Biochem. J. (1995) 312, 175–181 (Printed in Great Britain)

Ca^{2+}-dependent binding of annexin IV to surfactant protein A and lamellar bodies in alveolar type II cells

Hitoshi SOHMA,*‡ Norio MATSUSHIMA,† Tomoyuki WATANABE,* Akiko HATTORI,* Yoshio KUROKI* and Toyoaki AKINO*

*Department of Biochemistry, Sapporo Medical University School of Medicine, and †School of Health Sciences, Sapporo 060, Japan

Surfactant protein A (SP-A), a lung-specific glycoprotein in pulmonary surfactant, is synthesized and secreted from the alveolar type II cells. It has been shown that SP-A is a Ca^{2+}-binding protein with several binding sites and that the high-affinity site(s) is located in the C-terminal region of SP-A. In the present study we isolated the proteins from bovine lung soluble fraction that bind to SP-A in a Ca^{2+}-dependent manner using DEAE-Sephacel and SP-A-conjugated Sepharose 4B. At least three different protein bands with molecular masses of 24.5, 32, and 33 kDa were observed on SDS/PAGE. The main protein, with molecular mass of 32 kDa, was identified as annexin IV by the partial-amino-acid-sequence analyses and an immunoblot analysis with anti-(annexin IV) antiserum. We also found from the immunoblot analysis that the cytosolic fraction of isolated rat alveolar type II cells contains annexin IV. In addition, when rat lung cytosol was loaded on to the lung lamellar body-conjugated Sepharose 4B in the presence of Ca^{2+}, two proteins, with molecular masses of 32 and 60 kDa on SDS/PAGE respectively, were eluted with EGTA. The 32 kDa protein was shown to be annexin IV by an immunoblot analysis with the antiserum against annexin IV. The lung annexin IV augmented the Ca^{2+}-induced aggregation of the lung lamellar bodies from rats. However, the augmentation of aggregation of the lung lamellar bodies by annexin IV was attenuated when the lamellar bodies were preincubated with polyclonal anti-SP-A antibodies. SP-A bound to annexin IV under conditions where contaminated lipid was removed. These results suggest that SP-A bound to annexin IV based on protein–protein interaction, though both proteins are phospholipid-binding proteins. All these findings suggest that the interaction between SP-A and annexin IV may have some role in alveolar type II cells.

INTRODUCTION

Pulmonary surfactant is a mixture of lipids and proteins that function to stabilize alveoli by lowering the surface tension at the air/liquid interface [1,2]. For accomplishment of this function surfactant has to be synthesized and secreted by alveolar type II cells. Four different kinds of lung specific surfactant apoprotein, surfactant protein-A, -B, -C and -D (SP-A, -B, -C and -D), have been identified. SP-A, which is the most abundant protein in pulmonary surfactant, is a hydrophilic glycoprotein that has a molecular mass of 28–36 kDa on the basis of reducing SDS/PAGE. SP-A possesses some striking features: it has a collagen-like sequence in the N-terminal half [3], it forms oligomers through the interaction of the collagen-like domains [4] and its tertiary structure is similar to the complement factor C1q [5,6]. SP-A preferentially binds dipalmitoyl phosphatidylcholine (DPPC), an abundant fraction of surfactant lipids [7]. The domain having a carbohydrate with an N-glycosidic bond and a non-covalent phospholipid-binding region are located in the collagenase-resistant fragment (CRF) of SP-A, which is located in the residual C-terminal half with an apparent molecular mass of 20 kDa [8].

It has been shown that elevation of intracellular Ca^{2+} or cyclic AMP, or activation of protein kinase C, stimulates surfactant secretion from the type II cells [9]. However, the molecular mechanism for the secretion of the lamellar bodies from the type II cells is unclear at present. Ca^{2+} regulation of secretion in many cell types has been known [10]. SP-A is a Ca^{2+}-binding protein with several binding sites, and a high-affinity site is located in CRF [11,12]. Our previous study revealed that a Ca^{2+}-dependent conformational change in SP-A was observed on binding of Ca^{2+} to the CRF portion [13]. It has been demonstrated that SP-A causes Ca^{2+}-dependent aggregation of phospholipid liposomes [14] and facilitates the formation of a tubular myelin structure in the presence of Ca^{2+} and SP-B [15,16]. The biological activities of SP-A, however, requires a millimollar range of Ca^{2+} concentrations, in which both high- and low-affinity sites are occupied with Ca^{2+}. We anticipated that the high-affinity Ca^{2+}-binding site of SP-A may have distinct function in cytosol.

In the present study we have investigated the presence of Ca^{2+}-dependent SP-A binding protein in cytosol by SP-A–Sepharose 4B affinity chromatography. We identified annexin IV as the main protein involved in the binding to SP-A in a Ca^{2+}-dependent manner.

EXPERIMENTAL

Preparations of human SP-A

Surfactant was isolated from lung lavage of patients with alveolar proteinosis and delipidated with butan-1-ol by the method of Hawgood et al. [16]. SP-A was purified from the surfactant as described previously [17]. Briefly, the organic-solvent-insoluble precipitate was suspended in 5 mM Tris, pH 7.4, and dialysed against the same buffer. The dialysed sample was then centrifuged at 150000 *g* for 1 h and the supernatant was loaded on to a mannose–Sepharose 6B column [18] that had been equilibrated with 5 mM Tris (pH 7.4)/1 mM $CaCl_2$. SP-A was eluted with 2 mM EDTA. Further purification was accomplished by gel filtration with Sephacryl S300 and 5 mM Tris, pH 7.4.

Isolation of lamellar bodies from rat lung

Lamellar bodies were isolated from male Sprague–Dawley rat lungs by the method of Duck-Chong [19]. Briefly, the lungs were

Abbreviations used: SP-A, SP-B etc., surfactant protein A, B etc.; CRF, collagenase-resistant fragment of SP-A; TFA, trifluoroacetic acid; PVDF, poly(vinylidene difluoride); PTH, phenylthiohydantoin; CAPS, cyclohexylaminopropanesulphonic acid; DPPC, dipalmitoyl phosphatidylcholine.

