Role of the C-terminal domain of pulmonary surfactant protein A in binding to alveolar type II cells and regulation of phospholipid secretion

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Surfactant protein A (SP-A), with a reduced denatured molecular mass of 26–38 kDa, is characterized by a collagen-like sequence in the N-terminal half of the protein. This protein forms an oligomeric structure which is dependent upon this collagenous domain. SP-A has been demonstrated to function as an inhibitor of phospholipid secretion by primary cultures of alveolar type II cells via a cell surface receptor for the protein. However, the receptor-binding domain of SP-A has not been identified. The purpose of the present study was to investigate the role of the C-terminal domain of SP-A in binding to type II cells and regulation of phospholipid secretion. A monoclonal antibody to human SP-A, whose epitope was localized at the C-terminal domain of the protein, abolished the inhibitory activity of human SP-A on lipid secretion by type II cells, and attenuated the ability of human SP-A to compete with ^{125}I-(rat SP-A) for receptor binding. SP-A was then digested with collagenase and the collagenase-resistant fragment (CRF), which is the C-terminal domain of SP-A (thus lacking the N-terminal domain), was isolated. Gel filtration chromatography revealed that CRF exists as a monomer in solution containing Ca^{2+}. CRF had the ability to inhibit phospholipid secretion, although at a higher concentration than for SP-A, and was also able to compete with ^{125}I-(rat SP-A) for binding to type II cells. A direct binding study showed that CRF bound to type II cells in a concentration-dependent manner. The present study demonstrates that the non-collagenous, C-terminal, domain of SP-A is responsible for the protein's inhibitory effect on lipid secretion and its binding to type II cells.

INTRODUCTION

Pulmonary surfactant, which stabilizes alveoli by lowering the surface tension at the air–liquid interface, is a complex mixture of lipids and proteins that is synthesized and secreted by alveolar type II cells and is essential for normal respiration (King and Clements, 1972). Surfactant protein A (SP-A) is the major protein component of the surfactant complex (King et al., 1973) and is polymorphic (26–38 kDa) when analysed by electrophoresis under reducing and denaturing conditions. This polymorphism is a consequence of post-translational modifications, principally variable glycosylation of the precursor protein (Floros et al., 1985). The primary structures of canine (Benson et al., 1985), human (White et al., 1985), rat (Sano et al, 1987) and rabbit (Boggaram et al., 1988) SP-As have been determined, and this protein has the striking feature of including a collagen-like sequence. Studies on SP-A have shown that it can function as an inhibitor of phospholipid secretion by primary cultures of alveolar type II cells (Dobbs et al., 1987; Rice et al., 1987). SP-A has been reported to be a Ca^{2+}-binding protein (Haagsman et al., 1990); the presence of the Ca^{2+} ion is required for SP-A to show its inhibitory effect on surfactant lipid secretion (Dobbs et al., 1987). SP-A has also been demonstrated to enhance the uptake of phospholipid liposomes by alveolar type II cells (Wright et al., 1987). The action of SP-A on alveolar type II cells suggested the presence of a specific receptor, and Kuroki et al. (1988a) and Wright et al. (1989) have demonstrated the presence of a receptor for SP-A expressed on alveolar type II cells. The cell surface binding activity of rat SP-A is directly related to its capacity to inhibit surfactant lipid secretion by type II cells (Kuroki et al., 1988b), which suggests that the inhibitory effect of SP-A on lipid secretion occurs via a high-affinity receptor for this protein. However, the receptor-binding domain of SP-A has not been identified.

The purpose of the present study was to investigate the role of the C-terminal domain of SP-A in both its inhibitory effect on surfactant lipid secretion and its binding to type II cell monolayers.

