Characterization of the Ca^{2+} -dependent binding of annexin IV to surfactant protein A

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We have shown previously that surfactant protein A (SP-A) binds to annexin IV in a Ca^{2+} -dependent manner [Sohma, Matsushima, Watanabe, Hattori, Kuroki and Akino (1995) *Biochem. J.* **312**, 175–181]. Annexin IV is a member of the annexin family having four consensus repeats of about 70 amino acids and a unique N-terminal tail. In the present study, the functional site of both annexin IV and SP-A for the Ca^{2+} -dependent binding was investigated using mutant proteins. SP-A bound in a Ca^{2+} -dependent manner to an annexin-IV truncation mutant consisting of the N-terminal domain and the first three domains ($T_{N-1-2-3}$). SP-A also bound to T_{3-4} , but this interaction was not Ca^{2+} -dependent. SP-A bound weakly to the other truncation mutants (T_{N-1-2} , T_{2-3} and T_{2-3-4}). Each consensus repeat of annexin IV possesses a conserved acidic amino acid residue (Glu⁷⁰, Asp¹⁴², Glu²²⁶ and Asp³⁰¹) that putatively ligates

Ca^{2+} . Using annexin-IV DE mutants in which one, two or three residues out of the four Asp/Glu were altered to Ala by site-directed mutagenesis [Nelson and Creutz (1995) *Biochemistry* **34**, 3121–3132], it was revealed that Ca^{2+} binding in the third domain is more important than in the other Ca^{2+} -binding sites. SP-A is a member of the animal lectin group homologous with mannose-binding protein A. The substitution of Arg¹⁹⁷ of rat SP-A with Asp or Asn eliminated binding to annexin IV, whereas the substitution of Glu¹⁹⁵ with Gln was silent. These results suggest that the Ca^{2+} binding to domain 3 of annexin IV is required for the Ca^{2+} -dependent binding by SP-A and that Arg¹⁹⁷ of SP-A is important in this binding.

Key words: Ca^{2+} -binding protein, pulmonary surfactant, site-directed mutagenesis, truncation mutant.

INTRODUCTION

The annexins are a family of Ca^{2+} - and membrane-binding proteins found in most tissues [1]. The annexins have four (or eight for annexin VI) consensus 70–80-amino acid repeats (domains 1–4), in which Ca^{2+} - and membrane-binding sites are located [2]. It has been indicated that the annexins may be involved in several biological functions, such as exocytosis [3], anti-coagulation [4], anti-inflammation [5,6] and the formation of ion channels [7,8]. Members of the annexin family have been demonstrated to interact with specific proteins: annexin I binds to both S100C and apolipoprotein A-I [9,10]; annexin II to p11 (S100A) [11]; annexin VII to sorcin [12]; and annexin XI to calyculin [13]. The binding between annexins and annexin-binding proteins is Ca^{2+} -dependent, except in the case of annexin II, in which the binding is independent of Ca^{2+} . The binding of annexin II to p11 has been shown to be involved in the exocytotic process in chromaffin cells [14]. We identified annexin IV as a pulmonary surfactant protein A (SP-A)-binding protein that binds in a Ca^{2+} -dependent manner [15]. SP-A is a major component of proteins in pulmonary surfactant [16]. The interaction involves a protein–protein interaction, in spite of the abilities of both proteins to bind phospholipids. Annexin IV augmented the Ca^{2+} -dependent aggregation of lung lamellar bodies, the intracellular store of the surfactant [15]. This phenomenon seems to be similar to the Ca^{2+} -dependent aggregation of chromaffin granules by annexin IV [17].

SP-A is a member of the C-type animal lectin group homologous with mannose-binding protein A (MBP-A). SP-A has a molecular mass of 28–36 kDa on SDS/PAGE under reducing conditions, but forms an octadecamer of six trimer subunits

under native conditions. SP-A binds to both lipids [18] and Ca^{2+} [19,20]. Study *in vitro* suggests that SP-A has a role in the regulatory function of surfactant homeostasis, since both the inhibitory effect of agonist-stimulated secretion of surfactant and the augmentation of lipid uptake by alveolar type-II cells are caused by SP-A via SP-A receptor(s) [16,21]. The residues Glu¹⁸⁵ and Asn¹⁸⁷ of MBP-A, both of which ligate Ca^{2+} , have been demonstrated for sugar-binding specificity [22]. Rat SP-A has Glu¹⁹⁵ and Asn¹⁹⁷ at the positions corresponding to residues Glu¹⁸⁵ and Asn¹⁸⁷ of MBP-A. Mutational study has revealed the importance of the residues for the functional properties of SP-A [23]. Recently, decreased activity of bacterial phagocytosis by alveolar macrophages has been demonstrated in SP-A-deficient mice, suggesting the involvement of SP-A in host defence *in vivo* [24].

