Human surfactant protein A with two distinct oligomeric structures which exhibit different capacities to interact with alveolar type II cells

Akiko HATTORI, Yoshio KUROKI*, Hitoshi SOHMA, Yoshinori OGASAWARA and Toyoaki AKINO

Department of Biochemistry, Sapporo Medical University School of Medicine, South-1 West-17, Chuo-ku, Sapporo 060, Japan

The lung lavage fluids from patients with pulmonary alveolar proteinosis have been generally used as a source for human surfactant protein A (SP-A). We have recently found that a multimerized form of SP-A oligomer (alveolar proteinosis protein-I, APP-I) exists besides the normal-sized octadecamer (APP-II) in SP-As isolated from the patients. When analysed by Bio-Gel A15m column chromatography in 5 mM Tris buffer (pH 7.4), the apparent molecular masses of APP-I and APP-II were 1.65 MDa and 0.93 MDa, respectively. Gel-filtration analysis also revealed that APP-II is clearly separated from APP-I in the presence of 2 mM Ca^{2+} and 150 mM NaCl. We investigated the abilities of both SP-A oligomers to regulate phospholipid secretion and to bind to alveolar type II cells. Although APP-I inhibited lipid secretion, it was clearly a less effective inhibitor than APP-II. IC_{50} for inhibition of lipid secretion was apparently $0.23 \pm 0.08 \mu\text{g/ml}$ ($0.14 \pm 0.05 \text{ nM}$) and $0.055 \pm 0.019 \mu\text{g/ml}$ ($0.059 \pm 0.020 \text{ nM}$) for APP-I and APP-II, respectively. Both proteins bound to monolayers of type II cells in a concentration-dependent manner; however, APP-I clearly had a lower affinity

to bind to type II cells. The apparent dissociation constants were, $K_d = 2.31 \pm 0.70 \mu\text{g/ml}$ ($1.40 \pm 0.43 \text{ nM}$) and $0.89 \pm 0.22 \mu\text{g/ml}$ ($0.95 \pm 0.24 \text{ nM}$) for APP-I and APP-II, respectively. Excess unlabelled rat SP-A replaced 45 % of ^{125}I -APP-I and 77 % of ^{125}I -APP-II for type II cell binding. Although ^{125}I -APP-II competed with excess unlabelled APP-I or APP-II, ^{125}I -APP-I failed to compete and instead its binding rather increased in the presence of unlabelled APPs. The biotinylated APP-I bound to APP-I and APP-II coated on to microtitre wells in a concentration-dependent manner, indicating that APP-I interacts with APPs. This study demonstrates that the multimerized form of human SP-A oligomer exhibits the following attributes: (1) the reduced capacity to regulate phospholipid secretion from type II cells, and (2) lower affinity to bind to type II cells, and that the integrity of a flower-bouquet-like octadecameric structure of SP-A oligomer is important for the expression of full activity of this protein, indicating the importance of the oligomeric structure of mammalian lectins with collagenous domains.

INTRODUCTION

Alveolar type II cells synthesize and secrete pulmonary surfactant that prevents the alveoli from collapsing at the end of expiration. Surfactant protein A (SP-A) [1] is the major protein component of the surfactant complex and exhibits a reduced and denatured molecular mass of 35 kDa in humans. SP-A has been shown to have a flower-bouquet-like octadecameric structure similar to that of complement C1q [2]. SP-A interacts with lipids and specifically binds to dipalmitoylphosphatidylcholine (DPPC) [3]. SP-A has been shown to function as an inhibitor of phospholipid secretion from alveolar type II cells [4,5] and to bind to a high-affinity receptor on these cells [6,7]. This cell-surface binding activity is directly related to the capacity of SP-A to inhibit lipid secretion [8]. SP-A also enhances the uptake of lipids by type II cells [9].

The lung lavage fluids from patients with pulmonary alveolar proteinosis (PAP) [10,11] have been generally used as a source of human SP-A because large amounts of surfactant-rich materials are available for therapeutic purposes. We have recently found that in PAP patients there exists a multimerized form of SP-A (alveolar proteinosis protein, APP) [12]. Electron-microscopic study revealed that APP-II was observed as hexameric particles presumably consisting mainly of octadecamers which

appeared to possess an oligomeric structure similar to that of normal SP-A. APP-I, however, was observed as multimerized larger aggregates consisting of two or more octadecamers. Although APP-I and APP-II both had similar abilities to bind to DPPC and to induce phospholipid vesicle aggregation, APP-I abnormally affected phospholipid membrane organization [12]. Unlike APP-II, APP-I failed to form a tubular myelin structure *in vitro* in association with surfactant protein B. Since SP-A may play an important role in surfactant phospholipid homeostasis in the alveolar space, we investigated the abilities of SP-As with two distinct oligomeric structures to regulate phospholipid secretion and to bind to type II cells.