MATERIALS AND METHODS

Purification of SP-A

Surfactant was isolated from lung lavage of patients with alveolar proteinosis or of rats given an intratracheal instillation of silica (Dethloff et al., 1986) by the method of Hawgood et al. (1985) and delipidated by extraction with butan-1-ol (98 ml of butanol/2 ml of surfactant). The butanol-insoluble material was precipitated by centrifugation at 2000 *g* for 30 min. Residual solvent was evaporated under a gentle stream of nitrogen. SP-A was then purified as described previously (Kuroki et al., 1988a). Briefly, after the protein had been suspended in 5 mM Tris buffer (pH 7.4) and dialysed against the same buffer, the suspension was centrifuged at 150000 $g_{av.}$ for 1 h and the supernatant was applied to an affinity column of mannose–Sepharose 6B prepared by the method of Fornstedt and Porath (1975). The SP-A that bound to the column in the presence of 1 mM $CaCl_2$ was eluted with 2 mM EDTA. Further purification was accomplished by gel filtration over Bio-Gel A5m (Bio-Rad). The protein content was estimated by the method of Lowry et al. (1951) using BSA as the standard.

Collagenase treatment of SP-A

Human SP-A was suspended in 50 mM Tris buffer (pH 7.4)

Abbreviations used: SP-A, pulmonary surfactant protein A; CRF, the collagenase-resistant fragment of SP-A; DMEM, Dulbecco's modified Eagle's medium; PtdCho, phosphatidylcholine; PMA, phorbol 12-myristate 13-acetate.

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containing 2 mM $CaCl_2$. Collagenase (from *Clostridium histolyticum*; Advance Biofactures Corp.) was added (500 units/mg of protein) to the SP-A preparation and incubated at 37 °C for 24 h. The collagenase-resistant fragment (CRF) of SP-A was isolated by gel filtration using Bio-Gel A5m. The amino acid sequences of CRF were determined in a model 477A automated gas phase microsequencer (Applied Biosystems Inc., Foster City, CA, U.S.A.) with an on-line model 120A h.p.l.c. column.

Gel filtration

The molecular masses of SP-A and CRF were estimated by gel filtration chromatography. Analysis of CRF using a Bio-Gel A5m column (1.5 cm × 59 cm) was performed in 5 mM Tris buffer (pH 7.4) in the presence or absence of 2 mM $CaCl_2$ at 4 °C. Samples of the protein (0.5–1.0 mg) were applied to the column, and elution was monitored at an absorbance of 280 nm. Blue Dextran, thyroglobulin, ferritin and BSA were used as molecular mass standards.

Primary culture of rat alveolar type II cells and secretion of [^{3}H]phosphatidylcholine

Alveolar type II cells were isolated from adult male Sprague–Dawley rats by tissue dissociation with elastase (Worthington Biochemical Corp.) and purification on metrizamide density gradients by the method of Dobbs and Mason (1979). Type II cells were cultured with [^{3}H]choline (0.5 mCi/ml) overnight in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum. After washing the cells with medium, fresh medium (without radiolabel), protein and phorbol 12-myristate 13-acetate (PMA) were added and incubated for 3 h. Secretion was measured using [^{3}H]phosphatidylcholine as a marker for surfactant and was expressed as percentage secretion (radioactivity in medium/radioactivity in medium plus radioactivity in cells) (Sano et al., 1985). Lactate dehydrogenase activity in media was also measured (Fanestil and Barrows, 1965) to determine the amount of non-specific secretion. The lactate dehydrogenase activity released into the medium did not exceed 3% of the total cellular content in any experiment.

Preparation of ^{125}I-SP-A

Rat SP-A, human SP-A or CRF was iodinated by the method of Bolton and Hunter (1973) using the Bolton–Hunter reagent (Amersham Corp.). The specific radioactivity of the ^{125}I-labelled protein used ranged between 114 and 300 c.p.m./ng. In all preparations, more than 96% of the radioactivity was precipitated by treatment with 10% (w/v) trichloroacetic acid.

Receptor binding of SP-A

The study of binding of ^{125}I-SP-A to alveolar type II cells was carried out as described previously (Kuroki et al., 1988a). Briefly, monolayers of type II cells cultured for 20 h after isolation were incubated with 0.5 or 1.0 μg/ml labelled rat SP-A and the indicated amount of unlabelled proteins for 5 h at 37 °C, and then each dish was placed on ice and the medium was removed. Each monolayer was then washed on ice with ice-cold buffer (50 mM Tris buffer, pH 7.4, containing 0.1 M NaCl, 2 mM $CaCl_2$ and 1 mg/ml BSA). The cells were dissolved in 2 ml of 0.1 M NaOH, and the amount of ^{125}I-SP-A bound to the cells was determined using a γ-radiation counter.