‡ To whom correspondence should be addressed.

perfused through the pulmonary arteries with 0.25 M sucrose. The lungs were minced and homogenized with 1 M sucrose using a motor-driven Teflon pestle and glass homogenizer and then filtered through two layers of cheesecloth. It was layered under a sucrose gradient formed by consecutively layering 0.8–0.2 M sucrose in 0.1 M increments and then centrifuged at 80000 *g* at 4 °C for 3 h. The lamellar-body band was located at the 0.4/0.5 M sucrose interface. The lamellar bodies collected from the sucrose gradient (10 ml) were diluted with 90 ml of 20 mM Hepes, pH 7.5, and pelleted by centrifugation at 60000 *g* at 4 °C for 30 min. The lamellar-body pellets were resuspended in 0.24 M sucrose/20 mM Hepes (pH 7.5)/30 mM NaCl, probe-sonicated with a Sonifier cell disruptor (Heat Systems–Ultrasonics Inc.) for 10 s and stored in ice until use.

Affinity chromatography

Sepharose 4B was activated with CNBr by the method of Kohn and Wilchek [20]. Coupling was performed by incubation of the activated Sepharose 4B (3 ml) with human SP-A (1.5 mg in 0.1 M Na_2CO_3/10 μM EDTA) for 30 h at 4 °C for SP-A–Sepharose 4B preparation. Purified annexin IV (200 μg) was dialysed against 10 mM CHAPS/0.1 mM EGTA/10 mM Hepes, pH 7.5, and then against 0.1 M sodium bicarbonate, before coupling the annexin IV to Sepharose 4B. Coupling was performed by incubation of the activated Sepharose 4B (1 ml) with the annexin IV for 20 h at 4 °C. The method for coupling of the adrenal chromaffin granules to activated Sepharose 4B developed by Creutz [21] was used for preparing lung lamellar body–Sepharose 4B. Lamellar bodies in 0.24 M sucrose/20 mM Hepes (pH 7.5)/30 mM NaCl, containing 400 μg of proteins and 1.5 μmol of phospholipids were incubated with the activated Sepharose 4B (1 ml) for 2 h at 20 °C.

Preparation of SP-A binding protein from lung

All steps were carried out at 4 °C, except where indicated. Frozen bovine lung (50 g) was thawed into 500 ml of homogenizing buffer [10 mM Tris (pH 7.5)/10% sucrose/0.1 mM EGTA/2.5 μg/ml soybean trypsin inhibitor/0.1 mM PMSF] and homogenized three times for 20 s with the blender, with 30 s intervals between each homogenization. The homogenate was centrifuged at 1000 *g* for 10 min. The supernatant was collected, filtered though two layers of cheesecloth, and then centrifuged at 80000 *g* for 1 h. The supernatant was loaded on to DEAE-Sephacel (100 ml) that had been equilibrated with 20 mM Hepes (pH 7.5)/0.1 mM EGTA. After the column had been washed with the equilibration buffer, elution was performed with 500 ml of 0.1 M NaCl/20 mM Hepes (pH 7.5)/0.1 mM EGTA. The 0.1 M NaCl eluate was collected, and then $CaCl_2$ and $MgCl_2$ were added to final concentrations of 1 mM and 5 mM respectively and finally loaded on to SP-A-Sepharose 4B equilibrated with 20 mM Hepes (pH 7.5)/0.1 M NaCl/1 mM $CaCl_2$/and 5 mM $MgCl_2$. The column was washed with the equilibration buffer, then with 20 mM Hepes (pH 7.5)/0.5 M NaCl/0.5 mM $CaCl_2$, and then elution was carried out with 20 mM Hepes (pH 7.5)/0.1 M NaCl/1 mM EGTA. The peak fraction eluted with the elution buffer was collected (20 ml) and dialysed against 1 litre of 10 mM Hepes (pH 7.5)/0.1 mM EGTA for 12 h. The dialysed sample was centrifuged at 80000 *g* for 1 h, and the supernatant was loaded on to a Mono Q FPLC system equilibrated with 20 mM Hepes (pH 7.5)/0.1 mM EGTA. The column was washed with the equilibration buffer at a flow rate of 0.5 ml/min and then elution was carried out with linear gradient from 0 to 50% elution buffer [0.5 M NaCl/20 mM Hepes (pH 7.5)/0.1 mM EGTA] over 50 min at 20 °C.

Enzymic digestion of SP-A binding protein

The SP-A binding protein was digested with either trypsin (Sigma) or *Staphylococcus aureus* proteinase (Pierce). A 75 μg portion of protein in 0.15 ml of 0.1 M NaCl/20 mM Hepes (pH 7.5)/0.1 mM EGTA was incubated with 5 μg of trypsin at 37 °C for 3.5 h, or with 7.5 μg of lysyl-endopeptidase at 25 °C for 20 h. After incubation, 0.45 ml of 1% trifluoroacetic acid (TFA) was added to the protein solution to terminate the reaction. The peptide fragments generated were separated by reverse-phase HPLC using a Cosmosil 5C18P-300 column (4.6 mm × 150 mm; Nacalai Tesque, Kyoto, Japan) with a linear gradient of acetonitrile from 0 to 60% in 0.1% TFA over 60 min at a flow rate of 0.5 ml/min.

Amino-acid-sequence analysis

Amino acid sequences of the peptide fragments of the SP-A binding protein were determined using an Applied Biosystems model 477A protein/peptide sequencer equipped with an on-line phenylthiohydantoin (PTH) analyser (model 120A).

Immunoblots

For immunoblot analysis, the protein samples were resolved by SDS/PAGE and then transferred on to a poly(vinylidene difluoride) (PVDF) membrane (Immobilon-P, Millipore) for 20 h at 10 V in the presence of transfer buffer [10 mM cyclohexylaminopropanesulphonic acid (CAPS) (pH 11)/10% (v/v) methanol]. The blots were blocked with 2% dried Skimmilk in 10 mM PBS and then probed with rabbit anti-(annexin IV) antiserum that was kindly provided by Professor C. E. Creutz, Department of Pharmacology, University of Virginia, Charlottesville, VA, U.S.A. All blots were incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grobe, PA, U.S.A.) at 37 °C for 2 h. Finally, after washing, diaminobenzidine (1 mg/ml; Katayama chemicals, Osaka, Japan) and 0.03% H_2O_2 in 10 mM PBS were added as substrates.

