

Comparison of lipid aggregation and self-aggregation activities of pulmonary surfactant-associated protein A

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1. We compared the Ca^{2+} dependence of the self-aggregation of surfactant protein A (SP-A) with that of vesicle aggregation induced by SP-A. The Ca^{2+} concentration required for half-maximal activity of lipid aggregation was $0.74 \pm 0.29 \mu\text{M}$ ($n = 4$) for pig SP-A and $98 \pm 5 \mu\text{M}$ ($n = 2$) for dog SP-A. In contrast, the threshold concentration of Ca^{2+} required to induce self-association of both pig and dog SP-A was 0.5 mM. The Ca^{2+} concentration needed for half-maximal self-association was $2.36 \pm 0.15 \text{ mM}$ ($n = 4$) and $0.70 \pm 0.06 \text{ mM}$ ($n = 2$) for pig and dog SP-A respectively. 2. We also compared the effect of Ca^{2+} on the trypsin sensitivity of lipid-free and membrane-bound SP-A. At $1 \mu\text{M}$ Ca^{2+} , the tryptic digestion patterns of dog and pig lipid-free SP-A were quite different. Dog SP-A was very sensitive to proteolysis, being almost completely digested by 30 min, while pig SP-A was very resistant, even after 12 h. After protein aggregation of lipid-free SP-A (at 5 mM Ca^{2+}), the accessibility of the trypsin cleavage targets of the protein depended on the SP-A species (self-aggregated pig SP-A became more sensitive to degradation than its non-aggregated form, whereas self-aggre-

gated dog SP-A was less susceptible). In contrast, membrane-bound SP-A, from either pig or dog, was clearly protected from trypsin degradation at both low ($1 \mu\text{M}$) or high (1 mM) Ca^{2+} concentrations. The protection was slightly higher at 1 mM Ca^{2+} when the extent of lipid/SP-A aggregates was maximal. 3. On the other hand, vesicle aggregation activity of SP-A was decreased by 30–40% by removing the oligosaccharide moiety of the protein, whereas self-aggregation was not influenced by deglycosylation. The presence of mannan (at concentrations not lower than $10 \mu\text{g}/\mu\text{l}$) decreased vesicle aggregation induced by dog and pig SP-A by a mechanism that is independent of the binding of mannan to the carbohydrate-binding domain of SP-A. Self-aggregation of SP-A was not affected by the presence of sugars. 4. From these results, we conclude that: (1) the process of lipid aggregation induced by SP-A cannot be correlated with that of self-association of the protein occurring at supramillimolar concentrations of Ca^{2+} ; and (2) the N-linked carbohydrate moiety of SP-A and the ability of SP-A to bind carbohydrates are not involved in lipid aggregation.

INTRODUCTION

Pulmonary surfactant is a heterogeneous complex of lipid and proteins that serves to stabilize the alveoli and distal airways at low lung volumes. Dipalmitoylphosphatidylcholine (DPPC) is widely accepted to be the major lipid component of pulmonary surfactant. Besides DPPC, phosphatidylglycerol (PG) and specific protein components are required for the full biological activity of surfactant. At least three of these surfactant proteins (SP-A, SP-B and SP-C) potentiate the surface tension-reducing properties of the surfactant lipids (see [1,2] for reviews).

The most abundant surfactant-associated protein (SP-A) has a monomeric molecular mass of 30–40 kDa. Each SP-A subunit is characterized by an N-terminal region containing a collagen-like sequence and a C-terminal domain with a sequence similar to several Ca^{2+} -dependent carbohydrate-binding proteins, C-type lectins [3,4]. The functional form of SP-A is assembled through interactions in the collagen-like domain into a complex oligomer of 18 subunits [5–7]. In one of the initial steps of the assembly of SP-A, three subunits of SP-A probably form a triple-helical stem that is stabilized by interchain disulphide bonds. In the final stage of the assembly, the hexamers appear to be formed by lateral aggregation of the N-terminal half of the triple-helical stems [8].

SP-A interacts with surfactant phospholipids, such as DPPC

and PG [9–11], as well as with surfactant glycosphingolipids [12]. Several studies suggest that SP-A plays a major role in the intra-alveolar surfactant phospholipid organization: (i) SP-A induces phospholipid vesicle aggregation in the presence of Ca^{2+} [9,13]; (ii) SP-A mediates the formation of large ordered tubular aggregates known as tubular myelin, when added to DPPC, PG and SP-B mixtures in the presence of Ca^{2+} [14,15]; (iii) SP-A causes a Ca^{2+} -dependent increase of the phospholipid mixing activity of SP-B [16]; and (iv) SP-A promotes the formation of a stable surface film of phospholipids cooperating with the hydrophobic surfactant protein SP-B [17].