MATERIALS AND METHODS

Purification of human SP-A oligomers

Surfactant was isolated from the lung lavage fluids of patients with PAP, and delipidated by extraction with butanol by the method of Hawgood et al. [13]. The butanol-insoluble material was then precipitated by centrifugation at $1000 g_{av}$ for 30 min and residual solvent was evaporated under a gentle stream of nitrogen. The derived SP-A (APP) was then purified from the obtained pellet by the method described previously [14]. Briefly,

Abbreviations used: SP-A, surfactant protein A; PAP, pulmonary alveolar proteinosis; APP, alveolar proteinosis protein (SP-A isolated from individuals with PAP); PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; HRP, horseradish peroxidase; DPPC, dipalmitoylphosphatidylcholine.

* To whom all correspondence should be addressed.

the protein pellet was suspended in 5 mM Tris buffer (pH 7.4) and dialysed against the same buffer. The suspension was then centrifuged at $100\,000\ g_{av}$ for 1 h, and the supernatant was applied to an affinity column of mannose-Sepharose 6B as described by the method of Fornstedt and Porath [15]. The APP that bound to the column in the presence of 1 mM $CaCl_2$ was then eluted with 2 mM EDTA. Further purification of APP was accomplished by gel filtration over a Bio-Gel A5m column (Bio-Rad; $1.5\ cm \times 59\ cm$) equilibrated with 5 mM Tris buffer (pH 7.4). The purified APP was recovered as one peak under these conditions. The rat SP-A was also purified in a similar way to that described above by the method published previously [6]. The two distinct populations of APP oligomers were separated by gel-filtration chromatography over Bio-Gel A15m (Bio-Rad, $1.4\ cm \times 120\ cm$) that had been equilibrated with 5 mM Tris buffer (pH 7.4). The protein peaks corresponding to APP-I and APP-II were separately collected, concentrated and rechromatographed for further purification, and then used for the experiments. Blue dextran, thyroglobulin (669 kDa), ferritin (440 kDa) and catalase (232 kDa) were used as molecular-mass standards. To examine the elution profiles of APP-I and APP-II in the presence of Ca^{2+} , gel-filtration analysis was also performed in 5 mM Tris buffer (pH 7.4) containing 2 mM $CaCl_2$ and 150 mM NaCl.

Protein content was estimated using the method of Lowry et al. [16]. SDS/PAGE was performed according to the method of Laemmli [17].

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

Alveolar type II cells were isolated from adult male Sprague-Dawley rats by tissue dissociation with elastase, and purification was performed using metrizamide density gradients as described by the method of Dobbs and Mason [18]. Type II cells were cultured overnight with [3H]choline (0.5 $\mu Ci/ml$) in Dulbecco's modified Eagle's medium (DMEM) which contained 10% (v/v) fetal-calf serum. After washing the cells with DMEM which contained 1 mg/ml BSA, fresh DMEM (without radiolabel), protein and phorbol 12-myristate 13-acetate (PMA) were added and the mixture was incubated for 3 h. Surfactant secretion was measured using [3H]phosphatidylcholine as a marker and secretion was expressed as a percentage (radioactivity in medium/radioactivity in medium plus radioactivity in cells) [19].

Iodination of APP-I and APP-II

APP-I and APP-II were iodinated by the method of Bolton and Hunter [20] using Bolton-Hunter reagent (Amersham). The specific radioactivity of the ^{125}I -labelled protein used ranged from 204 to 642 c.p.m./ng. In all experiments, more than 96% of the radioactivity was precipitated by treatment with 10% (w/v) trichloroacetic acid.

Binding of APP-I and APP-II to alveolar type II cells

The study of the binding characteristics of ^{125}I -APP-I and ^{125}I -APP-II to alveolar type II cells was performed as described previously [6]. Briefly, monolayers of type II cells cultured for 20 h after isolation were incubated with labelled proteins of the appropriate concentrations for 5 h at 37 °C in DMEM containing 10% (v/v) fetal-calf serum. 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), and then finally the cells were dissolved in 2 ml of 0.1 M NaOH, and the amount of labelled protein was determined using a γ -radiation counter.

To examine the internalized pool of ^{125}I -APP, type II cells were treated with PBS containing 4 mg/ml trypsin and 1 mM EDTA on ice for 10 min after ^{125}I -APP binding at 37 °C as described previously [6]. After incubation with trypsin, the cells were harvested and centrifuged at 4 °C for 10 min. The radioactivities of the supernatant and the pellet were then counted.