Preparation of rat alveolar type II cells and their cytosol fractions

Alveolar type II cells were isolated from adult male Sprague–Dawley rats by tissue dissociation with elastase (Worthington Biochemical Corp.) and purified on metrizamide density gradients by the method of Dobbs and Mason [22]. The 5×10^7 cells were suspended in 5 ml of homogenization buffer [0.1 M NaCl/10 mM Hepes (pH 7.5)/0.1 mM EGTA/2.5 μg/ml soybean trypsin inhibitor/0.1 mM PMSF], and homogenized with 20 strokes in a Dounce homogenizer on ice. The homogenate was filtered through two layers of cheesecloth and centrifuged for 10 min at 1000 *g*. The supernatant was collected and centrifuged again at 80000 *g* for 1 h. The supernatant was collected as the cytosol.

Assay of annexin IV activity

Aggregating activity of annexin IV was assayed as described Creutz et al. [23] by measuring the increase in A_{400} nm of rat lung lamellar bodies on addition of $CaCl_2$.

Antibodies

Polyclonal antibodies against human SP-A and bovine brain G-protein α-subunit ($G_i\alpha$) [24] were prepared with rabbits. IgG fractions of the antisera were obtained using a Protein A–Sepharose CL-4B. The antibodies against human SP-A cross-

reacted with rat SP-A (results not shown). The IgG fraction was then dialysed against 10 mM PBS.

Other methods

Protein concentrations were determined by the method of Lowry et al. [25]. 0.1 %-SDS/PAGE was performed by the method of Laemmli [26]. The content of phospholipids of the lamellar bodies was determined by measuring phospholipid phosphorus [27].

RESULTS

In order to determine if a Ca^{2+}-dependent SP-A-binding protein exists in lung soluble fractions, SP-A–Sepharose 4B affinity chromatography was performed. Bovine lung soluble proteins were first loaded on to DEAE-Sephacel, the fractions eluted with 0.1 M NaCl were collected (results not shown), and then $MgCl_2$ and $CaCl_2$ to final concentrations of 5 mM and 1 mM respectively were added. The pooled fraction was loaded on to SP-A-Sepharose 4B (Figure 1a). The column was washed with two successive buffers containing 0.1 and 0.5 M NaCl, respectively (see the Experimental section), and the elution was performed with 1 mM EGTA (Figure 1a). A peak that contained proteins apparently bound to SP-A in a Ca^{2+}-dependent manner appeared. The fractions designated with solid bar were pooled (Figure 1a). The SDS/PAGE pattern of the pooled fraction shows a main band with an apparent molecular mass of 32 kDa and two other minor bands with apparent molecular masses of 24.5 and 33 kDa (Figure 1c, lane S). In order to isolate the 32 kDa main protein, the mixture was loaded on to a Mono Q equipped with an FPLC system (Figure 1b). Four peaks were eluted with a linear gradient of NaCl from 0 to 0.25 M. The 32 kDa protein was eluted at about 0.08 M NaCl with a single band on SDS/PAGE (Figure 1b, fraction 1, and Figure 1c, lane 1), while both fractions 3 and 4 contained proteins of 33 kDa at 0.09 and 0.1 M NaCl respectively (Figure 1b, and Figure 1c, lanes 3 and 4) and fractions 5 and 6 contained proteins of 25 kDa at 0.14 M NaCl (Figure 1b and Figure 1c, lanes 5 and 6).

For the purpose of identifying the purified 32 kDa main protein it was digested with either trypsin or *S. aureus* proteinase and the amino acid sequences of the fragments analysed (see the Experimental section). After the enzymic digestion, the mixture of peptide fragments was loaded on to an HPLC reverse-phase column (C_{18}) and elution was performed with linear gradient of acetonitrile from 0 to 50 % in 0.1 % TFA (results not shown).