In some experiments 0.2–20 μg/ml ^{125}I-(human SP-A) or ^{125}I-CRF was incubated with alveolar type II cells at 4 °C for 5 h, and the amount of labelled protein bound to the cell was determined as described above.

Monoclonal antibody

Monoclonal antibody PE10 was prepared against human SP-A as described previously (Kuroki et al., 1985).

Electrophoresis and immunoblotting

SDS/PAGE was performed according to the method of Laemmli (1970). Immunoblotting analysis was performed by the method of Towbin et al. (1979). Human SP-A and CRF were separated by SDS/PAGE and transferred electrophoretically to nitrocellulose. The nitrocellulose sheet was treated with 2% (w/v) skim milk in 10 mM phosphate-buffered saline (pH 7.4) to block non-specific binding, and subsequently with monoclonal antibody (1 μg/ml) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (2000 times dilution; Bio-Rad). Incubation with antibody was carried out at 37 °C for 90 min. Diaminobenzidine (1 mg/ml; Katayama Chemicals) was used as the substrate for the peroxidase reaction.

Statistical analysis

Statistical differences between results were assessed by Student's *t* test for independent samples.

RESULTS

Electrophoretic and immunoblotting analysis

SP-A was purified from lavage materials from rats or patients with alveolar proteinosis, and analysed by SDS/PAGE (Figure 1a, lanes B and C). Human SP-A was digested with bacterial collagenase and the isolated CRF was also analysed (Figure 1a, lane D). The N-terminal sequences of human CRF were determined; these were: (i) Gly-Pro-Pro-Gly-Leu-Pro- (beginning at Gly-75), (ii) Gly-Leu-Pro-Ala-His-Leu- (beginning at Gly-78) and (iii) Ala-His-Leu-Asp-Glu-Glu- (beginning at Ala-81). The data indicate that the collagenous domain of SP-A was digested and removed from the SP-A molecule. Sequence analysis of the peptide obtained by cyanogen bromide treatment of CRF revealed that the C-terminus of human CRF is preserved after collagenase digestion of SP-A (results not shown). These data indicate that the CRF obtained is the C-terminal domain of SP-A lacking the N-terminal collagenous domain.

Next, the specificity of monoclonal antibody PE10 was examined by immunoblotting analysis (Figure 1b). PE10 recognized non-glycosylated and glycosylated forms of SP-A with apparent molecular masses of 30 and 35 kDa (lane I) respectively, and oligomers with larger molecular masses. The monoclonal antibody also recognized non-glycosylated and glycosylated forms of CRF with apparent molecular masses of 16 and 20 kDa (lane H) respectively. The data indicate that the epitope for PE10 is localized at the C-terminal domain of SP-A.

Effect of the monoclonal antibody on the inhibition of phospholipid secretion by SP-A and on the binding of SP-A to type II cells

Human SP-A completely inhibited secretion of phospholipid by type II cells under both basal and PMA-stimulated conditions (Table 1). Monoclonal antibody PE10 abolishes the inhibitory

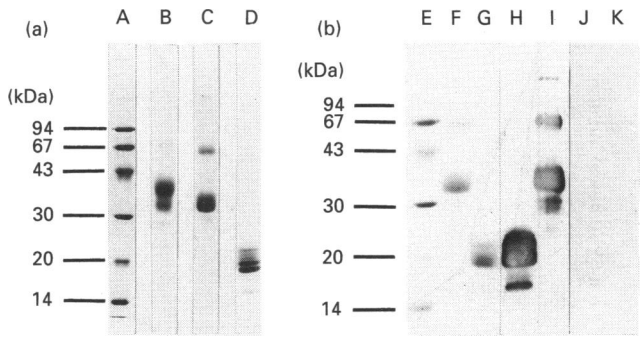

Figure 1 Electrophoretic and immunoblotting analysis of SP-A and CRF

(**a**) Samples (5 μg of protein/lane) of rat SP-A (lane B), human SP-A (lane C) and CRF (lane D) were analysed by SDS/PAGE using a 13% polyacrylamide gel under reducing conditions, and stained with Coomassie Blue. Lane A contains molecular mass standards. (**b**) Proteins were separated by SDS/PAGE, transferred electrophoretically to nitrocellulose and stained with Amido Black (lanes E, F and G). The nitrocellulose sheets were also probed for immunoreactive materials with monoclonal antibody PE10 (lanes H and I) or with control mouse IgG (lanes J and K) as described in the Materials and methods section. Lane E, molecular mass standards; lanes F, I and K, human SP-A; lanes G, H and J, human CRF.