The phospholipid vesicle aggregation activity of SP-A has been amply assessed by changes in turbidity [9,11,13,18–22], and has been seen by electron microscopy [15,23]. Typically, negatively charged vesicles were used for these studies. Some structural requirements of SP-A for lipid aggregation have been studied. Digestion with bacterial collagenase or reduction of SP-A with dithiothreitol strongly decreases lipid aggregation mediated by the protein [20] as well as the ability of SP-A to bind phospholipids [10,20], suggesting that these two processes are dependent on the integrity of the collagenous domain of SP-A (triple helix and intermolecular disulphide bond).

The requirement of the oligosaccharide moiety of SP-A for lipid aggregation is not fully clear. The carbohydrate domain of SP-A was proposed to be an important determinant in the ability

Abbreviations used: SP-A, surfactant protein A; CRD, carbohydrate recognition domain; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; PG, phosphatidylglycerol; OGP, n-octyl β -D-glucopyranoside.

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of SP-A to aggregate lipids, since deglycosylated SP-A did not aggregate phospholipids [23]. However, site-directed mutagenesis of SP-A reveals that the oligosaccharide moiety is not essential for lipid aggregation [24]. On the other hand, deglycosylation of the protein does not affect the lipid binding properties of SP-A [10,23]. Interestingly, McCormack et al. [25] recently reported that phospholipid aggregation is mediated by the C-terminal region of SP-A by a mechanism that is distinct from phospholipid binding.

Concerning the mechanism involved in the vesicle aggregation phenomenon, it has been proposed that this process could be mediated by Ca^{2+} -dependent protein-protein interaction between SP-A molecules [19]. These authors found a linear correlation between the extent of vesicle aggregation induced by SP-A in the presence of calcium and the extent of self-aggregation of SP-A lacking lipids at the same concentrations of calcium. The interaction between SP-A molecules in the presence of lipids was further proposed to occur mostly between the carbohydrate-binding domains and the oligosaccharide moieties of SP-A [23].

The present study analyses the aggregation of neutral and acidic vesicles mediated by SP-A and compares lipid aggregation and self-aggregation activities of SP-A. The possible mechanisms involved in the process of vesicle aggregation induced by SP-A are discussed.

EXPERIMENTAL

Isolation and N-glycosidase F treatment of SP-A

Pulmonary surfactant was prepared from pig or dog bronchoalveolar lavage as described previously [26]. Pig or dog SP-A was purified from isolated surfactant using sequential butanol and octyl glucoside extractions as described elsewhere [11].

Surfactant protein content was estimated by the method of Lowry using BSA as standard. However, the concentration of SP-A was always determined by quantitative amino acid analysis because the SP-A concentration was overestimated when determined by the method of Lowry. The amino acid analysis of SP-A was carried out on a Beckman System 6300 High Performance amino acid analyser. The protein hydrolysis was performed with 0.2 ml of 6 M HCl, containing 0.1% (w/v) phenol in evacuated and sealed tubes at 108 °C for 24 h. Norleucine was added to each sample as the internal standard.

Electrophoretic analysis of SP-A was performed under reducing conditions (50 mM dithiothreitol) by one-dimensional SDS/PAGE as described by Laemmli [27] using stacking and running gels of 4 and 12% acrylamide respectively. Gels were stained with Coomassie Brilliant Blue R 250.

SP-A was deglycosylated by treatment with 45 units of N-glycosidase F from *Flavobacterium meningosepticum* per mg of SP-A at 37 °C for 24 h in 15 mM sodium phosphate buffer, pH 7.4, containing 12 mM EDTA, 1.5 mM PMFS, 0.02% sodium azide and 20 mM n-octyl β -D-glucopyranoside (OGP). Control incubations contained all components except the enzyme. After incubation, OGP or EDTA were removed by dialysis of the reaction mixture against 5 mM Tris/HCl buffer, pH 7.4, for 48 h at 4 °C. The protein content in control and enzyme-treated samples was determined by quantitative amino acid analysis. SP-A was completely deglycosylated by N-glycosidase F, as shown by the change of mobility on SDS/PAGE under reducing conditions.