In the present study, we characterized the Ca^{2+} -dependent interaction between annexin IV and SP-A using several mutant proteins. Our results suggest that the Ca^{2+} -binding site in domain 3 of annexin IV is more important than the other sites for Ca^{2+} -dependency and that Arg¹⁹⁷ of rat SP-A is required for the binding.

MATERIALS AND METHODS

Materials

Bovine lung was obtained from the local slaughterhouse and native annexin IV was prepared as described previously [15]. Reagents were obtained from the following sources: restriction enzymes and DNA-modifying enzymes were from Gibco-BRL

Abbreviations used: SP-A, surfactant protein A; MBP-A, mannose-binding protein A; EIA, enzyme-linked immunoassay.

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and New England Biolabs; antibiotics and isopropyl β -D-thiogalactoside were from Sigma; molecular-mass standards, DEAE-Sephacel and Protein A-Sepharose 4B were from Amersham Pharmacia Biotech; DEAE-Toyopearl was from Tosoh, Tokyo, Japan; pET3d vector and BL21(3D) cells were from Strategene; trypsin was from Roche Diagnostic G.m.b.H.; horseradish peroxidase-conjugated goat anti-rabbit IgG was from Jackson ImmunoResearch Laboratories; *o*-phenylenediamine and diaminobenzidine were from Katayama Chemical Co., Osaka, Japan; and microtitre plates were from Dynatech Laboratories.

Preparation of annexin-IV mutants

To prepare recombinant annexin IV, fresh bovine liver was obtained from the local slaughterhouse and frozen immediately in liquid nitrogen. Total RNA was extracted from the liver by acid guanidinium thiocyanate/phenol/chloroform extraction method [25]. After synthesis of the first-strand cDNA, the annexin-IV gene was amplified by PCR with synthesized primers that were based on the sequence of bovine annexin IV reported by Hamman et al. [26]. *Nco*I and *Bam*HI restriction sites were introduced at the 5' and 3' termini of the coding sequence of bovine annexin-IV DNA, respectively. The two primers used were 5'-AGTTGCCATGGCAGCCAAGGGAGGC-3' and 5'-ATCGTGGATCCTTAATCCTCTCC-3' (restriction sites are underlined). An *Nco*I/*Bam*HI-digested wild-type annexin-IV DNA was subcloned into expression vector (pET-3d) by standard techniques. The DNA clone was used to transform *Escherichia coli* strain BL21(3D). To create the truncation mutants of annexin IV, DNA encoding each mutant was amplified by PCR using wild-type annexin IV as a template with the primers listed in Table 1 (the *Nco*I restriction site at the 5' end and the stop codon and *Bam*HI restriction site at the 3' end were introduced). The following subcloning and expression procedures, all performed at room temperature unless stated, were the same as those for wild-type annexin IV (see above). The method for the preparation of DE mutants of annexin IV (site-directed mutagenesis) is described elsewhere [27]. Purification of wild-type and DE mutants of annexin IV was based on Ca^{2+} -dependent binding to bovine brain lipids by the method of Nelson and Creutz [28]. Further purification was performed with DEAE-Toyopearl. To purify the truncation mutants of annexin IV, transformed *E. coli* were cultured in the presence of 1 mM isopropyl β -D-thiogalactoside for 3–4 h at 37 °C and harvested. The cells were suspended in buffer A [30 mM Tris/HCl (pH 7.5)/30 mM NaCl/1 mM dithiothreitol] and sonicated for about 20 min with a probe sonicator until the suspension became clear in ice. Inclusion bodies were pelleted by centrifuging the sample at 8000 *g* for 20 min. The pelleted inclusion bodies were suspended in 1 M sucrose and centrifuged at 8000 *g* for 20 min. The pellet was suspended in buffer B (2% Triton X-100/10 mM EDTA) and incubated overnight. The sample was centrifuged at 8000 *g* for 20 min and the pelleted inclusion bodies containing protein were solubilized with 1 ml of 8 M urea in buffer A. Refolding of the protein was performed by four successive dialyses against 4 M urea in buffer A for 12 h, 1 M urea in buffer A for 12 h, 30 mM urea in buffer A for 12 h and then buffer A for 12 h. Further purification was performed with DEAE-Toyopearl. The purified proteins were dialysed against 10 mM Hepes (pH 7.0) and 0.1 M NaCl and stored at –40 °C.