Table 1 The monoclonal antibody PE10 blocks the inhibitory effect of SP-A on lipid secretion by alveolar type II cells

The data are from three separate experiments, with duplicate samples in each experiment. Results are expressed as percentage secretion of [^{3}H]phosphatidylcholine ([^{3}H]PtdCho) (means $\pm$ S.D.) by alveolar type II cells. *$P < 0.001$ when compared with cells incubated in the presence of human SP-A + PMA.

Additions	Secretion of [^{3}H]PtdCho (% of total)
None	1.4 $\pm$ 0.1
PMA (0.1 μM)	11.2 $\pm$ 1.7
Human SP-A (1 μg/ml)	0.4 $\pm$ 0.1
Human SP-A + PMA	0.7 $\pm$ 0.1
PMA + control IgG (20 μg/ml)	11.5 $\pm$ 2.7
PMA + PE10 (20 μg/ml)	10.9 $\pm$ 1.0
Human SP-A + PMA + control IgG	0.9 $\pm$ 0.6
Human SP-A + PMA + PE10	11.2 $\pm$ 1.0*

Table 2 The monoclonal antibody PE10 attenuates the effect of human SP-A on the binding of ^{125}I-(rat SP-A) to alveolar type II cells

The data are from three separate experiments, with duplicate samples in each experiment. Type II cell monolayers were incubated with ^{125}I-(rat SP-A) (0.5 μg/ml) in the presence of human SP-A or antibody at 37 °C for 5 h, and the amount of ^{125}I-(rat SP-A) bound to the cells was determined as described in the Materials and methods section. Results are expressed as ng of protein bound to cells (means $\pm$ S.D.). *$P < 0.01$ when compared with cells incubated with ^{125}I-(rat SP-A) in the presence of human SP-A.

Additions	^{125}I-(Rat SP-A) bound (ng/dish)
None	63.1 $\pm$ 8.9
Human SP-A (5 μg/ml)	24.7 $\pm$ 5.0
Control IgG (50 μg/ml)	62.3 $\pm$ 11.6
PE10 (50 μg/ml)	62.8 $\pm$ 13.4
Human SP-A + control IgG	24.8 $\pm$ 5.0
Human SP-A + PE10	61.6 $\pm$ 9.3*

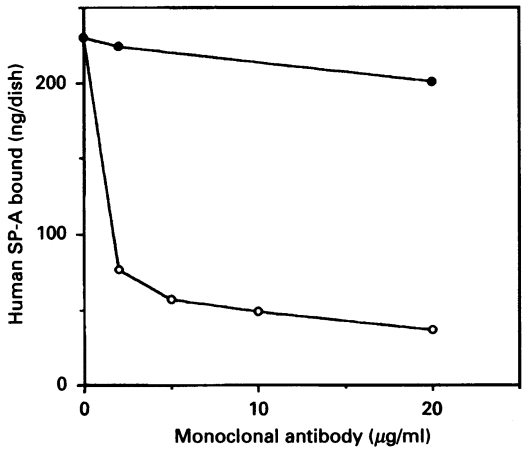

Figure 2 Effect of monoclonal antibody PE10 on the binding of human SP-A to type II cells

Type II cell monolayers were incubated with ^{125}I-(human SP-A) (1 μg/ml) in the presence of PE10 (○) or control monoclonal antibody (●) at concentrations of 0–20 μg/ml at 37 °C for 5 h, and the amounts of ^{125}I-(human SP-A) bound to cells were determined as described in the Materials and methods section. Data presented are from a representative one of three experiments.