In some experiments 0.5 $\mu g/ml$ of ^{125}I -APP-I or ^{125}I -APP-II was incubated with type II cells in the presence of excess amounts of unlabelled rat SP-A or APPs at 37 °C for 5 h, and the amount of labelled protein bound to the cells was determined as described above.

Trypsin treatment of APP-I and APP-II at 0 °C

APP-I and APP-II (8.3 μg each) were incubated with PBS (total volume 22 μl each) containing 4 mg/ml trypsin and 1 mM EDTA at 0 °C for 10 min. PMSF (100 mM, 2.4 μl) and 20% (w/v) SDS (8.1 μl) were added at final concentrations of 10 mM and 5%, respectively, to stop the reaction. An aliquot (16.4 μl) of sample buffer containing 8 M urea, 2% SDS, 0.01% Bromophenol Blue and 50 mM dithiothreitol was added. The proteins (3.4 μg as APP-I or APP-II per lane) were electrophoresed under reducing conditions at 4 °C using a 13% polyacrylamide gel, and then transferred to nitrocellulose sheets, and stained with Amido Black or immunostained with monoclonal anti-(human SP-A) antibody followed by horseradish peroxidase (HRP)-labelled anti-(mouse IgG). The SP-A was finally detected using diaminobenzidine as a substrate.

Binding of biotinylated APP-I to APPs

N-Hydroxysuccinimidobiotin (NHS-biotin, Pierce Chemicals) was used for introducing biotin moieties into APP-I. After APP-I was dialysed against 0.1 M $NaHCO_3$, 10 vol. of APP-I was mixed with 1 vol. of NHS-biotin solution (1 mg/ml in DMSO). The reaction mixture was incubated at room temperature for 3 h and then dialysed against 5 mM Tris buffer (pH 7.4).

To examine whether APP-I binds to APPs, unlabelled protein (50 μl , 1 $\mu g/ml$) was coated on to microtitre wells at 4 °C overnight. The unlabelled protein-coated wells were then incubated with various concentrations of biotinylated APP-I at 37 °C for 90 min after blocking the non-specific binding with PBS containing 0.5% (v/v) Triton X-100 and 3% (w/v) skimmed milk (buffer A). HRP-labelled avidin D (50 μl , 2 $\mu g/ml$ in buffer A) was then incubated at room temperature for 20 min, and the biotinylated APP-I bound to the solid-phase protein was detected by adding 100 μl of the substrate solution (0.1 M sodium citrate buffer, pH 4.6, containing 0.03% H_2O_2 and 1 mg/ml *o*-phenylenediamine). The reaction was finally stopped by the addition of 100 μl of 2 M sulphuric acid after incubation with the substrate solution for 20 min in the dark. The absorbance at 490 nm was read in a multiplate reader.

RESULTS

Analysis of two forms of human SP-A oligomers by gel filtration and electrophoresis

Purified samples of APP-I and APP-II were separately applied to the Bio-Gel A15m column. APP-I eluted at a position near that of Blue Dextran (Figure 1A) and APP-II eluted far behind Blue Dextran but ahead of thyroglobulin (Figure 1B). The apparent molecular masses of APP-I and APP-II in solution were calculated as 1.65 MDa and 0.93 MDa respectively. To examine whether APP-II forms larger aggregates similar to APP-I in the presence of Ca^{2+} , gel-filtration analysis was also performed in the

Figure 1 Gel-filtration chromatography of two forms of human SP-A oligomers

APP-I (A) and APP-II (B) were applied to a Bio-Gel A15m column (1.4 cm $\times$ 120 cm) equilibrated with 5 mM Tris buffer (pH 7.4) and monitored at 280 nm. The molecular-mass standards used are Blue Dextran (arrow 1), thyroglobulin (arrow 2), ferritin (arrow 3) and catalase (arrow 4).

Figure 2 Gel-filtration analysis of APP-I and APP-II in the presence of 2 mM Ca^{2+} and 150 mM NaCl

Aliquots (16 μg each) of purified APP-I (●) and APP-II (○) were separately applied to a Bio-Gel A15m column equilibrated with 5 mM Tris buffer (pH 7.4) containing 2 mM CaCl_2 and 150 mM NaCl. Eluted fractions (50 μl) were coated on to microtitre wells and reacted with anti-SP-A rabbit IgG followed by HRP-labelled anti-(rabbit IgG). APP-I and APP-II were finally detected with α -phenylenediamine as a substrate and measured at 490 nm.

presence of 2 mM Ca^{2+} and 150 mM NaCl (Figure 2). The peak of APP-II was clearly separated from that of APP-I. APP-I eluted at the position of the void volume under this condition.