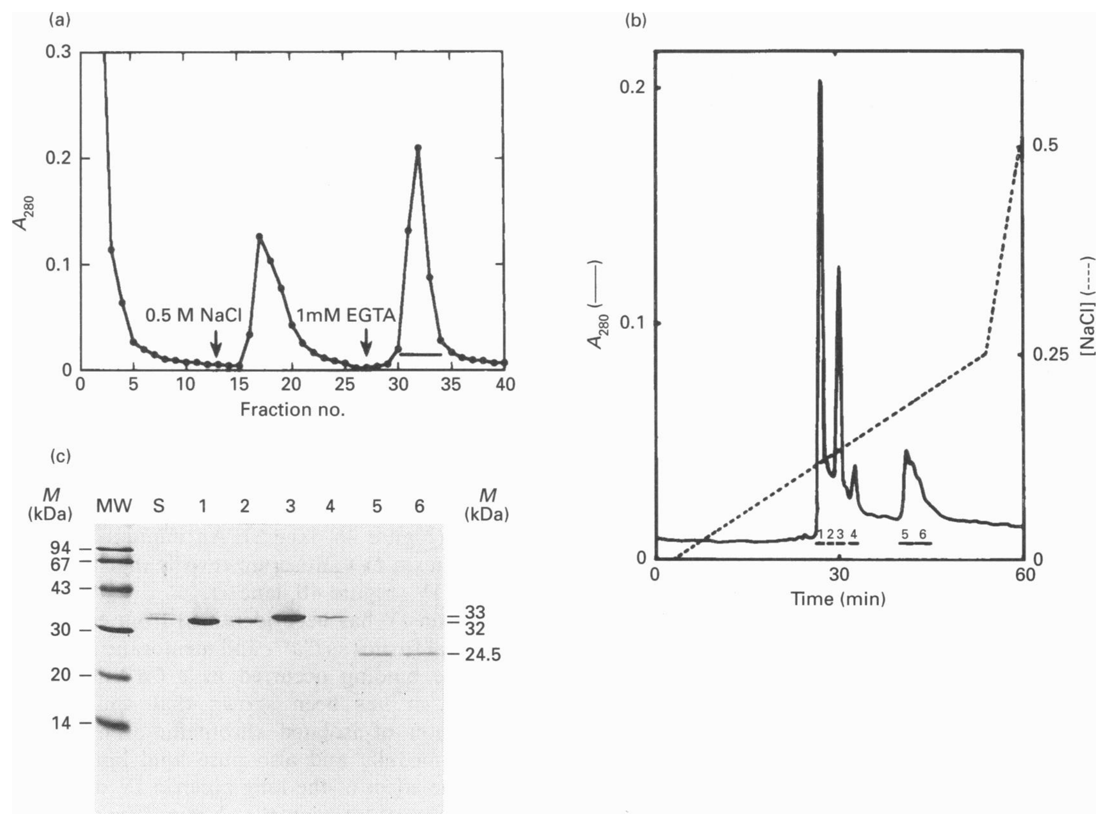

Figure 1 (a) SP-A–Sepharose 4B affinity chromatography, (b) separation of Ca^{2+}-dependent SP-A-binding proteins by FPLC Mono Q chromatography and (c) SDS/PAGE analysis of proteins obtained from SP-A–Sepharose 4B and Mono Q chromatography

(**a**) $MgCl_2$ and $CaCl_2$ were added to the DEAE-Sephacel pool (0.1 M NaCl eluate; see the Experimental section) to final concentrations of 1.0 and 5.0 mM respectively. The sample was loaded on to the SP-A–Sepharose 4B (3 ml) equilibrated with 20 mM Hepes (pH 7.5)/5 mM $MgCl_2$/1 mM $CaCl_2$/and 0.1 M NaCl and the column was washed with the equilibration buffer, then with 0.5 M NaCl/20 mM Hepes(pH 7.5)/0.5 mM $CaCl_2$. Finally elution was carried out with 1 mM EGTA/0.1 M NaCl/20 mM Hepes, pH 7.5. The peak fractions designated by a solid bar were pooled as Ca^{2+}-dependent SP-A-binding proteins. (**b**) The pooled fractions (20 ml) after the SP-A–Sepharose 4B chromatography was dialysed against 1 litre of 10 mM Hepes (pH 7.5)/0.1 mM EGTA. After centrifugation at 80000 ***g*** for 1 h, the sample was loaded on to Mono Q HR 5/5 equilibrated with 20 mM Hepes (pH 7.5)/0.1 mM EGTA and the column was washed with the equilibration buffer at flow rate of 0.5 ml/min and eluted with linear gradient of NaCl from 0 to 0.25 M. (**c**) Lane S, pooled fraction after SP-A–Sepharose 4B; lanes 1–6, fractions 1–6 of Mono Q chromatography designated with solid bars respectively; lane MW, molecular-mass (*M*) standards.

```
AAKGGTVKAA SGFNAAEDAQ TIRKAMKGLG TDEDAIINVL AYRSTAQRQE (1-50)
                          (T1)GLG TDEDAIINVL AYR

IRTAYKTTIG RDLMDDLKSE LSGNFEQVIL GMMTPTVLYD VQELRKAMKG (51-100)
                 (V1) LSGNFE

AGTDEGCLIE ILASRTPEEI RRINQTYQLQ YGRSLEDDIR SDTSFMFQRV (101-150)
      (V2) ILASRTPE

LVSISAGGRD ESNYLDDALM RQDAQDLYEA GEKKWGTDEV KFLTVLCSRN (151-200)
       (V3)SNYLDDALM RQDAQDLYE
                               (V4)A GEKKWGTDE

RNHLLHVFDE YKRIAQKDIE QSIKSETSGS FEDALLAIVK CMRNKSAYFA (201-250)
                         (T2)SETSGS FEDA

ERLYKSMKGL GTDDDTLIRV MVSRAEIDML DIRANFKRLY GKSLYSFIKG (251-300)

DTSGDYRKVL LIICGGDD(301-318)
```

Figure 2 Amino acid sequences of trypsin- and *S. aureus* proteinase-digested peptide fragments of the 32 kDa protein

Five of the sequences obtained are shown here and compared with bovine annexin IV [43]. T1, T2, tryptic peptites; V1–V3, peptides generated by *S. aureus* protease. Amino-acid-sequence homology was determined by reference to the SWISS-PROT protein-sequence database.

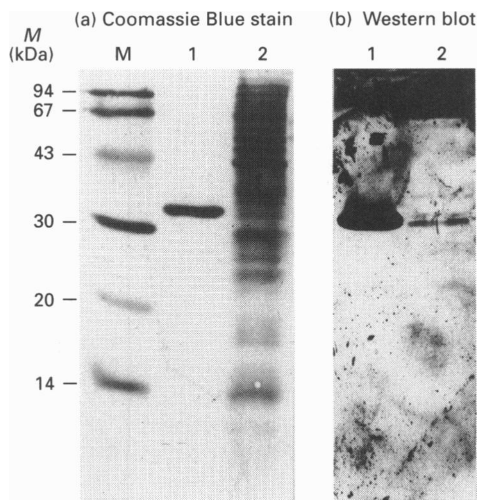

Figure 3 Western-blot analysis with anti-(annexin IV) antiserum

The isolated 32 kDa protein (0.5 μg, lane 1) and the cytosol fractions of the isolated rat alveolar type II cells (15 μg, lane 2) were subjected to SDS/PAGE and then transferred to a PVDF membrane. The blot was stained with Coomassie Blue (**a**) or incubated with rabbit anti-(annexin IV) antiserum (from Professor C. E. Creutz [30]; 1–250 dilution), then with goat anti-rabbit IgG conjugated to horseradish peroxidase and developed (**b**). Lane M, molecular-mass (*M*) standards.