SP-A self-association assay

Ca^{2+} -dependent self-association of SP-A was studied as described previously [21]. Briefly, the sample and the reference cuvettes

were first filled with 0.4 ml of 5 mM Tris/HCl buffer, pH 7.4. After 10 min equilibration at 37 °C, SP-A (20 μg) was added to the sample cuvette and the change in absorbance at 360 nm was monitored at 1-min intervals over 10 min. Next, Ca^{2+} (5 mM, final concentration) was added to both the sample and the reference cuvette and the change in absorbance was monitored again. Self-association of SP-A was reversed by adding EDTA (10 mM, final concentration).

Preparation of lipid vesicles

Unilamellar vesicles of DPPC, DPPC/dipalmitoylphosphatidylglycerol (DPPG) (7:3, w/w) and DPPC/egg-PG (7:3, w/w) were used. The different lipid vesicles were prepared as described previously [11] at a phospholipid concentration of 1 mg/ml by hydrating dry lipid films in a buffer containing 150 mM NaCl, 5 mM Tris/HCl, pH 7.4, and allowing them to swell for 1 h at temperature above the phase-transition temperature of the phospholipid vesicles. Next, the lipid dispersion was sonicated at the same temperature (above 45 °C) at 240 W with 10 bursts of 30 s (15 s between bursts) in an MSE tip Sonifer. The phospholipid concentration was determined by phosphorus determination according to Rouser et al. [28]. For vesicle-size analysis in solution, quasielastic light scattering (QELS) was used according to Koppel [29] on an Autosizer IIc Photon Correlation Spectrometer (Malvern Instruments, U.K.). Measurements were performed at 25 °C and 45 °C. Vesicle diameter for DPPC and for the binary mixtures DPPC/DPPG or DPPC/PG (7:3, w/w) was around 160–200 nm with a polydispersity index of 0.2, as reported previously [11].

Phospholipid vesicle aggregation assay

SP-A-induced phospholipid vesicle aggregation assays were performed at 37 °C in a Beckman DU-640 spectrophotometer, measuring the change in the absorbance at 400 nm as described previously [22]. Briefly, phospholipid vesicles (50 μg) were added to both the sample and the reference cuvette in a total volume of 0.5 ml of 5 mM Tris/HCl buffer, pH 7.4, 150 mM NaCl, with or without 50 μM EGTA. After 10 min equilibration at 37 °C, 5 μg of SP-A from a stock solution of the protein in 5 mM Tris/HCl buffer, pH 7.4, was added to the sample cuvette and the change in absorbance at 400 nm was monitored at 1-min intervals over 10 min. Next, Ca^{2+} (typically 1 mM final concentration) was added to both the sample and the reference cuvette and the change in absorbance was monitored again at 1-min intervals over 10 min. EDTA was used for reversing vesicle aggregation induced by SP-A.

Titration experiments

The calcium requirement for vesicle or self-aggregation was studied by titration experiments in which increasing amounts of a concentrated solution of CaCl_2 were added to the protein solution in the absence or presence of lipids. The assay buffer contained 50 μM EGTA. The Ca^{2+} concentration without adding CaCl_2 (contaminant Ca^{2+} in the experimental solution system) was 5 μM as measured by atomic absorption. The free Ca^{2+} concentration in each point of the titration experiments was estimated by a computer program (CHELATOR) [30] which also permits correction for ionic strength, temperature, pH and other competing ions.

Trypsin digestion

First, dog or pig SP-A (160 $\mu\text{g}/\text{ml}$) was incubated for 20 min at

37 °C with either DPPC or DPPC/DPPG (7:3, w/w) vesicles (1.6 mg/ml) in 5 mM Tris/HCl buffer, pH 7.4, 150 mM NaCl, 50 μ M EGTA, in the presence of either 1 μ M or 1 mM free Ca^{2+} . In addition, SP-A was incubated without lipids in the same conditions except that NaCl was not included in the buffer and that free Ca^{2+} concentrations were 1 μ M or 5 mM. Then, trypsin (Millipore, 246 units/mg; 20 μ g/ml) was added to the samples and the trypsin incubation was carried out at 37 °C at the indicated times. Digestion was stopped by adding 1 μ l of 1 M HCl (final pH 2.0). The mixtures were then dried under vacuum and analysed by SDS/electrophoresis under reducing conditions.

Data reported in the Figures of this paper were obtained from four different preparations of pig SP-A and from two different preparations of dog SP-A. For each preparation experiments were repeated at least twice.

