gel, electrotransferred to Immobilon, and stained with Coomassie blue (inset, right lane). Migration was compared to that of reduced globular protein standards (inset, left lane). Peptides migrating at 14 and 18 kDa were submitted for sequencing.

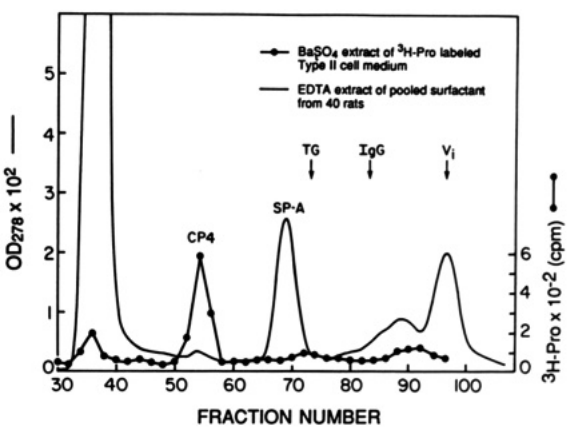

FIGURE 4: Gel filtration of native CP4. The EDTA extract of pooled surfactant from 40 rats was applied to a 1.5 × 165 cm column of 4% agarose (Bio-Rad A-15m) and eluted with extraction buffer at room temperature (solid line). Barium sulfate adsorbed and citrate-eluted pneumocyte-derived CP4 was chromatographed under the same conditions (closed circles). Identity of the radiolabeled pneumocyte CP4 peak was confirmed by SDS–PAGE and fluorography (data not shown). Molecular mass standards include thyroglobulin (TG) and IgG. The included volume (V_i) is also indicated.

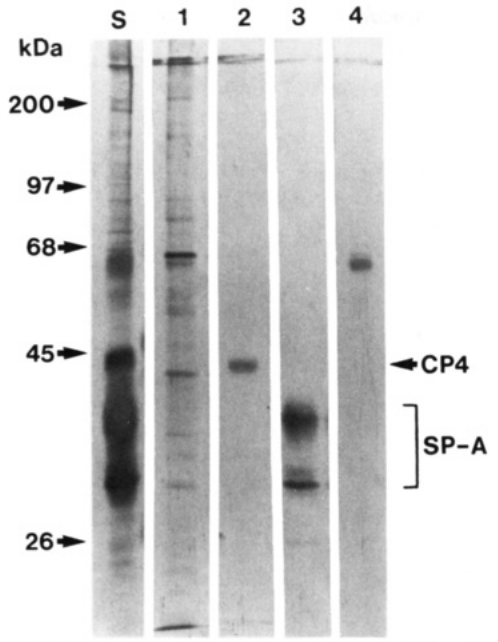

FIGURE 5: SDS–PAGE of surfactant proteins resolved by gel filtration on 4% agarose (Figure 4). Equal aliquots from each peak fraction were subjected to SDS–PAGE on 5%/10% gels following reduction with DTT. Proteins were visualized by silver staining. (Lane S) Starting material containing SP-A and smaller amounts of CP4, reduced; (lane 1) void fraction 39; (lane 2) fraction 55 containing CP4; (lane 3) fraction 69 containing SP-A; (lane 4) fraction 85. The positions of rat SP-A and pneumocyte CP4 at right.