More than ten peptide peaks from each sample appeared on the column. Each peak fraction was freeze-dried and the amino acid sequence was analysed. Five peptide sequences were determined (Figure 2, T1, T2, and V1–V3), and they were referred to the SWISS-PROT protein-sequence database for sequence homology analysis. It was found that they were in complete agreement with corresponding parts of amino acid sequence of annexin IV (Figure 2; [43]). It was also demonstrated that the 32 kDa protein was recognized by an immunoblot analysis with the specific antiserum against annexin IV (Figure 3, lane 1). Moreover, the immunobolt analysis revealed that the cytosol fractions of isolated alveolar type II cells from rat lung contains annexin IV (Figure 3, lane 2). After Mono Q chromatography, the yield of annexin IV from 50 g of bovine lung was estimated to be about 200 μg.

It is probable that annexin IV will also bind to SP-A included in the lamellar bodies in a Ca^{2+}-dependent manner, since the lamellar bodies in the alveolar type II cells contains SP-A [28]. To examine this possibility, the lamellar bodies isolated from rat lung by the method of Duck-Chong [19] were conjugated to Sepharose 4B by the method developed by Creutz [21] for the conjugation of adrenal chromaffin granules to activated Sepharose 4B. The soluble proteins from rat lung were loaded on to the lamellar-body–Sepharose 4B in the presence of Ca^{2+}. The column was washed with buffer containing 2 mM Ca^{2+} and then eluted with 1 mM EGTA (Figure 4). The proteins that were bound to the lamellar bodies in a Ca^{2+}-dependent manner were eluted from lamellar-body-Sepharose 4B (Figure 4a). The SDS/PAGE pattern shows that the peak fraction contained a main band of molecular mass 32 kDa and a minor band of 60 kDa (Figure 4b, lane 1). An immunoblot analysis with the anti-(annexin IV) antiserum reveals that this 32 kDa protein is annexin IV (Figure 4b, lane 1).

Annexin IV has been identified from experiments conducted on several tissues as Ca^{2+}- and membrane (lipid)-binding protein [29]. The binding occurred in a Ca^{2+}-dependent manner. In addition, it has been shown that annexin IV induces the aggregation of isolated chromaffin-granule membranes from adrenal medulla and also pure lipid liposomes [30]. We next tested the effect of the lung annexin IV on the Ca^{2+}-dependent aggregation of lung lamellar bodies (Figure 5a). When annexin IV was added to the rat lung lamellar bodies containing 110 μmol of phospholipids, little change in the turbidity was observed in the absence of Ca^{2+}. Turbidity of the lamellar bodies was increased by the addition of Ca^{2+} (2 mM). The Ca^{2+}-induced increase in turbidity was greater in the presence of annexin IV than buffer alone (Figure 5a, traces a and d). The augmented aggregation by annexin IV was, however, attenuated by the presence of polyclonal anti-(human SP-A) antibodies (Figure 5a, trace c) which cross-reacted with rat SP-A (results not shown). The inhibition increased with rises in the concentration of anti-

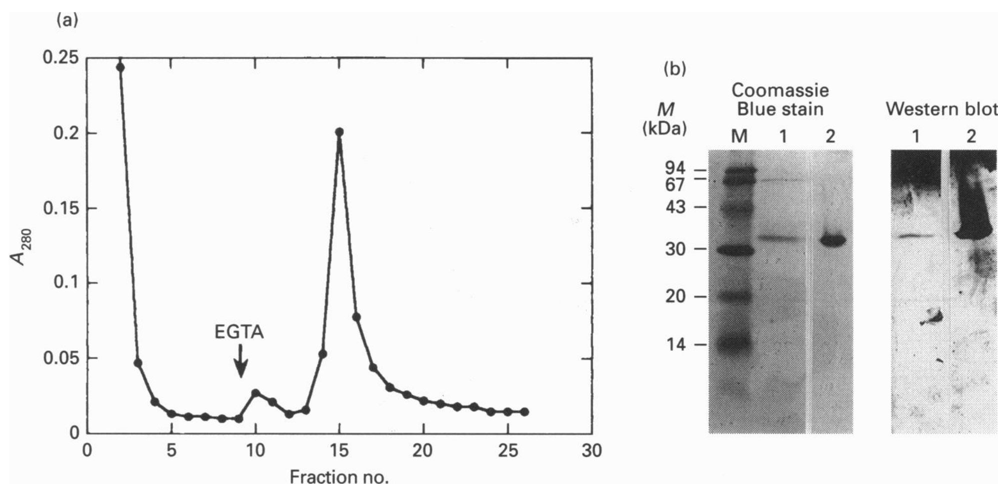

Figure 4 (a) Lung-lamellar-body–Sepharose 4B affinity chromatography and (b) SDS/PAGE and immunoblot analyses of the peak fraction (fraction number 15)

(**a**) Rat lung cytosol was extracted as described in the Experimental section. $CaCl_2$ (1 M) was added to the cytosol to final concentration of 1 mM. The sample was loaded on to lamellar-body–Sepharose 4B equilibrated with 0.24 M sucrose/20 mM Hepes (pH 7.5)/2 mM $CaCl_2$/1 mM $MgCl_2$/30 mM NaCl. After washing with the equilibration buffer, elution was performed with 0.24 M sucrose/20 mM Hepes (pH 7.5)/1 mM EGTA/30 mM NaCl. (**b**) Aliquots of fraction number 15 (lane 1) and lung annexin IV (0.5 μg) prepared in the present study (lane 2) were subjected to SDS/PAGE and transferred to a PVDF membrane. The blot was stained with Coomassie Blue (left panel) or incubated with rabbit anti-(annexin IV) antiserum (from Professor C. E. Creutz [30], 1–250 dilution), then with goat anti-rabbit IgG conjugated to horseradish peroxidase and developed (right panel). Lane M, molecular-mass (*M*) standards.

SP-A antibodies; with 21 μg/ml of the antibodies the turbidity was about the control level even in which no annexin IV was contained (Figure 5b). A control experiment with anti-[bovine G-protein α-subunit ($G_i\alpha$)] antibodies that does not detect SP-A did not affect the Ca^{2+}-induced increase in turbidity with annexin IV (Figure 5a, trace b). The increased turbidity by Ca^{2+} decreased on the addition of EGTA. Thus annexin IV augmented the aggregation of lung lamellar bodies in a Ca^{2+}-dependent manner.