Preparation of rat SP-A

Surfactant was isolated from lung lavage of male Sprague-Dawley rats that had been given an intratracheal instillation of

silica (10 mg) in saline 4 weeks before lavage. The surfactant was delipidated with butan-1-ol by the method of Hawgood et al. [29]. SP-A was purified from the surfactant as described previously [30]; the organic solvent-insoluble precipitate was suspended in Tris/HCl (5 mM, pH 7.4) and dialysed against the same buffer. The dialysed sample was then centrifuged at 150 000 *g* for 1 h and the supernatant loaded on to a mannose-Sepharose 6B column [31] that was equilibrated with Tris/HCl (5 mM, pH 7.4) and CaCl_2 (1 mM). SP-A was eluted with EDTA (2 mM). Further purification was accomplished by gel filtration with Bio Gel A5m (Bio Rad, 1.5 cm $\times$ 59 cm) with Tris/HCl (5 mM, pH 7.4). The purified proteins were dialysed against 10 mM Hepes (pH 7.0)/0.1 M NaCl and stored at –40 °C.

Preparation of SP-A mutants

The mutant cDNAs for SP-A were produced by PCR with the overlap-extension technique as described previously [23]. The expression of recombinant proteins was performed using the baculovirus system. Recombinant SP-A was purified with mannose-Sepharose 6B from the culture media as described previously [23]. The purified proteins were dialysed against 10 mM Hepes (pH 7.0)/0.1 M NaCl and stored at –40 °C.

Enzyme-linked immunoassay (EIA) of SP-A binding

Binding experiments with recombinant wild-type annexin IV and its mutants to SP-A were performed using microtitre plates. SP-A binding to the truncation mutants of annexin IV was performed with various concentrations of SP-A in 20 mM Hepes (pH 7.5)/100 mM NaCl/1 mM MgCl_2 and either 1 mM CaCl_2 or 1 mM EGTA. Wild-type annexin IV or annexin-IV mutant was coated on to a well of microtitre plates (100 ng of protein in 50 μ l/well) for 12 h at 4 °C, and blocked with 10 mg/ml BSA for 1 h. For Ca^{2+} titration, SP-A (100 ng of protein in 50 μ l/well) in 50 mM Hepes (pH 7.0)/100 mM NaCl/1 mM MgCl_2 and a calcium buffer (consisting of 2.5 mM EGTA and various amounts of CaCl_2) was incubated for 1 h. For the experiments shown in Figures 4–7 (see below), 50 $\times$ stock solutions of CaCl_2 buffered with EGTA were prepared, and the final free Ca^{2+} concentration was ascertained using a Ca^{2+} electrode. Subsequent washes and incubations contained the highest $[\text{Ca}^{2+}]$ used during the SP-A incubations, except that washes also contained 0.02% Tween 20. Bound SP-A was detected with polyclonal rabbit anti-rat SP-A IgG (10 μ g/ml in PBS) and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:4000 dilution with PBS) and finally *o*-phenylenediamine (1 mg/ml) in 50 mM sodium citrate (pH 4.85) and 0.03% (v/v) H_2O_2 was used as the substrate. Reactions were monitored for 5 min at room temperature and quenched by adding 50 μ l of 2 M H_2SO_4 , and absorbance at 492 nm was measured.

Antibodies

Polyclonal anti-rat SP-A antibody was prepared from a rabbit. IgG fraction of the antiserum was then obtained with a Protein A-Sepharose 4B column (Pharmacia).

Other methods

Protein concentrations were determined by the BCA protein assay reagent kit (Pierce) or the method of Bradford [32] using BSA as standard. PAGE in the presence of SDS (0.1% w/v) (SDS/PAGE) was performed using the method of Laemmli [33].

Table 1 Synthetic oligonucleotide primers for wild-type and truncation mutants of bovine annexin IV

Restriction sites (*Nco*I/*Bam*HI) are underlined. An additional Ala codon (GCT) was added after the initial for T₂₋₃ and T₂₋₃₋₄ to avoid a frame shift. See text for details of the mutants.