When APP-I and APP-II were analysed using SDS/PAGE under reducing conditions, the proteins exhibited main bands with apparent molecular masses of 36 kDa and 62 kDa (Figure 3A, lanes a and b). Bands of higher molecular masses were observed under non-reducing conditions; in fact, most of the APP-I and some of the APP-II did not even enter the stacking gel (Figure 3A, lanes c and d). ^{125}I -labelled APP-I and APP-II were also examined by electrophoresis (Figure 3B). The major forms of the radiolabelled proteins correlated well with the major forms observed using the unlabelled protein. There is little evidence to

Figure 3 Electrophoretic analysis of APP-I and APP-II, and their iodinated forms

(A) Samples (5 μg of protein per lane) of APP-I (lanes a and c) and APP-II (lanes b and d) were analysed on SDS/13%-polyacrylamide gels under reducing (lanes a and b) and non-reducing conditions (lanes c and d). Proteins were stained with Coomassie Blue. (B) Preparations (5×10^5 c.p.m.) of ^{125}I -APP-I (lanes e and g) and ^{125}I -APP-II (lanes f and h) were analysed by electrophoresis in 13% polyacrylamide gels under reducing (lanes e and f) and non-reducing conditions (lanes g and h) and then autoradiographed. St., molecular mass standards.

suggest that iodination of the protein caused any marked protein degradation.

Effects of APP-I and APP-II on surfactant secretion from alveolar type II cells

Both APP-I and APP-II inhibited the secretion of [^3H]phosphatidylcholine from alveolar type II cells in a manner dependent upon APP concentration (Figure 4). However, the inhibitory strength of the two APPs were different. The IC_{50} of inhibition of

Figure 4 Inhibition of lipid secretion from alveolar type II cells by APP-I and APP-II

Freshly isolated rat alveolar type II cells were radiolabelled with [^3H]choline overnight. After non-adherent cells and unincorporated radiolabels were removed by washing, the secretagogue PMA (100 nM) and 0.03, 0.1 and 1.0 $\mu\text{g/ml}$ of APP-I (●) and APP-II (○) were incubated with the cells for 3 h. Media and cells were harvested and percentage secretion was defined as radioactivity in medium/radioactivity in (medium + cells). Maximum secretion stimulated by PMA was $8.3 \pm 1.7\%$ (mean $\pm$ S.E.M., $n = 4$). Results are expressed as percentages of the PMA-stimulated secretion. The data shown are means $\pm$ S.E.M., $n = 4$. Basal secretion was $20 \pm 5\%$ of the PMA-stimulated secretion (mean $\pm$ S.E.M., $n = 4$).

Figure 5 Trypsin treatment of APP-I and APP-II at 0 °C

APP-I and APP-II (3.4 µg each per lane) were incubated with PBS containing 4 mg/ml trypsin and 1 mM EDTA at 0 °C for 10 min. PMSF (100 mM) and 20% SDS were added at final concentrations of 10 mM and 5%, respectively, to stop the reaction. The proteins were electrophoresed under reducing conditions at 4 °C using a 13% polyacrylamide gel, and then transferred to nitrocellulose sheets, and stained with Amido Black (lanes a, b, c and d) or immunostained with monoclonal anti-(human SP-A) antibody (lanes e and f), as described under the Materials and methods section. Lanes a, c and e, APP-I; lanes b, d and f, APP-II. Lanes a and b, trypsin absent (–); lanes c, d, e and f, trypsin present (+).

Figure 6 Binding of APP-I and APP-II to alveolar type II cells

(A) Concentration-dependent binding of APP-I and APP-II to type II cell monolayers. Type II cell monolayers were incubated with 0.2–10 µg/ml ¹²⁵I-APP-I (●) or ¹²⁵I-APP-II (○) at 37 °C for 5 h, and then the amounts of the protein bound to cells were determined. The results represent the specific binding calculated by the slope peeling method. Data presented are means ± S.E.M. ($n = 3$) at 0.2–2 µg/ml and means of two experiments at 5 and 10 µg/ml. (B) Scatchard plot analysis. Data corresponding to 0.2–5 µg/ml ¹²⁵I-APP-I (●) or ¹²⁵I-APP-II (○) were replotted by the method of Scatchard. Abbreviations: B, bound; F, free.