We next loaded purified human SP-A that had already been delipidated with butan-1-ol (see the Experimental section) on to annexin IV–Sepharose 4B in the presence of Ca^{2+}. This annexin IV was dialysed against buffer containing detergent (10 mM CHAPS) and EGTA in order to remove contaminated lipids prior to the coupling of the annexin IV to Sepharose 4B. Although a fraction containing SP-A that was unbound to the column was eluted in the washing step, the SP-A bound to the column was eluted with EGTA (Figure 6). The protein peak eluted with EGTA was identified as SP-A by an immunoblot analysis with the monoclonal antibody against human SP-A [31] (result not shown).

These results (Figures 5 and 6) suggest that direct binding, i.e. a protein–protein interaction between SP-A and annexin IV, occurred.

DISCUSSION

It has been shown that SP-A binds Ca^{2+} [11,12] as well as phospholipid [32,33], especially DPPC [7]. The lipid binding to SP-A occurs in a Ca^{2+}-dependent manner. SP-A has been shown to have an inhibitory effect on agonist-stimulated phospholipid secretion [34,35] and to enhance uptake of phospholipids [36] by a primary culture of alveolar type II cells. The binding of SP-A to the specific cell-surface receptor(s) on alveolar type II cells is required for these functions [17,28,36]. Thus SP-A in the alveolar space is likely to play a regulatory role in the turnover of the phospholipids. The Ca^{2+}-binding experiments with delipidated SP-A have shown that SP-A possesses a high-affinity Ca^{2+}-binding site in the non-collagenous C-terminal domain [12]. In the present study, on the other hand, the cytosolic Ca^{2+}-dependent SP-A binding proteins in lung were isolated by affinity chromatography conducted on SP-A coupled to Sepharose 4B (Figure 1). The main protein with apparent molecular mass of 32 kDa on SDS/PAGE was identified as annexin IV (Figures 2 and 3). Annexin IV, a member of the annexin family of homologous proteins that bind lipids in a Ca^{2+}-dependent fashion, has been identified as the cytosolic protein [30]. Annexin IV has been shown to interact with chromaffin granules from adrenal medulla and to cause aggregation of the granules and also of pure lipid membranes [30,38]. The question arises as to whether the binding between SP-A and annexin IV is direct or indirect, since both SP-A and annexin IV bind lipids. It is possible that lipids contaminated in the extracted proteins were sandwiched between SP-A and annexin IV during the SP-A–Sepharose 4B chromatography. This uncertainty can be somewhat dispelled by considering the experiment with delipidated SP-A and annexin IV–Sepharose 4B (Figure 6). In this experiment isolated annexin IV had been dialysed against buffer containing 10 mM CHAPS/0.1 mM EGTA to remove contaminated lipids prior to the coupling of annexin IV to CNBr-activated Sepharose 4B. Neither the SP-A (100 μg) after treatment with butan-1-ol nor the annexin IV (100 μg) after dialysis against buffer containing detergent contained detectable phospholipid phosphorus (results not shown), suggesting that each delipidation procedure was complete. This experiment revealed that SP-A possessed an ability to bind to annexin IV in a Ca^{2+}-dependent manner (Figure 6), although about 70% of SP-A was collected in the breakthough and 30% was bound to the column. Although it is unclear why the apparent stoichiometric binding of SP-A to the immobilized annexin IV is low, annexin IV covalently coupled to Sepharose 4B may undergo conformational change that affects the binding. In addition, annexin IV augmented the aggregation of the lung lamellar bodies in a Ca^{2+}-dependent manner, and the augmented aggregation was inhibited by the presence of polyclonal anti-SP-A antibodies (Figure 5). These results clearly

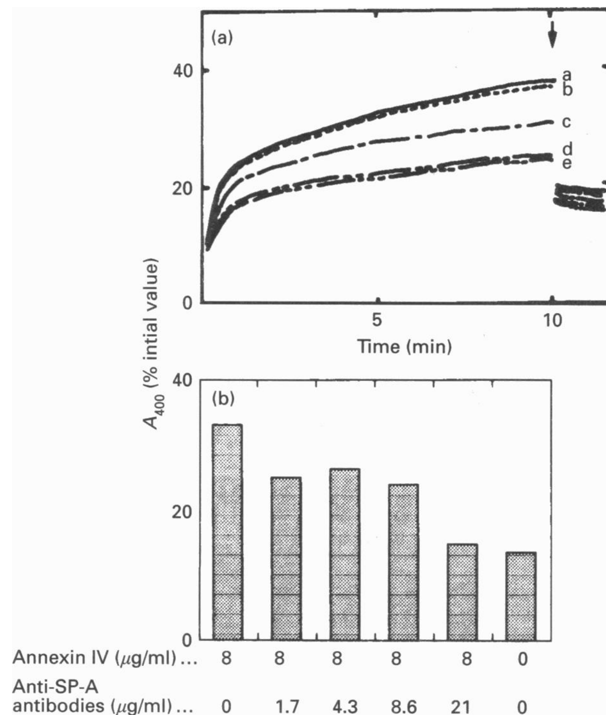

Figure 5 Ca^{2+}-dependent aggregation of rat lung lamellar bodies by annexin IV at 25 °C

(**a**) Lamellar bodies containing 110 nmol of phospholipid and 19 μg of protein, annexin IV (8 μg/ml) or buffer and anti-SP-A antibodies (IgG) (10 μg/ml) or anti-[bovine G-protein α-subunit ($G_i\alpha$)] antibodies (IgG) (13 μg/ml) or 10 mM PBS were preincubated in a 1 ml cuvette in 0.24 M sucrose/20 mM Hepes (pH 7.5)/30 mM NaCl for 5 min at 25 °C. At zero time, 2 μl portions of $CaCl_2$ (1 M) to final concentration of 2 mM were added to the cuvette. At 10 min, 20 μl of EGTA (0.2 M) was added to the cuvette (indicated by an arrow). Trace a, annexin IV and PBS; trace b, annexin IV and anti-$G_i\alpha$ antibodies; trace c, annexin IV and anti-SP-A antibodies; trace d, buffer and anti-SP-A antibodies; trace e, buffer and PBS. (**b**) Turbidity at time 5 min after addition of 2 mM $CaCl_2$ with various concentrations of anti-SP-A antibodies. Lamellar bodies containing 200 nmol of phospholipid and 13 μg of protein, 8 μg/ml annexin IV and various concentrations of anti-SP-A antibodies were preincubated in the 1 ml cuvette under the same conditions as in (**a**). Final concentrations of annexin IV and anti-SP-A antibodies in the mixture are indicated in the Figure.