	Sense primer (5' → 3')	Anti-sense primer (5' → 3')
Wild type	AGTTGCCATGGCAGCCAAGGGAGGC	ATCGTGGATCCTTAATCATCTCC
T _{N-1-2}	AGTTGCCATGGCAGCCAAGGGAGGC	ATCGTGGATCCTTAATGCTTTCATCCCTG
T _{N-1-2-3}	AGTTGCCATGGCAGCCAAGGGAGGC	ATCGTGGATCCTTATGCAGATTTGTCCTC
T ₂₋₃	AGTTGCCATGGCTATGACACCCACGGTG	ATCGTGGATCCTTATGCAGATTTGTCCTC
T ₂₋₃₋₄	AGTTGCCATGGCTATGACACCCACGGTG	ATCGTGGATCCTTAATCATCTCC
T ₃₋₄	AGTCGCCATGGATGAAAGCAATTATCTGG	ATCGTGGATCCTTAATCATCTCC

RESULTS

Binding of SP-A to the truncation mutants of annexin IV

To examine the SP-A-binding site of annexin IV, we first prepared five kinds of truncation mutant of annexin IV in which one or two consensus domain(s) were truncated. These were depicted as T_{N-1-2}, representing the N-terminal domain and the first two consensus domains (Ala¹-Asn¹⁶³); T_{N-1-2-3}, the N-terminal do-

Figure 1 Truncation mutants of annexin IV

(A) Schematic illustration of truncation mutants of annexin IV. Four repeats of consensus 70–80 amino acids of annexin IV are depicted with rectangles and numbered from 1 to 4. The truncation mutants are shown by two-headed arrows. (B) SDS/PAGE patterns of the truncation mutants stained with Coomassie Brilliant Blue. Lanes 1–5, T_{N-1-2}, T_{N-1-2-3}, T₂₋₃, T₂₋₃₋₄ and T₃₋₄, respectively; lanes 6 and 7, wild-type and native annexin IV, respectively. M, molecular-mass standards.

Figure 2 Binding of SP-A to immobilized truncation mutants of annexin IV

Microtitre wells were coated with 100 ng of wild-type or truncation mutants of annexin IV. After blocking, each well was incubated with various concentrations of rat SP-A in the presence of either 1 mM Ca²⁺ (●) or 1 mM EGTA (○). Bound SP-A was detected with rabbit anti-rat SP-A IgG as described in the Materials and methods section. (A) T_{N-1-2}; (B) T_{N-1-2-3}; (C) T₂₋₃; (D) T₂₋₃₋₄; (E) T₃₋₄; (F) wild-type annexin IV. Data represent means ± S.E.M. in triplicate assays representing one of three experiments.

main and the first three consensus domains (Ala¹-Ala²⁴⁷); T₂₋₃ (Met⁸³-Ala²⁴⁷); T₂₋₃₋₄ (Met⁸³-C-terminus); and T₃₋₄ (Asp¹⁶⁰-C-terminus). Apparent molecular masses of the truncation mutants on SDS/PAGE for T_{N-1-2}, T_{N-1-2-3}, T₂₋₃, T₂₋₃₋₄ and T₃₋₄ were about 18, 28, 19, 28 and 18 kDa, respectively (Figure 1B). Using these

Figure 3 Binding of SP-A to immobilized DE mutants of annexin IV (one-site mutants)

Microtitre wells were coated with 100 ng of wild-type or DE mutants of annexin IV. After blocking, each well was incubated with rat SP-A in the presence of various concentrations of free Ca^{2+} . Bound SP-A was detected with rabbit anti-rat SP-A IgG. Wild-type annexin IV ($\blacktriangle$), DE2 ($\bullet$), DE3 ($\blacksquare$) and DE4 ($\blacklozenge$). Non-specific binding in the presence of 1 mM EGTA was subtracted from each point. Data represent means $\pm$ S.E.M. in triplicate assays representing one of three experiments.

mutants and wild-type annexin IV we examined Ca^{2+} -dependent binding by SP-A with the EIA method (see the Materials and methods section). The binding assay was performed with varying concentrations of native rat SP-A in the presence of either 1 mM CaCl_2 or 1 mM EGTA (Figure 2). SP-A bound to $\text{T}_{\text{N-1-2-3}}$ in a Ca^{2+} -dependent manner, as did wild-type annexin IV (Figures 2B and 2F), suggesting that domain 4 of annexin IV is not involved in the binding. SP-A also bound to T_{3-4} . However, in this case, Ca^{2+} -sensitivity was lacking: SP-A bound to T_{3-4} even in the presence of EGTA (Figure 2E). These results suggest that the SP-A-binding site is located on the C-terminal region of annexin IV. On the other hand, only a fractional binding of SP-A to the other truncation mutants, $\text{T}_{\text{N-1-2}}$, T_{2-3} and T_{2-3-4} , was observed (Figures 2A, 2C and 2D). There seems to be inconsistency in the fact that SP-A only bound weakly to both T_{2-3} and T_{2-3-4} , even though both these mutants possess domain 3. Therefore, domains 1 and 2 might have other functional properties with regard to Ca^{2+} -dependent binding by SP-A (see the Discussion section).