action of SP-A on lipid secretion, as shown in Table 1, whereas control IgG failed to affect the activity of SP-A. Since the binding of SP-A to its cell surface receptor is directly related to the inhibitory activity of the protein on surfactant secretion by type II cells (Kuroki et al., 1988b), the effect of the monoclonal antibody on SP-A binding to type II cells was also examined. When ^{125}I-(rat SP-A) at 0.5 μg/ml was incubated with alveolar type II cells in the presence of 5 μg/ml human SP-A, excess human SP-A competed with labelled rat SP-A for binding to type II cells, and reduced binding of the latter by approx. 61% (Table 2). The inclusion of PE10 at 50 μg/ml in the buffer containing labelled rat SP-A and human protein resulted in the loss of the ability of human SP-A to compete with ^{125}I-(rat SP-A) for binding to type II cells. Next, we directly examined whether various concentrations (2–20 μg/ml) of PE10 blocked the binding of ^{125}I-(human SP-A) to type II cells. Binding was reduced by increasing concentrations of PE10, whereas control monoclonal antibody had almost no effect on SP-A binding (Figure 2). This indicates that PE10 may recognize the site of interaction of human SP-A with type II cells. Recognition by PE10 of the CRF, i.e. of the C-terminal domain of human SP-A, suggests that the site in the SP-A molecule responsible for binding to type II cells is located in the C-terminal domain.

Analysis of CRF by gel permeation chromatography

When gel filtration chromatography was performed in the absence of Ca^{2+}, human SP-A was eluted with an apparent molecular mass greater than 700 kDa (Figure 3a), and CRF was eluted with an apparent molecular mass of 100 kDa (Figure 3b), indicating that there had been no contamination by undigested SP-A in the preparation of CRF. In the presence of 2 mM Ca^{2+}, CRF was eluted with a molecular mass of 20 kDa (Figure 3c); this indicates that Ca^{2+} affects the size of the CRF molecule, and that CRF exists as a monomer in Ca^{2+}-containing solutions.

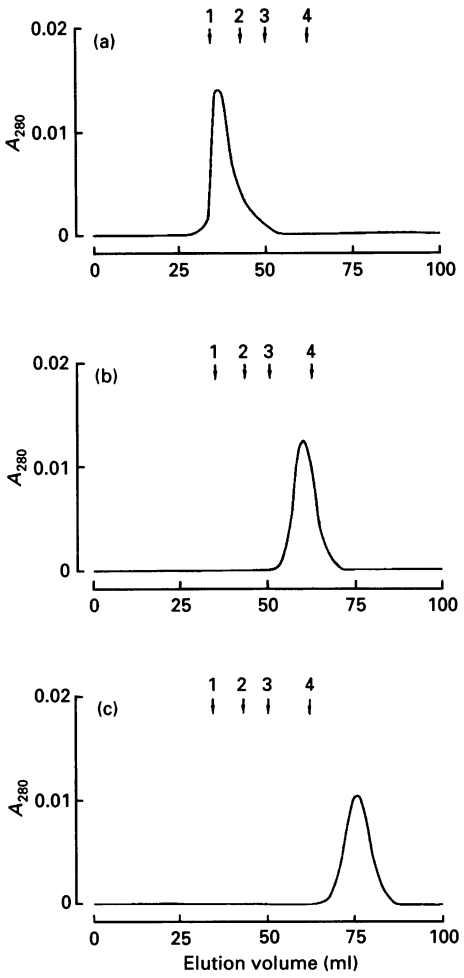

Figure 3 Gel filtration analysis of native human SP-A and CRF

Samples of human SP-A (**a**) and CRF (**b** and **c**) were analysed by gel filtration using 1.5 cm × 59 cm column of Bio-Gel A5m at 4 °C in the absence (**a** and **b**) or presence (**c**) of 2 mM Ca^{2+}. The molecular mass markers are: 1, Blue Dextran; 2, thyroglobulin (670 kDa); 3, ferritin (436 kDa); 4, BSA (67 kDa).

Table 3 CRF retains the ability to inhibit phospholipid secretion by type II cells

The data are from three separate experiments, with duplicate samples in each experiment. Results are expressed as percentage secretion of [^{3}H]phosphatidylcholine ([^{3}H]PtdCho) (means ± S.D.) by alveolar type II cells. $^*P < 0.05$; $^{**}P < 0.02$ when compared with cells incubated in the presence of PMA.