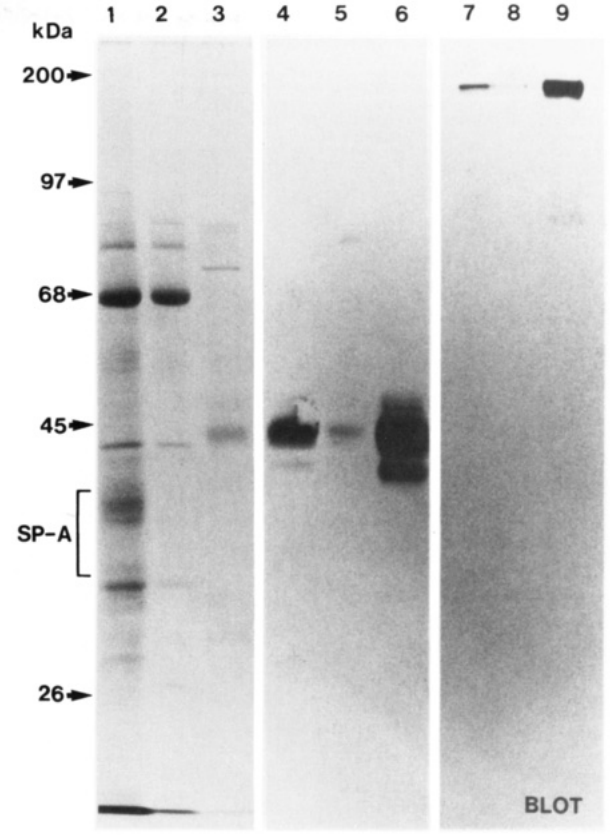

FIGURE 6: Immunoblots of lavage and surfactant CP4. Proteins were resolved by SDS–PAGE on 5%/10% gels, with (lanes 1–6) or without (lanes 7–9) prior reduction with DTT. Silver-stained proteins are at left (lanes 1–3), and immunoblots reacted with antibodies to purified lavage CP4 are at right (lanes 4–9). (Lanes 1, 4, and 7) Unwashed high-speed pellet of lavage; (lanes 2, 5, and 8) high-speed supernatant of lavage; (lanes 3, 6, and 9) barium sulfate adsorbed and citrate-eluted lavage proteins.

DISCUSSION

This paper describes the purification and biochemical characterization of CP4, a collagenous surfactant-associated glycoprotein. Our data provide strong evidence that this protein is identical with a collagenous glycoprotein that we previously isolated from cultures of type II pneumocytes (Persson et al., 1988). The pneumocyte-derived and lung proteins show identical migration on SDS–PAGE in the presence and absence of sulfhydryl reduction, coelute from reverse-phase and gel-filtration columns, and show similar profiles of relatively basic isoforms by isoelectric focusing. All three proteins are efficiently adsorbed to barium salts and can be selectively eluted with 200 mM sodium citrate. In addition, the pneumocyte and surfactant-associated proteins specifically react with antibodies to purified lavage CP4, and the pneumocyte-derived protein cross reacts with antibodies to surfactant proteins (Persson et al., 1988).

We have previously deduced the presence of a collagen-like domain in pneumocyte CP4 on the basis of its sensitivity to purified bacterial collagenase, the identification of radioactive hydroxyproline in the newly synthesized protein, and the inhibitory effects of 2,2′-dipyridyl on CP4 secretion (Persson et al., 1988). The existence of such a domain has now been substantiated by compositional analysis and partial amino acid sequencing of the purified lung protein.

Our studies establish that CP4 is immunologically and chemically different from SP-A. CP4 is not recognized by polyclonal antibodies to rat SP-A (Persson et al., 1988), and SP-A does not cross react with antibodies to rat CP4. The proteins show different behavior in a variety of chromatographic systems, have different subunit and nonreduced molecular masses by SDS–PAGE and molecular sieve chromatography, and have different charge properties. They also show different cyanogen bromide and proteolytic cleavage patterns (Persson et al., 1988). Finally, there are significant differences in amino acid composition, in the primary sequence of their collagenous domains, and in their patterns of posttranslational modification. Notably, CP4 contains hydroxylysine and acid-labile components consistent with hydroxylysine glycosides.

On the other hand, there are several similarities between CP4 and SP-A. Both proteins are hybrid glycoproteins that contain collagenous and noncollagenous sequences, both have similar-sized collagenase-resistant domains (Persson et al., 1988), and both are assembled and secreted by type II cells as high molecular mass disulfide-bonded complexes. In addition, the synthesis and secretion of both proteins decrease dramatically during the first few hours of primary culture of isolated rat pneumocytes (Crouch et al., 1987; unpublished data).