Ca^{2+} -dependent binding of SP-A to the DE mutants of annexin IV (one- and two-site mutations)

Annexins have the consensus sequence for a high-affinity type-II Ca^{2+} -binding site (Lys/Arg-Gly/Arg-Xaa-Gly-Thr-Xaa³⁸-Asp/Glu) in each consensus repeat, as determined from the annexin-V crystal structure [34]. To verify the Ca^{2+} -dependent regulatory site on annexin IV, we next used annexin-IV DE mutants, which had the conserved Asp/Glu residues changed to Ala [27], for an SP-A-binding assay with the EIA method. As shown in Figure 3, the estimated EC_{50} values of Ca^{2+} concentrations for both DE2 and DE4 were similar to that of wild-type annexin IV (approx. 5×10^{-6} M free Ca^{2+} for both DE2 and DE4, and 3×10^{-6} M free

Figure 4 Binding of SP-A to immobilized DE mutants of annexin IV (two-site mutants)

Microtitre wells were coated with 100 ng of wild-type annexin IV. After blocking, each well was incubated with rat SP-A in the presence of various concentrations of free Ca^{2+} . Bound SP-A was detected with rabbit anti-rat SP-A IgG. (A) Wild-type annexin IV ($\blacktriangle$), DE12 ($\blacklozenge$), DE13 ($\blacksquare$) and DE14 ($\bullet$). (B) Wild-type annexin IV ($\blacktriangle$), DE23 ($\bullet$), DE24 ($\blacksquare$) and DE34 ($\blacklozenge$). Non-specific binding in the presence of 1 mM EGTA was subtracted from each point. Data represent means $\pm$ S.E.M. in triplicate assays representing one of three experiments.

Ca^{2+} for wild-type annexin IV). On the other hand, the estimated EC_{50} of Ca^{2+} -concentration for DE3 (approx. 10^{-5} M free Ca^{2+}) was slightly higher than those for the other DE mutants (Figure 3). Because DE1 was not available in this study, we next used other mutants in which substitutions were performed in two domains (DE12, 13 and 14) to test the importance of the Ca^{2+} -binding site in domain 1 (Figure 4A). Estimated EC_{50} values of Ca^{2+} concentrations for DE12 and DE14 were similar to that for wild-type annexin IV (approx. 4.0×10^{-6} , 3.5×10^{-6} and 3.2×10^{-6} M free Ca^{2+} for DE12, DE14 and wild-type annexin IV, respectively). On the other hand, the estimated EC_{50} of Ca^{2+} concentration for DE13 (approx. 10^{-5} M free Ca^{2+}) was slightly higher than those for the other DE mutants and wild-type annexin IV (Figure 4A). From these results it appears that only the substitution on domain 3 can cause the shift of EC_{50} to a higher Ca^{2+} concentration. To verify these results we examined SP-A binding to other DE two-site mutants (DE23, DE24 and

Figure 5 Binding of SP-A to immobilized DE mutants of annexin IV (three-site mutants)

Microtitre wells were coated with 100 ng of wild-type annexin IV. After blocking, each well was incubated with rat SP-A in the presence of various concentrations of free Ca²⁺. Bound SP-A was detected with rabbit anti-rat SP-A IgG. Wild-type annexin IV (▼), DE234 (●), DE134 (■), DE124 (◆) and DE123 (▲). Non-specific binding in the presence of 1 mM EGTA was subtracted from each point. Data represent means $\pm$ S.E.M. in triplicate assays representing one of three experiments.

DE34; Figure 4B). The estimated EC₅₀ of Ca²⁺ concentration for DE24 was similar to that for wild-type annexin IV (approx. 4.0×10^{-6} and 3.2×10^{-6} M free Ca²⁺ for DE24 and wild-type annexin IV, respectively). On the other hand, estimated EC₅₀ values of Ca²⁺ concentrations for DE23 and DE34 (9.0×10^{-6} M free Ca²⁺ for both proteins) were slightly higher than those for DE24 and wild-type annexin IV (Figure 4B).