RESULTS

Figure 1 shows that the addition of pig SP-A to either DPPC or DPPC/DPPG (7:3, w/w) vesicles in 5 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl results in a marked increase in light absorbance due to lipid aggregation. After addition of Ca^{2+} (3 mM, final concentration) the light absorbance of SP-A/lipid aggregates increases by an additional 20–25%. Interestingly, the addition of EDTA (6 mM) dissociates the aggregate completely, suggesting that vesicle aggregation occurring after addition of pig SP-A is also dependent on Ca^{2+} . In fact, the presence of 1 mM EDTA in the assay buffer prevents lipid aggregation induced by adding SP-A (Figure 1). Considering that the endogenous Ca^{2+} concentration (i.e. without adding Ca^{2+} to our experimental aqueous system) was 5 μ M, as measured by atomic absorption, the results suggest that pig SP-A induces lipid aggregation at very low concentrations of Ca^{2+} . Figure 1 also shows that pig SP-A induces aggregation of DPPC/DPPG (7:3, w/w) in the presence, but not in the absence, of 150 mM NaCl.

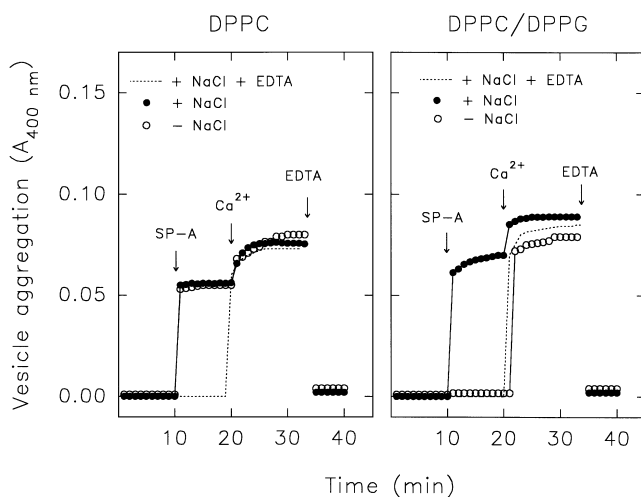


Figure 1 Physiological ionic strength is required for SP-A-induced aggregation of acidic but not neutral vesicles at low calcium concentrations

Sample and reference cuvettes were filled with 70 μ g/ml of either DPPC or DPPC/DPPG (7:3, w/w) vesicles in 5 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl (●), or 4 mM NaCl (○), or 150 mM NaCl and 1 mM EDTA (broken line). After 10 min equilibration at 37 °C, pig SP-A (final concn. 6 μ g/ml) was added to the sample cuvette. Next, Ca^{2+} (final concn. 3 mM) was added to both the sample and the reference cuvette. The process was reversed by addition of 6 mM EDTA to both cuvettes. A representative experiment of four experiments is shown. Four different preparations of pig SP-A were used.

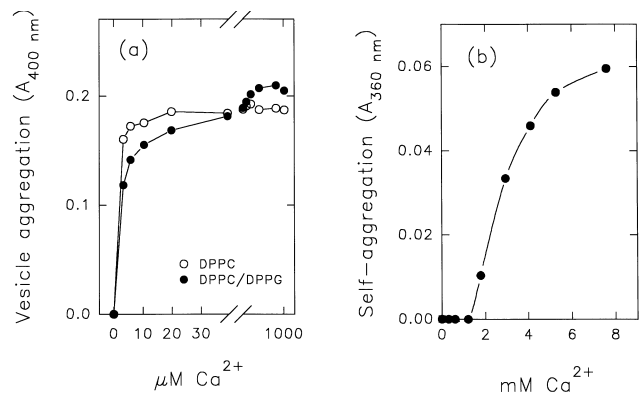


Figure 2 Ca^{2+} dependence of vesicle aggregation and self-aggregation

Experiments were done at 37 °C as described in the Experimental section. (a) Final concentrations of pig SP-A and phospholipids were 15 μ g/ml and 150 μ g/ml respectively. (b) Final concentration of SP-A was 44 μ g/ml. A representative experiment of four experiments from four different preparations of pig SP-A is shown.