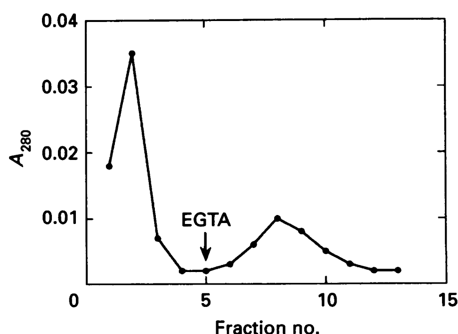

Figure 6 Annexin IV–Sepharose 4B affinity chromatography

Purified and delipidated SP-A (50 μg) was dialysed against 5 mM Tris, pH 8.0. After 1 mM $CaCl_2$ had been added to the sample, it was loaded on to annexin IV–Sepharose 4B equilibrated with 10 mM Hepes (pH 7.5)/1 mM $CaCl_2$. Elution was performed with 10 mM Hepes (pH 7.50)/1 mM EGTA.

suggest that the protein–protein interaction occurs between SP-A and annexin IV.

In the alveolar type II cells, SP-A has been shown to be distributed over the phospholipids in the lamellar bodies [37], although SP-A constitutes only 1% of total lamellar-body protein [32]. On the other hand, SP-A is an abundant protein in extracellular sufactant [28]. The experiment with the lamellar-body-conjugated Sepharose 4B and lung cytosol revealed that annexin IV strongly bound to the lamellar bodies in a Ca^{2+}-dependent manner (Figure 4). For preparing a lamellar-body-conjugated Sepharose 4B, lamellar bodies containing 1.5 μmol of phospholipid and 400 μg of protein were incubated with the CNBr-activated Sepharose 4B (1 ml) in the present study. The amount of SP-A in the lamellar body was estimated to be about 6 μg on the basis of the findings by Oosterlaken-Dijksterhuis et al. [32]. The yield of annexin IV from the lamellar-body–Sepharose 4B was estimated to be about 20 μg from 10 g of rat lung (Figure 4); thus the apparent stoichiometric ratio of annexin IV to SP-A was higher than 1:1. For preparing SP-A–Sepharose 4B, 1.5 mg of purified SP-A was incubated with the CNBr-activated Sepharose 4B (3 ml), and the yield from the SP-A–Sepharose 4B was estimated to be about 200 μg from 50 g of bovine lung (Figure 1); the apparent stoichiometric ratio of annexin IV to SP-A was less than 1:1. It has been shown that annexins are a family of proteins that bind to phosphatidylcholine and that the addition of negatively charged phospholipids to phosphatidylcholine enhances the biological activities of annexin IV in a Ca^{2+}-dependent manner [38]. As the lung lamellar bodies contain negatively charged phosphatidylglycerol as the second major phospholipid [39,40], the phospholipid components of the lamellar bodies may be advantageous for annexin IV binding to SP-A in the lamellar bodies and might have resulted in the augmentation of the apparent stochiometric ratio of annexin IV to SP-A.

Soluble proteins that bind to the chromaffin granules in a Ca^{2+}-dependent manner have been identified [30]. Two-dimensional gel analysis of the 33 kDa SP-A-binding protein from Mono Q (Figure 1b, fraction 3, and Figure 1c, lane 3) revealed that the pI of fraction 3 was approx. 6.5, while that of annexin IV (Figure 1b, fraction 1, and Figure 1c, lane 1) was approx. 5.5 (results not shown). These results are similar to those of chromaffin-granule-binding proteins, chromobindin 6 (epidermal-growth-factor receptor kinase substrate) and chromobindin 4 (annexin IV) respectively [30]. Two-dimensional gel analysis with the 24.5 kDa SP-A binding protein (Figure 1b, fractions 5 and 6, and Figure 1c, lanes 5 and 6) showed two spots appearing from each fraction with the pI values of 6.6 and 6.1 respectively (results not shown). It is not known whether these two spots correspond to those of the chromaffin granule-binding proteins, 26 kDa chromobindins 2 and 3 (unknown proteins) with pI values of 6.8 and 5.7 respectively [30]. On the other hand, the 24.5 and 33 kDa SP-A-binding proteins were unobserved in the eluate of the lamellar-body-Sepharose 4B (Figure 4).

Synexin (annexin VII), a family of annexins and a potent stimulator for the aggregation of chromaffin granules, facilitates the fusion between chromaffin granules and lipid liposomes [23,41]. On account of these properties of annexins (IV and VII), it has been suggested that they are are involved in the process of exocytosis of chromaffin granules from the cells. Annexin IV from the lung also augmented the aggregation of lamellar bodies by the presence of Ca^{2+} (Figure 5). Interestingly, annexin IV, out of the several annexins, was preferentially adsorbed to both SP-A–Sepharose 4B and lamellar-body–Sepharose 4B in a Ca^{2+}-dependent manner (Figures 1 and 5). Annexin IV was also detected in the cytosol of the isolated alveolar type II cells

(Figure 2). Thus it is anticipated that annexin IV may be involved in the secretion process of the lamellar bodies from the type II cells. On the other hand, a protein with molecular mass of 47 kDa that is likely to be synexin was not observed with either the SP-A– or lamellar body–Sepharose 4B chromatography (Figures 1 and 4). Therefore it is unclear whether synexin binds to the lamellar bodies or not, although the existence of synexin has been reported in the lung [42].

The secretion of phosphatidylcholine from the type II cells has shown to be stimulated by several agonists directed to the elevation of the intracellular Ca^{2+} concentration, activation of protein kinase C or activation of adenylate cyclase [9]. In response to second messengers, several protein factors are believed to be involved. The role of SP-A binding proteins other than annexin IV needs to be investigated in the next step.