Ca²⁺-dependent binding of SP-A to the DE mutants of annexin IV (three-site mutations)

To verify further the importance of the Ca²⁺-binding site in domain 3, a binding assay was performed with three-site mutants (DE234, DE134, DE124 and DE123; Figure 5). Interestingly, SP-A bound to DE124 in a Ca²⁺-dependent manner as well as to wild-type annexin IV (the estimated EC₅₀ of Ca²⁺ concentration of 3.0×10^{-6} M was shown for both), whereas SP-A bound weakly to DE123 and DE234. The extents of the binding of SP-A to DE134, DE123 and DE234 were smaller than of binding to DE124 and wild-type annexin IV, even in the presence of 1 mM Ca²⁺ (Figure 5). These results suggest that the conformational change induced by binding of Ca²⁺ to domain 3 on annexin IV is important for the binding by SP-A.

Ca²⁺-dependent binding of SP-A mutants to annexin IV

SP-A is a member of the C-type animal lectin group and it is homologous with serum MBP-A. MBP-A binds mannose specifically. Simultaneous mutation of Glu¹⁸⁵ $\rightarrow$ Gln and Asn¹⁸⁷ $\rightarrow$ Asp diminished the specificity [22]. Both Glu¹⁸⁵ and Asn¹⁸⁷ have been shown to ligate Ca²⁺ from the crystal structure [35,36]. From sequence alignment, Glu¹⁹⁵ and Arg¹⁹⁷ of SP-A correspond with Glu¹⁸⁵ and Asn¹⁸⁷ of MBP-A, respectively. Next, we examined Ca²⁺-dependent binding of the SP-A mutants to wild-type

Figure 6 Binding of SP-A mutants to annexin IV

Microtitre wells were coated with 100 ng of wild-type annexin IV. After blocking, each well was incubated with rat SP-A mutants in the presence of various concentrations of free Ca²⁺. Bound SP-A was detected with rabbit anti-rat SP-A IgG. Wild-type SP-A (EPR, ▼), Arg¹⁹⁷ $\rightarrow$ Asn (EPN, ▲), Arg¹⁹⁷ $\rightarrow$ Asp (EPD, ◆), Glu¹⁹⁵ $\rightarrow$ Gln (QPR, ■) and Glu¹⁹⁵ $\rightarrow$ Gln, Arg¹⁹⁷ $\rightarrow$ Asp (QPD, ●). Non-specific binding in the presence of 1 mM EGTA was subtracted from each point. Data represent means $\pm$ S.E.M. in triplicate assays representing one of three experiments.

annexin IV (Figure 6). In the present study we used four kinds of SP-A mutant (SP-A^{Glu195Gln}, SP-A^{Arg197Asn}, SP-A^{Arg197Asp} and SP-A^{Glu195Gln,Arg197Asp}). The Ca²⁺-titration curves of the binding of SP-A^{Glu195Gln} and wild-type SP-A to annexin IV were similar to each other (estimated EC₅₀ of 5.5×10^{-6} and 4.0×10^{-6} M free Ca²⁺ for SP-A^{Glu195Gln} and wild-type SP-A, respectively; Figure 6). On the other hand, the other SP-A mutants (SP-A^{Arg197Asn}, SP-A^{Arg197Asp} and SP-A^{Glu195Gln,Arg197Asp}) did not appear to bind to annexin IV (Figure 6). Thus Arg¹⁹⁷ is likely to be an important residue in the Ca²⁺-dependent binding to annexin IV.

DISCUSSION

Annexins are a family of Ca²⁺- and phospholipid-binding proteins, and the Ca²⁺- and phospholipid-binding sites are located in the C-terminal consensus repeats [37]. Moreover, members of the annexin family have been shown to bind to various proteins: annexins I, II, VII and XI to S100C, p11, sorcin and calcyclin, respectively [9,11–13,20]. p11 and calcyclin are members of the S100 protein family, S100A10 and S100A6, respectively. Both S100 protein and sorcin possess high-affinity Ca²⁺-binding sites known as EF-hand structures. SP-A is also a Ca²⁺-binding protein and a high-affinity site is located in the C-terminal portion containing carbohydrate-recognition domain; however, SP-A has no EF-hand motif [19,20]. The binding sites of annexins I, II, VII, and XI for the EF-hand proteins have been identified on the N-terminal portion [9,11–13]. On the other hand, the SP-A-binding site of annexin IV was located on the C-terminal portion (Figure 2). Recently, apolipoprotein A-I has been identified to bind to annexins I and VII, and binding to annexin I occurs in the C-terminal domain [10]. Thus not only the N-terminal unique domain of annexins but also the C-terminal consensus domain could participate in the Ca²⁺-dependent interaction with the annexin-binding proteins.