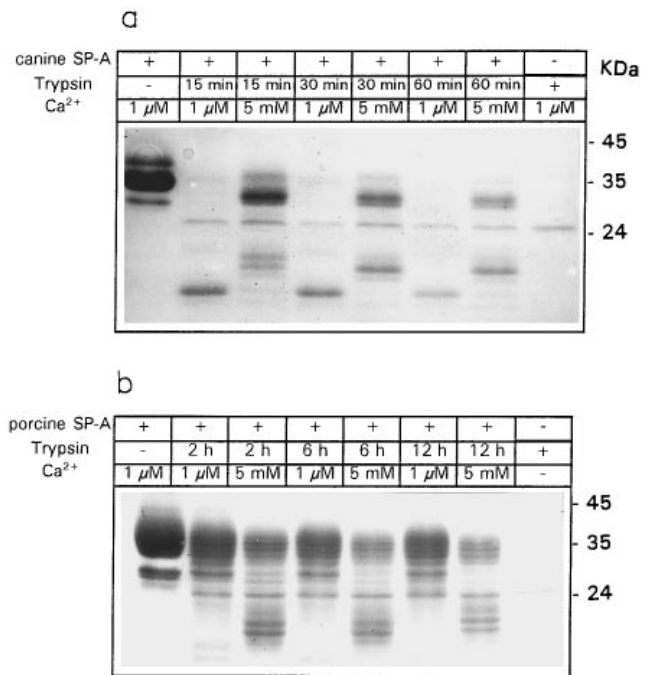


Figure 3 Trypsin sensitivity of dog (a) and pig (b) lipid-free SP-A in their non-aggregated and aggregated forms

Proteolytic digestion of SP-A was done at the indicated times as described in the Experimental section. Molecular-mass markers in kDa are indicated to the right of the gel.

However, ionic strength has no influence on the aggregation of neutral vesicles of DPPC. These results further confirm previous studies [11] which showed that the binding of negatively charged vesicles to SP-A was abrogated at low ionic strength. However, the interaction of neutral vesicles with SP-A was not dependent on the ionic strength. The lack of interaction of acidic vesicles with SP-A at low ionic strength and Ca^{2+} concentration and the subsequent abolition of vesicle aggregation is interpreted to arise from electrostatic repulsion between the negative charge of the phospholipids and the negative surface charge on the protein.

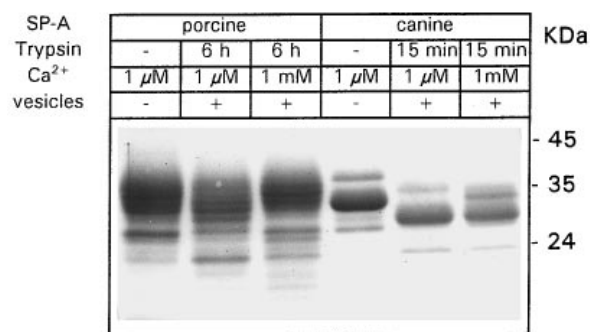


Figure 4 Trypsin sensitivity of membrane-bound SP-A

Dog or pig SP-A was first incubated with DPPC/DPPG (7:3, w/w) vesicles at 37 °C for 20 min in the presence of Ca²⁺ (1 μ M or 1 mM). Then, the proteolytic digestion of either lipid-bound SP-A or lipid/SP-A aggregates was done at the indicated times. Similar results were obtained with DPPC vesicles. Molecular-mass markers in kDa are indicated to the right of the gel.

The aggregation of vesicles is dependent on the lipid/protein ratio. Titration of SP-A with DPPC vesicles resulted in a hyperbolic plot which reached saturation at a lipid/protein weight ratio around 10:1. Titration of a preparation of DPPC vesicles with SP-A gave a similar saturating lipid/protein weight ratio (results not shown). The same results were obtained with negatively charged vesicles. According to these results, we used a

lipid/SP-A weight ratio of 10:1 or higher in all experiments reported here.

Calcium dependence of the vesicle and self-aggregation processes

Figure 2(a) shows the calcium dependence of vesicle aggregation induced by pig SP-A. At very low concentrations of calcium, aggregation of neutral and acidic vesicles is observed, reaching a maximum level at 20 μ M Ca²⁺. The Ca²⁺ concentration required for half-maximal vesicle aggregation induced by pig SP-A ($k_a^{Ca^{2+}}$) is $0.76 \pm 0.24 \mu$ M ($n = 4$) for DPPC, $0.74 \pm 0.29 \mu$ M ($n = 4$) for DPPC/DPPG (7:3, w/w) and 0.74μ M ($n = 1$) for DPPC/PG (7:3, w/w). The Ca²⁺ activation constant ($k_a^{Ca^{2+}}$) for SP-A-dependent vesicle aggregation does not depend on the lipid composition of the vesicles. The extent of aggregation of DPPC/PG (7:3, w/w) or DPPC/phosphatidylinositol (7:3, w/w) vesicles, which might contain co-existing fluid and gel domains at 37 °C, was lower than that of DPPC or DPPC/DPPG (7:3, w/w) vesicles [22]. This suggests the influence of the physical state of the vesicles on the aggregation process, as previously reported elsewhere [11].