[³H]phosphatidylcholine secretion by APP-II was apparently 0.055 ± 0.019 µg/ml (mean ± S.E.M., $n = 4$). In contrast, that by APP-I was apparently 0.23 ± 0.08 µg/ml. Since the apparent molecular masses of APP-I and APP-II in solution are different, the IC_{50} values of APP-I and APP-II were calculated as a function of molar concentration; the results being 0.14 ± 0.05 nM and 0.059 ± 0.02 nM respectively. When secretion experiments were performed in the presence of 0.2 M mannose, both proteins inhibited lipid secretion from type II cells; the IC_{50} values of APP-I and APP-II being 0.22 and 0.04 µg/ml respectively (means of two experiments). This suggests that the inhibitory effects on

lipid secretion of both isoforms of APP are not due to a lectin property against mannose ligands and may occur via a receptor expressed on type II cells that binds to rat SP-A [8]. These results demonstrate that APP-I is less effective than APP-II in inhibiting lipid secretion from alveolar type II cells.

Trypsin treatment of APP-I and APP-II at 0 °C

To examine whether both APPs exhibit equivalent sensitivities to the protease attack, we treated the proteins of APP-I and APP-II with 4 mg/ml trypsin at 0 °C and analysed APPs by electrophoresis. The control lanes of trypsin (–) exhibited significant bands with higher molecular masses in addition to the bands of 36 and 62 kDa (Figure 5, lanes a and b); it is likely that the aggregates of APPs were not well resolved because the electrophoresis was performed at 4 °C. After trypsin treatment, the bands with higher molecular masses, 62 and 36 kDa, disappeared when stained with Amido Black, except the band of trypsin (approx. 24 kDa), in both protein samples (Figure 5, lanes c and d). When the nitrocellulose membranes were immunostained with anti-(human SP-A) monoclonal antibody, only broad bands between 24 and 30 kDa were detected (Figure 5, lanes e and f). This indicates that trypsin treatment degrades both APP-I and APP-II to an equivalent degree.

Binding of APP-I and APP-II to alveolar type II cells

Since the binding of SP-A to type II cells is directly responsible for its capacity to inhibit surfactant lipid secretion [8], we investigated the concentration-dependent binding of APP-I and APP-II to these cells using ¹²⁵I-labelled APP-I and APP-II. Specific binding was calculated using the slope peeling method described by Goldstein and Brown [21]. Both APP-I and APP-II bound to monolayers of alveolar type II cells in a concentration-dependent manner with the amount of binding reaching a maximum at 5 µg/ml (Figure 6A). The amounts of APP-I bound to the cells, however, were clearly less than those of APP-II at lower concentrations.

The total binding was operationally subdivided into two separate pools; one pool of APP is protease labile and is likely to exist at the cell surface; the second pool of APP becomes refractory to the action of trypsin, which is presumed to be an internalized pool. From 9 to 12% of total binding of each protein became resistant to trypsin. This suggests that the internalized and surface-bound pools may be almost equivalent between APP-I and APP-II, since the control experiments without cells indicate that the sensitivities of APP-I and APP-II to the trypsin attack (4 mg/ml at 0 °C) are almost the same as described above.

We next determined the binding constants. When the binding of these proteins to type II cells was further analysed using the method of Scatchard [22], APP-I and APP-II exhibited quite different Scatchard plots, although both plots yielded curves with positive slopes indicative of positive co-operativity (Figure 6B). When these same data were analysed using Hill plots, the coefficients n_H were 1.34 and 1.50 for APP-I and APP-II respectively, and the apparent dissociation constants K'_d were 2.31 ± 0.70 µg/ml (mean ± S.E.M., $n = 3$) and 0.89 ± 0.22 µg/ml respectively. When calculated as an oligomeric molecular mass, K'_d became 1.40 ± 0.43 nM (mean ± S.E.M., $n = 3$) and 0.95 ± 0.24 nM for APP-I and APP-II respectively.

We also examined whether rat SP-A competed with labelled APP-I or APP-II for type II cell binding. When 0.5 µg/ml of ¹²⁵I-APP-I or ¹²⁵I-APP-II was incubated with various concentrations of unlabelled rat SP-A, rat SP-A competed with ¹²⁵I-APP-II more effectively than ¹²⁵I-APP-I (Figure 7). A concentration of 50 µg/ml of rat SP-A replaced 77% of the labelled APP-II but

Figure 7 Competition of ^{125}I -APP-I and ^{125}I -APP-II with unlabelled rat SP-A for type II cell binding

Type II cell monolayers were incubated with $0.5\text{ }\mu\text{g/ml}$ ^{125}I -APP-I (●) or ^{125}I -APP-II (○) in the presence of various concentrations of rat SP-A at $37\text{ }^{\circ}\text{C}$ for 5 h, and the amounts of the radiolabelled protein which bound to cells were determined. The amounts of ^{125}I -APP-I and ^{125}I -APP-II which bound in the absence of rat SP-A (control binding) were 52.2 and 109.4 ng/dish respectively. The results are expressed as percentages of control binding. Data presented are from a representative one of three experiments.