The study using the truncation mutants of annexin IV showed that SP-A bound both $T_{N-1-2-3}$ and T_{3-4} (Figure 2). Since domain 3 is common between $T_{N-1-2-3}$ and T_{3-4} , the binding site of annexin IV might be located in domain 3. On the other hand, the reason why SP-A only bound weakly to both T_{2-3} and T_{2-3-4} , in spite of the fact that both contain domain 3, is not clear (Figure 2). As SP-A bound to $T_{N-1-2-3}$ in a Ca^{2+} -dependent manner and $T_{N-1-2-3}$ is a form of T_{2-3} supplemented with the N-terminal region and domain 1, the N-terminal portion might have a role in the Ca^{2+} -dependent interaction with SP-A. We speculate that domain 2 might have an inhibitory effect in the interaction with SP-A. The reason why SP-A bound weakly to T_{N-1-2} in a Ca^{2+} -dependent manner is unclear, although T_{N-1-2} lacks domain 3. The truncation mutants were all retrieved under denaturing conditions with urea from the isolated inclusion bodies of the *E. coli*, and then renatured by dialysis under native conditions (see the Materials and methods section). Thus the mutants used in this study might not have had their original conformations, even though they were soluble in neutral buffer (Figure 2). However, the CD spectra of the mutants showed two minimum ellipticities at 208 and 222 nm, indicating the presence of α -helical structures. Estimated α -helix contents were about 70–80% for all the truncation mutants and 80% for wild-type annexin IV by the method of Chen et al. [38] (results not shown). These results suggest that the mutants had their original conformations. On the other hand, the possibility of low amounts of the immobilized proteins on the microtitre wells can be ruled out since the amounts of proteins immobilized were not altered when determined with polyclonal antibody against annexin IV (results not shown).

As annexin IV binds to SP-A in a Ca^{2+} -dependent manner, it is conceivable that the conformation of the Ca^{2+} -bound state of annexin IV is required for the binding. Each 70–80 amino acid tandem repeat of annexin IV contains a consensus sequence of Lys/Arg-Gly/Arg-Xaa-Gly-Thr and an Asp or Glu that is 39 residues downstream in the sequence. The sequence forms a high-affinity type-II Ca^{2+} -binding site, based on the crystal structure of annexin V [34]. Although the initial Lys of the consensus sequence of domain 3 of both annexins V and IV is substituted for Gly, this domain on annexin V is still a Ca^{2+} -binding site [39]. Thus domain 3 of annexin IV might become a Ca^{2+} -binding site even though only two occupied Ca^{2+} -binding sites in domains 1 and 4 have been identified in the crystal structure of annexin IV [40]. Mutational experiments directed at type-II Ca^{2+} -binding sites (Asp/Glu $\rightarrow$ Ala) were first performed with annexin II, and the alteration affected the Ca^{2+} - and lipid-binding properties [41,42]. In this study, the DE mutants of annexin IV, which had substitution(s) of the conserved acidic residue(s) to Ala [27], were used to examine which Ca^{2+} -binding site(s) was important for the Ca^{2+} -dependent binding by SP-A. The single-site mutation in either domain 2 or 4 appeared to be silent in the Ca^{2+} -sensitive binding by SP-A, whereas the mutation in domain 3 slightly altered EC_{50} to a higher Ca^{2+} concentration (Figure 3). The two-site mutational study suggests that the type-II Ca^{2+} -binding site in domain 1 is not involved in the Ca^{2+} -dependent binding by SP-A and that only the Ca^{2+} -binding in domain 3 is important (Figure 4). However, even when the point mutation occurred in domain 3, Ca^{2+} -dependent binding and a similar extent of binding to wild-type annexin IV at a high free Ca^{2+} concentration was observed (Figures 3 and 4). Furthermore, the three-site mutational study indicates that only the type-II Ca^{2+} -binding site in domain 3 is sufficient for the Ca^{2+} -dependent binding by SP-A, and that the other three-site mutants with a mutation in domain 3 tend to diminish binding by SP-A (Figure 5). Taking these results together with those for truncation-

mutant annexin IV (Figure 2), both Ca^{2+} binding to the type-II Ca^{2+} -binding site in domain 3 and the conformational change in domain 1 induced by the Ca^{2+} binding in domain 3, or type-III Ca^{2+} -binding sites [43], might be required for SP-A-annexin-IV binding. On the other hand, as the immobilized proteins on the microtitre wells were comparable among the mutants when determined with polyclonal anti-annexin IV antibody (results not shown), the possibility of unequal immobilizations depending on the mutants can be ruled out.