Additions	Secretion of [^{3}H]PtdCho (% of total)
None	1.5 ± 0.9
PMA (0.1 μM)	10.9 ± 2.9
PMA + human SP-A (1 μg/ml)	0.5 ± 0.1
PMA + human SP-A (100 μg/ml)	0.4 ± 0.2
PMA + human CRF (10 μg/ml)	9.1 ± 0.6
PMA + human CRF (20 μg/ml)	6.0 ± 0.6*
PMA + human CRF (50 μg/ml)	3.6 ± 1.2**
PMA + human CRF (100 μg/ml)	2.9 ± 1.3**

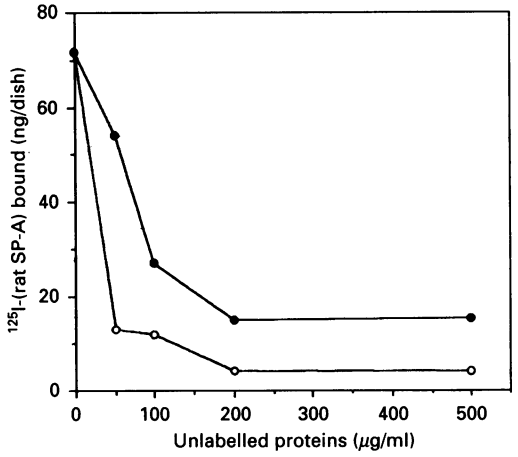

Figure 4 Effect of CRF on the binding of ^{125}I-labelled rat SP-A to type II cells

Type II cell monolayers were incubated with ^{125}I-(rat SP-A) (1 μg/ml) in the presence of human SP-A (○) or human CRF (●) at concentrations of 50–500 μg/ml at 37 °C for 5 h, and the amount of ^{125}I-(rat SP-A) bound to cells was determined as described in the Materials and methods section. Data presented are from a representative one of three experiments.

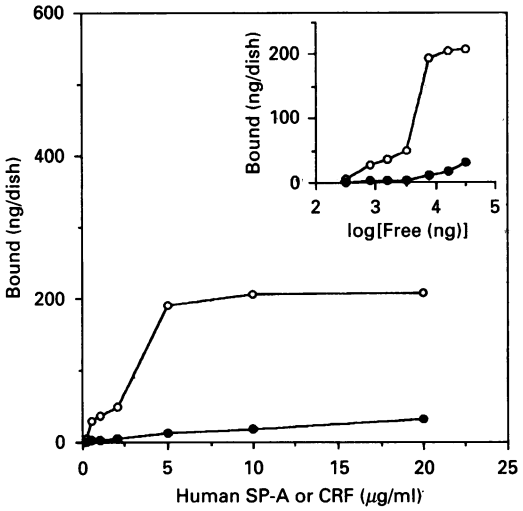

Figure 5 Concentration-dependent binding of human SP-A and CRF to type II cells

Various concentrations of ^{125}I-(human SP-A) (○) or ^{125}I-CRF (●) were incubated with type II cell monolayers at 4 °C for 5 h, and the amounts of the protein bound to cells were determined as described in the Materials and methods section. The results represent the specific binding calculated by the slope peeling method. Data presented are from a representative one of three experiments. Inset: Klotz plot of the binding data.

CRF can inhibit phospholipid secretion by type II cells and can compete with ^{125}I-(rat SP-A) for binding to type II cells

We investigated whether human CRF can affect secretion of surfactant lipid by type II cells. CRF at 1 μg/ml failed to inhibit surfactant lipid secretion; at this concentration native SP-A exhibited maximal inhibition. However, CRF at higher concentrations was able to inhibit phospholipid secretion (Table 3); 20 μg/ml CRF was required to give half-maximal inhibition.

Next, we examined whether CRF competes with ^{125}I-(rat SP-A) for binding to type II cells. Human SP-A at 50 μg/ml reduced binding of labelled rat SP-A by approx. 82% (Figure 4), indicating that human SP-A can compete with rat SP-A for

binding and may interact with the receptor expressed on type II cells. When ^{125}I-(rat SP-A) was incubated with type II cells in the presence of various concentrations of CRF, its binding was reduced in a dose-dependent manner (Figure 4), although CRF was still less effective in competing with ^{125}I-(rat SP-A) when compared with native human SP-A.