Table 1 Competition of ^{125}I -APP-I and ^{125}I -APP-II with excess unlabelled APPs for type II cell binding

Type II cells were incubated with radiolabelled APP-I or APP-II ($0.5\text{ }\mu\text{g/ml}$) in the absence (control) or the presence of APP-I or APP-II ($20\text{ }\mu\text{g/ml}$). Control (100%) binding was 49.6 ± 6.2 ng/dish for ^{125}I -APP-I or 100 ± 10.1 ng/dish for ^{125}I -APP-II (mean $\pm$ S.E.M., $n = 3$). Data shown are means $\pm$ S.E.M. ($n = 3$).

Unlabelled protein	Radiolabelled APP bound (% of control)	
	APP-I	APP-II
APP-I	118 ± 8	63 ± 5
APP-II	125 ± 5	26 ± 3

only 45% of the labelled APP-I. We also examined whether unlabelled APP-I or APP-II ($20\text{ }\mu\text{g/ml}$) competed with the iodinated APP-I or APP-II ($0.5\text{ }\mu\text{g/ml}$) (Table 1). Unlabelled APP-II replaced approx. 74% of ^{125}I -APP-II but unlabelled APP-I reduced the binding of ^{125}I -APP-II only by 37%. In reverse, unlabelled APP-I or APP-II failed to compete with ^{125}I -APP-I for type II cell binding. Cell binding rather increased in the presence of excess unlabelled APPs. The results indicate that APP-I has a lower affinity to bind to type II cells than APP-II, and that APP-I exhibits high non-specific binding.

Binding of biotinylated APP-I to APPs

Excess unlabelled APP-I and APP-II increased the binding of ^{125}I -APP-I to type II cells as described above; this raised a possibility that APP-I may interact with APP-I and APP-II. To pursue this idea, we examined whether APP-I binds to APPs. The biotinylated APP-I was incubated with APP-I, APP-II, rat SP-A and BSA coated on to microtitre wells, and the APP-I bound to the solid-phase protein was determined by HRP-labelled avidin D (Figure 8). The biotinylated APP-I bound to APP-I and APP-

Figure 8 Binding of biotinylated APP-I to APPs

APP-I (●), APP-II (○), rat SP-A (△) and BSA (□) were coated on to microtitre wells ($50\text{ }\mu\text{l}$, $1\text{ }\mu\text{g/ml}$). The biotinylated APP-I at 0 – $5\text{ }\mu\text{g/ml}$ was then added into the wells and incubated at $37\text{ }^{\circ}\text{C}$ for 90 min followed by incubation with HRP-labelled avidin D ($50\text{ }\mu\text{l}$, $2\text{ }\mu\text{g/ml}$). The biotinylated APP-I bound to the unlabelled proteins coated on to the wells was finally detected using α -phenylenediamine as substrate, as described in the Materials and methods section. Data presented are from a representative one of three experiments.

II coated on to microtitre wells in a concentration-dependent manner. The biotinylated APP-I appears to exhibit some binding to rat SP-A, but its binding is clearly less than that to APPs. The data clearly indicate that APP-I interacts with APPs.

DISCUSSION

The present study demonstrates that multimerized larger aggregates of human SP-A oligomer (APP-I) isolated from patients with PAP exhibit a reduced capacity to regulate lipid secretion from type II cells compared with the normal SP-A oligomer (APP-II). The mechanism by which these larger aggregates of SP-A oligomers form is unknown. This may be a consequence of the structural modification of the protein caused by long-term accumulation in the alveoli. A previous study from this laboratory showed that the collagenase-resistant fragment of APP (a mixture of APP-I and APP-II) existed as a monomer when analysed by Bio-Gel A5m column chromatography [23]. This suggests that the collagen-like domain is involved in the formation of large aggregates of APP oligomer (APP-I) since larger molecular masses of the collagenase-resistant fragment of APP were not observed after the digestion of the collagen-like domain.