Both proteins also show biochemical similarities to the rat, bovine, and human hepatic mannose-binding proteins (Table I) (Colley & Baenziger, 1987a,b; Drickamer et al., 1986; Drickamer, 1988; Ezekowitz et al., 1988; Kawasaki et al., 1985; Oka et al., 1987) and to bovine conglutinin (Davis & Lachmann, 1984; Strang et al, 1986). All of these proteins contain short collagenous and comparatively large collagenase-resistant domains and are assembled as high molecular mass complexes with disulfide-bonded subunits. Like the mannose-binding proteins and bovine conglutinin, CP4 contains hydroxylysine and hydroxylysine glycosides (Table I) (Colley & Baenziger, 1987a,b; Kawasaki et al., 1985). However, unlike these proteins, CP4 and SP-A contain asparagine-linked (endoglycosidase F and neuraminidase sensitive) oligosaccharides (Persson et al., 1988; Possmayer, 1988).

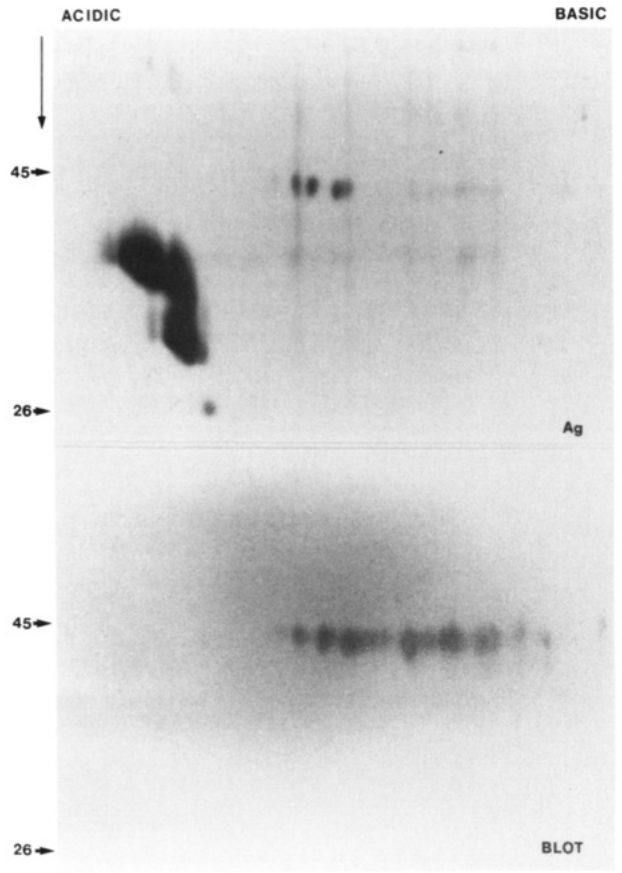

FIGURE 7: EDTA-extracted surfactant-associated proteins were resolved by IEF in the first dimension (acidic, left, and basic, right; measured pH range, 3.5–8.0), followed by SDS–PAGE on 10% acrylamide gels in the second dimension (arrow). Parallel gels were silver stained (top) or electrotransferred to nitrocellulose and visualized by immunoblotting with antibodies to purified rat lavage CP4 (bottom).

We have shown that CP4 can interact with other components of crude surfactant via divalent cation dependent mechanisms (Persson et al., 1988). Thus, the report by Rannels et al. (1987) that SP-A and several other proteins synthesized by rat pneumocytes contain γ-carboxyglutamic acid (Gla) residues led us to examine the binding of CP4 to barium sulfate. Insoluble barium salts have been widely used for the isolation of prothrombin and other vitamin K dependent calcium-binding proteins (Nelsestuen, 1984), and barium sulfate binding properties are believed to be conferred by Gla. As shown in Figures 1 and 2, CP4 is markedly enriched by extraction of lavage or pneumocyte culture medium with barium sulfate. However, our attempts to chemically identify Gla in lung CP4 have been unsuccessful. If Gla is present, it must be present at an average concentration of less than one per chain. We, therefore, suggest that barium binding of CP4 involves non-Gla-dependent mechanisms or requires only small numbers of modified glutamic acid residues per molecule or macromolecular complex. Interestingly, recent studies have also failed to demonstrate the presence of Gla in canine SP-A (Wallin et al., 1988).