Figure 2(b) shows the calcium dependence of the self-aggregation process of pig SP-A. The Ca²⁺ concentration required for half-maximal self-association is 2.36 ± 0.15 mM ($n = 4$). This value is much higher than that for half-maximal vesicle aggregation given above. The threshold concentration of calcium required to induce self-association of pig SP-A is 0.5 mM, similar to that reported for other SP-A species (dog or human) [19]. On

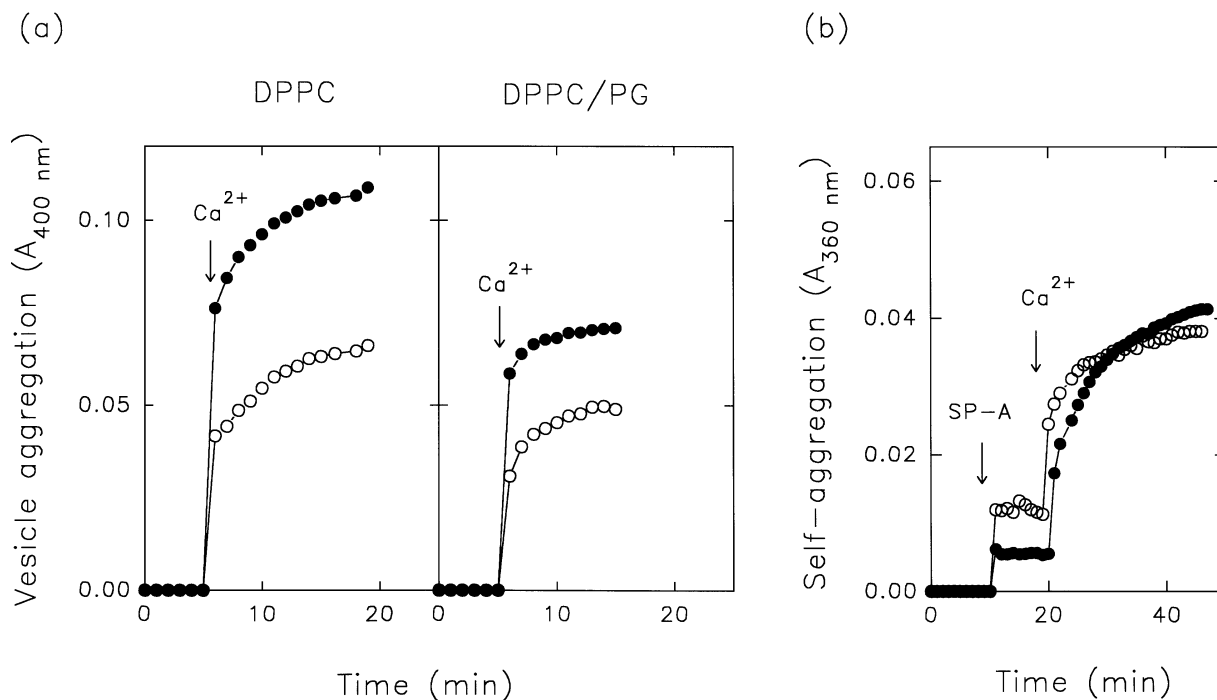


Figure 5 Effect of deglycosylation of pig SP-A on vesicle aggregation (a) and self-aggregation (b)

(a) Sample and reference cuvettes were filled with 70 μ g/ml of either DPPC or DPPC/PG (7:3, w/w) vesicles in 5 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl and 50 μ M EGTA. After 10 min equilibration at 37 °C, 6 μ g/ml SP-A was added to the sample cuvette. Next, vesicle aggregation was started by addition of Ca²⁺ (final concn. 1 mM) to both the sample and the reference cuvette. (b) Self-aggregation was done as described in the Experimental section. Final concentrations of pig SP-A and Ca²⁺ were 40 μ g/ml and 5 mM respectively. The results shown are from a representative one of four experiments. Two different preparations of pig SP-A were used.