We performed the binding experiments at $37\text{ }^{\circ}\text{C}$ because these proteins, especially APP-I, are likely to precipitate at $0\text{ }^{\circ}\text{C}$ in the presence of calcium ions. Thus, we examined two pools of ^{125}I -APPs by trypsin treatment after type II cell binding. From 9 to 12% of total binding of each protein became resistant to trypsin, suggesting that the internalized and surface-bound pools do not appear to be different between APP-I and APP-II. Since $\sim 30\%$ of rat SP-A is trypsin-resistant [6], the internalized pool of APPs appears to be smaller than that of rat SP-A. Although the interpretation of the binding constants presented in this study may not be able to lead to an exact understanding of the kinetics of cell-surface receptor binding, we can confidently conclude that APP-II interacts with type II cells with a higher affinity than APP-I and that the latter inhibits lipid secretion less effectively. When the abilities of APP-I and APP-II to inhibit lipid secretion

from type II cells were compared as a function of molar concentrations, the IC_{50} of lipid secretion by APP-I was 2.4-times higher than that by APP-II. The apparent K'_d of APP-I was also found to be higher than that of APP-II when the binding of these proteins to type II cells was analysed. These results are consistent with the previous observation that the receptor-binding activity of SP-A is directly related to its capacity to inhibit lipid secretion from type II cells [8].

Rat SP-A inhibits lipid secretion below the basal level at $1 \mu\text{g/ml}$ and the IC_{50} of rat SP-A for inhibition is $\sim 0.1 \mu\text{g/ml}$ [4]. Rat SP-A binds to type II cells with an apparent K'_d of $\sim 1 \mu\text{g/ml}$ [6]. In our study APP-II also potently inhibited lipid secretion below the basal level at $1 \mu\text{g/ml}$ with an IC_{50} of $0.055 \mu\text{g/ml}$, and a K'_d for type II cell binding of $0.89 \mu\text{g/ml}$. Our results indicate that the capacity of APP-II to regulate lipid secretion from alveolar type II cells is close to that of normal SP-A isolated from animals. APP-II may self-aggregate in DMEM, resulting in the formation of larger aggregates similar to APP-I, since SP-A has been shown to aggregate in the presence of millimolar concentrations of Ca^{2+} [24]. However, APP-II was clearly separated from APP-I in the presence of 2 mM Ca^{2+} and 150 mM NaCl (see Figure 2). Although we do not know the exact molecular sizes of APPs or normal SP-A in DMEM at 37°C , and it is uncertain whether normal SP-A with octadecamers changes its oligomeric structure in DMEM at 37°C , the present study is consistent with our recent study that APP-II was observed as an octadecamer (flower-bouquet-like structure) similar to normal SP-A and possesses the ability to form a tubular myelin structure *in vitro* like normal SP-A [12]; in contrast, APP-I does not appear to form a flower-bouquet-like structure but forms the multimerized large aggregate [12]. Since the flower-bouquet-like octadecamer is the basic structure of normal SP-A [2], the present study supports the idea that the integrity of the flower-bouquet-like octadecameric structure of SP-A is important in order for this protein to be fully active.

When the labelled APP-I or APP-II was incubated with type II cells in the presence of excess unlabelled rat SP-A, rat SP-A competed with both the labelled human proteins but did so with ^{125}I -APP-II more effectively than with ^{125}I -APP-I. Rat SP-A replaced 77% of APP-II but only 45% of APP-I. This may simply mean that both human proteins bind to the same receptor as rat SP-A but that APP-I exhibits higher non-specific binding to type II cells than APP-II. When ^{125}I -APP-I or ^{125}I -APP-II was then co-incubated with unlabelled APP-I or APP-II, the unlabelled proteins competed reasonably well with ^{125}I -APP-II for type II cell binding. However, the unlabelled APPs failed to reduce the binding of ^{125}I -APP-I to type II cells and they even increased labelled APP-I binding. This inconsistent result could be explained by the binding study of the biotinylated APP-I to the proteins; APP-I bound to APPs. When ^{125}I -APP-I was incubated with type II cells in the presence of excess unlabelled APPs, it is likely that the binding of ^{125}I -APP-I to unlabelled APP molecules, which had bound to SP-A receptor on type II cells, could occur since APP-I can interact with APPs. Unlabelled rat SP-A competed with ^{125}I -APP-I for type II cell binding but did so less effectively than with ^{125}I -APP-II; this may be due to the interaction of APP-I with rat SP-A since APP-I also appears to bind to rat SP-A albeit to a lesser extent. It is unclear how the labelled APP-I interacts with APPs or why APP-I binds to APPs to a greater extent than to rat SP-A. APP-I may cause aggregation of itself and APPs since significant self-aggregation is observed in APP-I [12].