We further studied the concentration-dependent binding of human SP-A or CRF to type II cells using ^{125}-labelled human SP-A or CRF. Specific binding was calculated using the slope-peeling method described by Goldstein and Brown (1974) (Figure 5). Human SP-A bound to type II cells in a concentration-dependent manner and reached saturation at 5 μg/ml, demonstrating that human SP-A can bind to rat alveolar type II cells with high affinity. ^{125}I-CRF also bound in a concentration-dependent manner; however, the amounts that bound were quite small.

DISCUSSION

Alveolar type II cells produce and secrete pulmonary surfactant, a complex mixture of lipids and proteins (King and Clements, 1972). The macromolecular structure of SP-A is hexameric, with six globular domains connected by short stalks to a common stem (Voss et al., 1988). This protein is capable of binding phospholipids (Ross et al., 1986; Kuroki and Akino, 1991), carbohydrates (Haagsman et al., 1987) and calcium (Haagsman et al., 1990). Dobbs et al. (1987) and Rice et al. (1987) have reported that SP-A inhibits phospholipid secretion by alveolar type II cells. More recent studies have demonstrated that disulphide bond formation is required for SP-A to inhibit lipid secretion (Kuroki et al., 1988c). Using ^{125}I-labelled SP-A as a probe, this molecule has been demonstrated to bind to a high-affinity receptor on alveolar type II cells (Kuroki et al., 1988a; Wright et al., 1989). Although the oligosaccharide moiety of SP-A does not appear to be involved in receptor binding (Kuroki et al., 1988b,c), the binding domain of SP-A has not yet been identified.

In the present study monoclonal antibody PE10, which recognizes CRF, was found to block the inhibitory activity on phospholipid secretion and the receptor binding activity of human SP-A. The results obtained from this study led us to isolate the non-collagenous C-terminal domain of SP-A and investigate its role. We used CRF isolated from human SP-A, because denaturation with a detergent and a reducing agent was required for complete digestion of rat SP-A with bacterial collagenase. The results demonstrated that human CRF was able to inhibit surfactant lipid secretion and to compete with ^{125}I-(rat SP-A) for binding to type II cells, although much higher concentrations of the protein were required than of native SP-A.

Wright et al. (1989) studied the binding of CRF to alveolar type II cells. CRF bound to these cells, but the amount bound was much lower than for SP-A. When 1 μg/ml SP-A or CRF was incubated with type II cells, 766 pg of SP-A/μg of cell protein bound to the cells, whereas only 99 pg of CRF bound/μg. In the present study, CRF was able to compete with ^{125}I-(rat SP-A) for binding to type II cells, although a vast excess of CRF was required in comparison with native human SP-A. Our result from the competition study essentially confirms the result of Wright et al. (1989) that the C-terminal domain retains its capacity to bind to the cell surface. However, in their study it was not determined whether the C-terminal domain had an inhibitory effect on lipid secretion. In the present study, CRF was demonstrated to possess the ability to inhibit lipid secretion by type II cells, which appeared to be coupled to the binding of the protein to the cells. Competition experiments with human SP-A and CRF for ^{125}I-(rat SP-A) binding and secretion experiments with CRF suggest that multiple receptor populations may be present and that the affinities of CRF and native SP-A for binding to type II cells may be different. We examined the binding of ^{125}I-(human SP-A) and ^{125}I-CRF to type II cells at 4 °C. An analysis of the binding of human SP-A as described by Klotz (1982) (Figure 5, inset) revealed that half-maximal binding occurred at 1.5×10^{-9} M, assuming the molecular mass of native SP-A to be approx. 1.6 MDa (Kuroki et al., 1988a; Pison et al., 1992). There are approx. 5.4×10^4 binding sites/cell for the human SP-A oligomer. In contrast, an analysis of CRF binding (Figure 5, inset) indicates that the curve obtained has not reached an inflection point; obviously, it is not yet near the saturation value. In this situation the Scatchard plot would be an even less reliable method of analysis (Klotz, 1982). Even if we assume that the binding of CRF to type II cells reaches saturation at 20 μg/ml, half-maximal binding would occur at a concentration higher than 4.6×10^{-7} M. Thus we may make only tentative estimates of the binding constants of CRF from the data obtained in the present study, but we can conclude that the binding affinity of CRF is far lower than that of native SP-A. Since CRF is unable to form an oligomer and exists as a monomer in solution, as shown in Figure 3, loss of the integrity of the macromolecular structure of SP-A might change the affinity of this protein for binding to type II cells and result in the requirement of a vast excess CRF to inhibit lipid secretion.