Although CP4 can be selectively extracted from washed rat surfactant, we have found that a significant fraction of CP4 is soluble under our conditions of surfactant isolation. Quantitation of CP4 in lavage by immunoblotting or densitometry of silver-stained gels indicates that approximately 90% of the total CP4 is in the 48000*g* supernatant of rat bronchoalveolar lavage; comparable relative recoveries were obtained following sequential barium sulfate extraction and reverse-phase HPLC. By contrast, approximately 50% of the total CP4 in bovine lavage is associated with the surfactant pellet under comparable conditions of lavage (unpublished data). Thus, unlike SP-A and the hydrophobic proteins, CP4 could also function as a soluble protein within the alveoli or distal airways. Alternatively, CP4 could dissociate from surfactant during the isolation and wash procedures.

Phelps and Taeusch (1985) have described a basic charge train of 45-kDa polypeptides, termed "class D" proteins, in crude surfactant from several species. Our studies indicate that CP4 isoforms are the major class D proteins of surfactant. We, therefore, propose that CP4 be renamed SP-D in accordance with suggested nomenclature for the other surfactant-associated proteins (Possmayer, 1988). The biologic function(s) of this protein and the nature of its interactions with other insoluble components in bronchoalveolar lavage remain to be elucidated.

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Primary Structure and Androgen Regulation of a 20-Kilodalton Protein Specific to Rat Ventral Prostate†

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ABSTRACT: Nuclear and cytosolic forms of a 20-kdalton rat ventral prostate protein were purified and partially sequenced from their N-termini. Isolated nuclei were treated with micrococcal nuclease and extracted in 0.6 M NaCl, and proteins were separated by affinity chromatography on Matrex gel green A, ammonium sulfate fractionation, and fast protein liquid chromatography on Superose 12. The 43 amino acid N-terminal sequence of the nuclear 20-kdalton protein was identical with the cytosolic protein except it lacked 7 N-terminal amino acids present in the cytosolic form. The DNA sequence of a full-length complementary DNA clone isolated from a ventral prostate gt11 library extended the N-terminal sequence of the cytosolic form by an additional nine amino acids from the predicted initiation methionine. The cDNA included the nucleotide sequence for the 43 amino acid N-terminal sequence of the purified 20-kdalton protein and predicted molecular weights of 16 686, 17 521, and 18 650, respectively, for the nuclear, cytoplasmic, and nonprocessed proteins. Northern blot analyses of reproductive tract tissue RNAs using the 20-kdalton protein cDNA as probe revealed a single mRNA species of 0.92 kb detectable only in extracts of rat ventral prostate. Expression of the 0.92-kb mRNA was androgen dependent since the mRNA was undetectable in extracts obtained 4 days after castration and was restored 16 h after restimulation with androgen.

Rat ventral prostate synthesizes two proteins in abundance; prostatein (Lea et al., 1979), known also as prostatic binding protein (Heyns et al., 1978; Heyns & Moor, 1977), α-protein (Chen et al., 1979), and estramustine binding protein (Forsgren et al., 1979), is androgen dependent, secreted into prostatic fluid, and binds several steroids with low affinity and high capacity. Prostatein has been reported to function in seminal fluid as a cholesterol carrier protein (Chen et al., 1982). The high concentration of prostatein and its dependence on androgen have made it a useful protein in studies on androgen action in the prostate (Wilson et al., 1981; Perry et al., 1985).

A second abundant protein of rat ventral prostate is a 20-kdalton glycoprotein identified by cell-free translation of ventral prostate messenger RNA and hybridization arrest of translation using cloned complementary DNA (Parker et al., 1978; Chamberlin et al., 1983). The 20-kdalton protein was subsequently purified and shown to be a glycoprotein secreted by rat ventral prostate (Chamberlin et al., 1983; Wang et al., 1986). It is androgen regulated, like prostatein, but does not bind dihydrotestosterone.

Secretory proteins of other regions of the rat prostate include a protein secreted by the dorsal/lateral prostate known as probasin (Matuo et al., 1982a) which has a calculated molecular weight of 18 936 and contains 160 amino acids (R. Matusik, personal communication). Dorsal prostate produces, in large quantities, an androgen-regulated protein referred to as DPI (Wilson & French, 1980).

In this report, we describe the purification of the 20-kdalton protein and partial N-terminal amino acid sequence analysis

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