Ca^{2+} -sensitive binding to chromaffin granules was significantly affected by mutation in the type-II Ca^{2+} -binding site in domain 4 of annexin IV, whereas the mutation in domain 3 was silent [27]. In a comparison of the results of the annexin-IV DE mutants binding to chromaffin granules with those of SP-A binding to annexin-IV DE mutants, the Ca^{2+} -binding site(s) of annexin IV required for the granules and for SP-A are distinct.

The three-dimensional structure of the C-terminal portion of MBP-A (carbohydrate-recognition domain) has been demonstrated by crystallographic studies [22,36]. It was demonstrated that both Glu¹⁸⁵ and Asn¹⁸⁷ of MBP-A ligate Ca^{2+} from the crystal structure [22,36]. Furthermore, simultaneous mutations Glu¹⁸⁵ $\rightarrow$ Gln and Asn¹⁸⁷ $\rightarrow$ Asp altered sugar-binding specificity of MBP-A [22]. From the amino acid-sequence alignment of rat SP-A and MBP-A [22], Glu¹⁹⁵ and Arg¹⁹⁷ of rat SP-A correspond with Glu¹⁸⁵ and Asn¹⁸⁷ of MBP-A, respectively. Simultaneous mutations Glu¹⁹⁵ $\rightarrow$ Gln and Arg¹⁹⁷ $\rightarrow$ Asp of SP-A also altered the functional properties of SP-A [23]. Recently, single mutation Glu¹⁹⁵ $\rightarrow$ Ala has been shown to diminish binding to mannose-Sepharose [44], whereas single substitution of Arg¹⁹⁷ with Asp or Asn attenuated the ability of lipid liposome aggregation but did not affect the other functional properties of SP-A [45]. In the present study, it is striking that the single substitution Arg¹⁹⁷ $\rightarrow$ Asp or Asn almost eliminated the binding to annexin IV, whereas Glu¹⁹⁵ $\rightarrow$ Gln was silent (Figure 6). These results might have been caused by the distinct conformational changes in the mutants compared with wild-type SP-A. However, this possibility can be ruled out since the CD spectra of the SP-A mutants were similar to that of wild-type SP-A (estimated α -helix and β -sheet contents about 21–26% and 26–33%, respectively, by the method of Chen et al. [38], results not shown). Therefore, these results suggest clearly that Arg¹⁹⁷ is important in the Ca^{2+} -dependent binding to annexin IV. On the other hand, Ala is substituted for Arg¹⁹⁷ in human SP-A [46]. We first identified annexin IV as a human SP-A-binding protein [15]. Although the reason why annexin IV binds to both human and rat SP-A in a Ca^{2+} -dependent manner is unclear, the adjacent basic residue Arg¹⁹⁹ in human SP-A (Gln is substituted for Arg¹⁹⁹ in rat SP-A) may have the role of Arg¹⁹⁷ in rat SP-A. Further study will be required to solve this problem.

Two lines of evidence demonstrated the presence of annexin IV in alveolar type-II cells: immunoblotting with the cytosolic fraction of isolated type-II cells from rats [15] and immunohistochemical studies with rabbit lung [47]. To test if lung lamellar bodies contain annexin IV, we performed immunoblot analysis with anti-annexin IV antibody, and an annexin IV-reactive band with molecular mass of 32 kDa was visualized (Sohma, H., unpublished work), suggesting that annexin IV was one of the constituents of lamellar bodies. It is possible that a complex between SP-A and annexin IV is formed in lung lamellar bodies. Lung lamellar bodies have been determined to possess large amounts of saturated neutral phospholipid (dipalmitoylphosphatidylcholine, DPPC) and acidic phospholipids (phosphatidylglycerol) [48]. Since SP-A binds preferentially to DPPC [18], whereas annexin IV binds to phosphatidylcholine membranes containing acidic phospholipid such as phosphatidyl-

serine or phosphatidylglycerol [49], the SP-A–annexin-IV complex might cross-link natural and acidic phospholipids within lamellar bodies.

The properties *in vitro* of the SP-A–annexin-IV interaction presented here may provide a clue as to the possible role of annexin IV in alveolar type-II cells.

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