Since SP-A has been shown to bind phospholipids (Ross et al., 1986; Kuroki and Akino, 1991), there was a possibility that this property of the protein had something to do with its binding to type II cells. However, the cell-type specificity and saturation kinetics made it unlikely that the binding reflected a non-specific affinity for phospholipids (Kuroki et al., 1988a). Recent studies from this (Kuroki et al., 1992) and another (Childs et al., 1992) laboratory have revealed that SP-A also binds to glycolipids such as galactosylceramide and asialo-G_{M2}. We, however, found that galactosylceramide and asialo-G_{M2} failed to compete with ^{125}I-SP-A for binding to type II cells (results not shown). Given all of these facts, the binding of SP-A or CRF to type II cells does not appear to be due to the association of the protein with lipids.

Ross et al. (1986) have shown that SAP-18, which lacks both the hydrophobic amino acids Gly^{81}–Val^{117} and the N-terminal collagenous region of SP-A, failed to inhibit phospholipid secretion by alveolar type II cells. However, they used SAP-18 at 10 μg/ml for the inhibition study. In the present study, CRF at 10 μg/ml exhibited almost no effect on lipid secretion by type II cells; half-maximal inhibition was seen at a concentration of approx. 20 μg/ml. It is possible that, during preparation, the CRF was contaminated with a trace of undigested SP-A; this could have inhibited lipid secretion and competed with ^{125}I-SP-A at higher concentrations. We purified CRF using gel filtration chromatography, by which CRF can be completely separated from native SP-A. In the present study, the molecular size of CRF in solution changed to approx. 20 kDa in the presence of Ca^{2+}, indicating that CRF existed as a monomer in the culture medium. CRF possesses a binding site for Ca^{2+} (Haagsman et al., 1990), and the binding of Ca^{2+} to CRF causes a conformational change, which could alter the size of the molecule. Since much higher concentrations of CRF were required for the inhibition of lipid secretion and for competition with ^{125}I-SP-A, it is obvious that oligomeric forms of SP-A participate actively in receptor binding. These results may suggest multiple receptors for SP-A on alveolar type II cells. There is also a possibility that the carbohydrate recognition domain on CRF caused that molecule to act as a lectin for lipid secretion by type II cells; this would

result in the inhibition of secretion, since concanavalin A effectively inhibits surfactant lipid secretion (Rice and Singleton, 1988). However, CRF, unlike concanavalin A, was able to compete with ^{125}I-SP-A for binding to type II cells. In addition, CRF was also able to inhibit lipid secretion even in the presence of 0.1 M mannose (results not shown), suggesting that the action of CRF on type II cells is not due to a lectin property, at least not against a mannose ligand. Concanavalin A does not compete with ^{125}I-SP-A for binding to type II cells (Kuroki et al., 1988b). A recent study using colloidal gold immunocytochemistry in conjunction with electron microscopy has also shown that the lectin activity of SP-A is not required for binding to type II cells (Thorkelsson et al., 1982).

Although the relationship between the inhibitory action of SP-A on phospholipid secretion and receptor occupancy is unclear, the results of the present study suggest that the binding site of the SP-A molecule for a receptor expressed on type II cells is located in the C-terminal domain. The present study also indicates that the intermolecular disulphide bond in the N-terminal region of SP-A is not directly involved in the binding of SP-A to type II cells, although it appears to be essential for formation of the oligomeric structure (Haagsman et al., 1989; Haas et al., 1991). Although it is unclear which portion in the C-terminal domain binds to type II cells, the C-terminal region from Gly^{200} appears to be more important than the hydrophobic region (Gly^{81}–Val^{117}) of CRF, since monoclonal antibody PE10, which blocks SP-A activity, recognizes the C-terminal part of CRF (N. Hiraike and T. Akino, unpublished work). The epitope for PE10 is now under investigation.

In conclusion, the present study demonstrates that the non-collagenous, C-terminal, domain of SP-A is responsible for the inhibitory effect of this protein on lipid secretion and for binding to its receptor.

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