There arose a possibility that the ability of APP-I to inhibit phosphatidylcholine secretion is caused by the contaminated APP-II in the APP-I fraction. When we analysed APP-I and APP-II, which had been purified in 5 mM Tris buffer ($\text{pH } 7.4$) in the presence of 2 mM Ca^{2+} and 150 mM NaCl by gel filtration over Bio-Gel A15m, we found that APP-I was clearly separated from APP-II, although a very small amount of APP-II appears to exist in the peak of APP-I (see Figure 2). In spite of this small contamination of APP-II in the APP-I fraction, APP-I is clearly less effective than APP-II in inhibiting lipid secretion.

The data presented provide evidence that the multimerized large aggregate form of human SP-A oligomer does not correctly regulate lipid secretion and does not bind to type II cells as easily as normal SP-A oligomer. We believe that this impaired performance is due to the lack of integrity of the octadecameric structure of the abnormal SP-A. If SP-A's role is to prevent futile accumulation of extracellular surfactant by regulating lipid secretion from type II cells, then the abnormal APP oligomer with its impaired ability to inhibit surfactant secretion may cause the excess accumulation of surfactant in the alveoli of patients with PAP. Since SP-A belongs to the group of mammalian lectins with collagenous domains in which the proteins possess the unique oligomeric structure such as a flower-bouquet and a crucifix [25], the present study demonstrates the importance of the oligomeric structure of group III C-type lectins for the expression of the function.

REFERENCES

- 1 Possmayer, F. (1988) *Am. Rev. Respir. Dis.* **138**, 990–998
- 2 Voss, T., Eistetter, H., Schäfer, K. P. and Engel, J. (1988) *J. Mol. Biol.* **201**, 219–227
- 3 Kuroki, Y. and Akino, T. (1991) *J. Biol. Chem.* **266**, 3068–3073
- 4 Dobbs, L. G., Wright, J. R., Hawgood, S., Gonzalez, R., Venstrom, K. and Nellenbogen, J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1010–1014
- 5 Rice, W., Ross, G. F., Singleton, F. M., Dingle, S. and Whitsett, J. A. (1987) *J. Appl. Physiol.* **63**, 692–698
- 6 Kuroki, Y., Mason, R. J. and Voelker, D. R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5566–5570
- 7 Wright, J. R., Borchelt, J. D. and Hawgood, S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5410–5414
- 8 Kuroki, Y., Mason, R. J. and Voelker, D. R. (1988) *J. Biol. Chem.* **263**, 17596–17602
- 9 Wright, J. R., Wager, R. E., Hawgood, S., Dobbs, L. and Clements, J. A. (1987) *J. Biol. Chem.* **262**, 2888–2894
- 10 Rosen, S. H., Castleman, B. and Liebow, A. A. (1958) *N. Engl. J. Med.* **258**, 1123–1142
- 11 Hook, G. E. R., Bell, D. Y., Gilmore, L. B., Nadeau, D., Reasor, M. J. and Talley, F. A. (1978) *Lab. Invest.* **39**, 342–357
- 12 Hattori, A., Kuroki, Y., Katoh, T., Takahashi, H., Shen, H.-Q., Suzuki, Y. and Akino, T. (1996) *Am. J. Respir. Cell Mol. Biol.*, in the press
- 13 Hawgood, S., Benson, B. and Hamilton, R. J. (1985) *Biochemistry* **24**, 184–190
- 14 Kuroki, Y., Tsutahara, S., Shijubo, N., Takahashi, H., Shiratori, M., Hattori, A., Honda, Y., Abe, S. and Akino, T. (1993) *Am. Rev. Respir. Dis.* **147**, 723–729
- 15 Fornstedt, N. and Porath, J. (1975) *FEBS Lett.* **57**, 187–191
- 16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 17 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 18 Dobbs, L. G. and Mason, R. J. (1979) *J. Clin. Invest.* **63**, 378–387
- 19 Sano, K., Voelker, D. R. and Mason, R. J. (1985) *J. Biol. Chem.* **260**, 12725–12729
- 20 Bolton, A. E. and Hunter, W. M. (1973) *Biochem. J.* **133**, 529–539
- 21 Goldstein, J. L. and Brown, M. S. (1974) *J. Biol. Chem.* **249**, 5153–5162
- 22 Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51**, 660–672
- 23 Murata, Y., Kuroki, Y. and Akino, T. (1993) *Biochem. J.* **291**, 71–76
- 24 Haagsman, H. P., Sargeant, T., Hauschka, P. V., Benson, B. J. and Hawgood, S. (1990) *Biochemistry* **29**, 8894–8900
- 25 Thiel, S. and Reid, K. B. M. (1989) *FEBS Lett.* **250**, 78–84