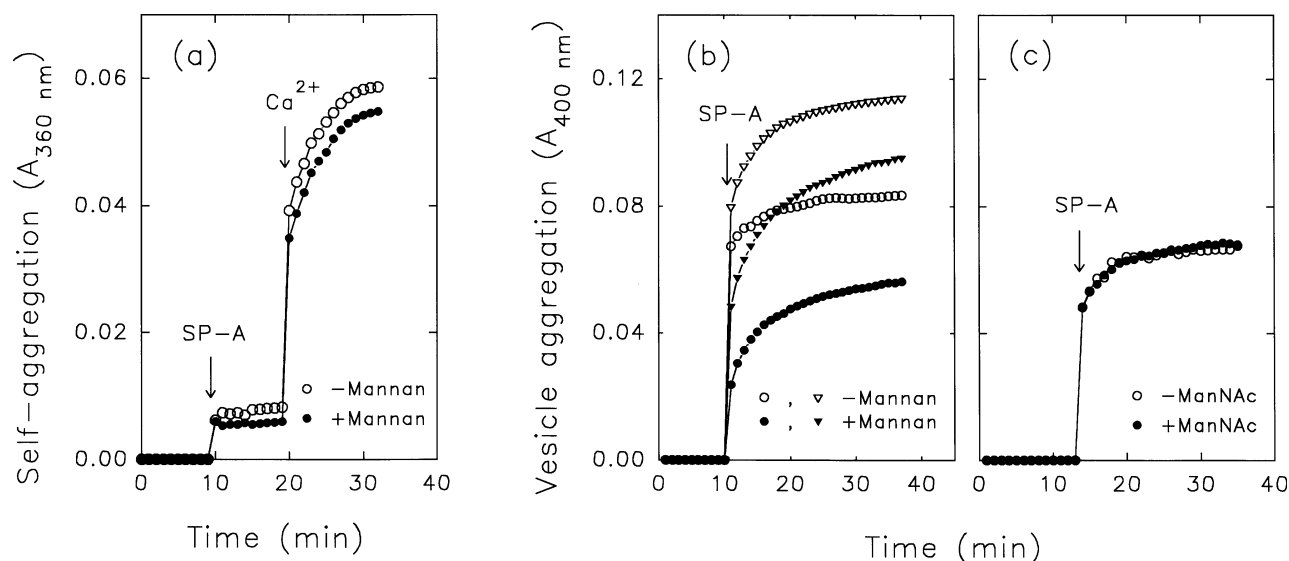


Figure 6 Effect of the presence of carbohydrates on self-association (a) and vesicle aggregation (b,c) at pH 7.4

Self-association (a) was done as described in the Experimental section. Final concentrations of dog SP-A, Ca^{2+} and mannan were 50 $\mu\text{g}/\text{ml}$, 5 mM and 10 $\mu\text{g}/\mu\text{l}$ respectively. For vesicle aggregation, sample and reference cuvettes were filled with either 80 $\mu\text{g}/\text{ml}$ (triangles) or 40 $\mu\text{g}/\text{ml}$ (circles) of DPPC/DPPG (7:3, w/w) vesicles in 5 mM Tris/HCl buffer, pH 7.4, 150 mM NaCl, 1 mM Ca^{2+} , with or without 10 $\mu\text{g}/\mu\text{l}$ mannan (b) or 100 mM *N*-acetylmannosamine (c). After 10 min equilibration at 37 °C, vesicle aggregation was started by addition of 8 $\mu\text{g}/\text{ml}$ dog SP-A. The phospholipid/protein ratio was 10:1 (triangles) or 5:1 (circles). Results presented are from a representative one of three experiments. Similar results were obtained with pig SP-A.

the other hand, the Ca^{2+} requirement for vesicle aggregation induced by SP-A is not fully clear. Ross et al. [20] reported that the threshold concentration of Ca^{2+} required for vesicle aggregation induced by dog SP-A was higher than 3 mM. That value is above the free Ca^{2+} concentration in the alveolar space of adult animals (approx. 1.5 mM) [31]. In contrast, Efrati et al. [18] found that the threshold concentration of Ca^{2+} needed for dog SP-A-induced vesicle aggregation was 0.5 mM. To determine whether the high difference between Ca^{2+} activation constants for vesicle and self-aggregation observed with pig SP-A occurred also in other SP-A species, we repeated the experiments with dog SP-A and DPPC/DPPG (7:3, w/w) vesicles. The Ca^{2+} concentration required for half-maximal vesicle aggregation induced by dog SP-A was $98 \pm 5 \mu\text{M}$ ($n = 2$), whereas that required for half-maximal self-association was $0.70 \pm 0.06 \text{ mM}$ ($n = 2$). Thus independent of SP-A species, these two processes have very different Ca^{2+} requirements, showing that lipid aggregation activity of SP-A cannot be mediated by self-aggregation of the protein induced by supramillimolar concentrations of Ca^{2+} .