In summary, annexin IV was identified to be the protein that preferably binds to SP-A in a Ca^{2+}-dependent manner.

We are grateful to Professor Carl E. Creutz for providing us with the specific antiserum against annexin IV and also for his valuable advice. This research was in part supported by Grants in Aid for Scientific Research from the Ministry of Education, Japan.

REFERENCES

1 King, R. J. and Clements, J. A. (1972) Am. J. Physiol. **223**, 707–714
2 King, R. J., Klass, D. J., Gikas, E. G. and Clements, J. A. (1973) Am. J. Physiol. **224**, 788–795
3 White, R. T., Damm, D., Miller, J., Spratt, K., Schilling, J., Hawgood, S., Benson, B. and Cordell, B. (1985) Nature (London) **317**, 361–363
4 Ross, G. F., Notter, R. H., Meuth, J. and Whitsett, J. A. (1986) J. Biol. Chem. **261**, 14283–14291
5 Sano, K., Fisher, J., Mason, R. J., Kuroki, Y., Schilling, J., Benson, B. and Voelker, D. R. (1987) Biochem. Biophys. Res. Commun. **144**, 367–374
6 Boggaram, V., Quing, K. and Mendelson, C. R. (1988) J. Biol. Chem. **263**, 2939–2947
7 Kuroki, Y. and Akino, T. (1991) J. Biol. Chem. **266**, 3068–3073
8 Haagsman, H. P., White, R. T., Schilling, J., Lau, K., Benson, B. J., Golden, J. et al. (1989) Am. J. Physiol. **257**, L421–L429
9 Mason, R. J. (1992) in Pulmonary Surfactant: From Molecular Biology to Clinical Practice (Robertson, B., Van Golde, L. M. G. and Batenburg, J. J., eds.), pp. 295–311, Elsevier Science Publishers B.V., Amsterdam
10 DeLisle, R. C., and Williams, J. A. (1986) Annu. Rev. Physiol. **48**, 225–238
11 Haagsman, H. P., Sargeant, T., Hauschka, P. V., Benson, B. J. and Hawgood, S. (1990) Biochemistry **29**, 8894–8900
12 Sohma, H., Watanabe, T., Kuroki, Y., Yoshino, H., Matsushima, N., Yazawa, M. et al. (1992) Biochim. Biophys. Acta **1159**, 150–154
13 Sohma, H., Hattori, A., Kuroki, Y. and Akino, T. (1993) Biochem. Mol. Biol. Int. **30**, 329–336
14 Benson, B. J., Williams, M. C., Hawgood, S. and Sargeant, T. (1984) Prog. Respir. Res. **18**, 83–92
15 Suzuki, Y., Fujita, Y. and Kogishi, K. (1989) Am. Rev. Respir. Dis. **140**, 75–81
16 Hawgood, S., Benson, B. and Hamilton, R. L., Jr. (1985) Biochemistry **24**, 184–190
17 Kuroki, Y., Mason, R. J. and Voelker, D. R. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 5566–5570
18 Fornstedt, N. and Porath, J. (1975) FEBS Lett. **57**, 187–191
19 Duck-Chong, C. G. (1978) Life Sci. **22**, 2025–2030
20 Kohn, J., and Wilchek, M. (1982) Biochem. Biophys. Res. Commun. **107**, 878–884
21 Creutz, C. E. (1981) Biochem. Biophys. Res. Commun. **103**, 1395–1400
22 Dobbs, L. G. and Mason, R. J. (1979) J. Clin. Invest. **63**, 378–387
23 Creutz, C. E., Pazoles, C. J. and Pollard, H. B. (1978) J. Biol. Chem. **253**, 1860–1868
24 Sohma, H., Hashimoto, H., Ohguro, H. and Akino, T. (1992) Biochem. Biophys. Res. Commun. **184**, 175–182
25 Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
26 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
27 Bartlett, G. R. (1959) J. Biol. Chem. **234**, 466–468
28 Phizackerley, P. J. R., Town, M. H. and Newman, G. E. (1979) Biochem. J. **183**, 731–736
29 Creutz, C. E. (1992) Science **258**, 924–931
30 Creutz, C. E., Zaks, W. J., Hamman, H. C., Crane, S., Martin, W. H., Gould, K. L., Oddie, K. M. and Parsons, S. J. (1987) J. Biol. Chem. **262**, 1860–1868
31 Kuroki, Y., Fukada, Y., Takahashi, H. and Akino, T. (1985) Biochim. Biophys. Acta **836**, 201–209
32 Oosterlaken-Dijksterhuis, M. A., van Eijk, M., van Buel, B. L. M., van Golde, L. M. G. and Haagsman, H. P. (1991) Biochem. J. **274**, 115–119
33 Hawgood, S., Benson, B. J. and Hamilton, R. L., Jr. (1985) Biochemistry **24**, 184–190
34 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
35 Rice, W. R., Ross, G. F., Singleton, F. M., Dingle, S. and Whitsett, J. A. (1987) J. Appl. Physiol. **63**, 692–698
36 Wright, J. R., Borchelt, J. D. and Hawgood, S. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5410–5414
37 Coalson, J. J., Winter, V. T., Martin, H. M. and King, R. J. (1986) Am. Rev. Respir. Dis. **133**, 230–237
38 Junker, M., and Creutz, C. E. (1994) Biochemistry **33**, 8930–8940
39 Montfoort, A., van Golde, L. M. G. and van Deenen, L. L. M. (1971) Biochim. Biophys. Acta **231**, 335–352
40 Adachi, H., Hayashi, H., Sato, H., Dempo, K., and Akino, T. (1989) Biochem. J. **262**, 781–786
41 Creutz, C. E. (1981) J. Cell Biol. **91**, 247–256
42 Chander, A. and Wu, R.-D. (1990) FASEB J. **A840**, 3327
43 Hamman, H. C., Gaffey, L. C., Lynch, K. R. and Creutz, C. E. (1988) Biochem. Biophys. Res. Commun. **156**, 660–667

Received 18 April 1995/27 June 1995; accepted 11 July 1995