Trypsin sensitivity of SP-A in lipid/protein aggregates or in its lipid-free self-associated form

Figure 3 shows the tryptic digestion patterns of dog (Figure 3a) and pig (Figure 3b) lipid-free SP-A, in the presence of 1 μM or 5 mM Ca^{2+} , at the indicated times. Dog SP-A is very sensitive to proteolysis in its non-aggregated form (at 1 μM Ca^{2+}) being almost completely digested by 30 min, while pig SP-A is very resistant, even after 12 h. Interestingly, the effect of protein aggregation on the susceptibility to proteolysis of dog and pig SP-A in their self-associated forms (reached at 5 mM Ca^{2+}) is opposite. After aggregation (at 5 mM Ca^{2+}), pig SP-A becomes more sensitive to degradation than its non-aggregated form, whereas dog SP-A is less susceptible. These results suggest that aggregation seems to modify protein conformation since the

accessibility of trypsin cleavage targets of SP-A changes, being more or less exposed depending on SP-A species.

Membrane-bound SP-A from either pig or dog is clearly protected from trypsin degradation at both low (1 μM) or high (1 mM) concentrations of Ca^{2+} (Figure 4). The protection is independent of SP-A species and is slightly higher when lipid/SP-A aggregates are completely formed at 1 mM Ca^{2+} .

Involvement of the carbohydrate moiety and the carbohydrate-binding domain of SP-A in vesicle aggregation and self-aggregation

To investigate whether the oligosaccharide chains of SP-A are required for vesicle aggregation and self-aggregation processes, deglycosylation of the protein was carried out using N-glycosidase F. After deglycosylation treatment, no glycosylated SP-A could be detected by Coomassie Blue staining. Deglycosylation of pig SP-A decreases aggregation of neutral and acidic vesicles by 30–40% (Figure 5a). However, self-aggregation of the protein is not influenced by removing asparagine-linked carbohydrate (Figure 5b). Interestingly, although the extent of aggregation decreases with deglycosylated pig SP-A, the Ca^{2+} concentration required for half-maximal vesicle aggregation ($k_a^{\text{Ca}^{2+}}$) is similar for the two proteins, control and deglycosylated.

To find out if the binding of mannan to the carbohydrate-binding domain of dog or pig SP-A has any influence on vesicle aggregation and self-aggregation phenomena, we studied the effect of the presence of this homopolysaccharide on these two processes. The binding of SP-A to mannan was shown to occur at pH values higher than 4.5 and at Ca^{2+} concentrations higher than 10 μM [32]. Figure 6(a) shows that the presence of mannan (at a concentration of 10 $\mu\text{g}/\mu\text{l}$) does not influence self-association of the protein, but decreases vesicle aggregation induced by dog or pig SP-A at pH 7.4 (Figure 6b). Lower concentrations of mannan do not influence lipid aggregation. The inhibitory

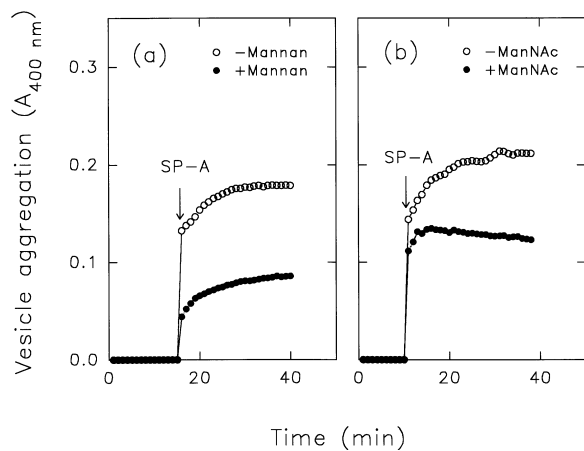


Figure 7 Effect of mannan (a) or *N*-acetylmannosamine (b) on vesicle aggregation at pH 4.0

Sample and reference cuvettes were filled with 80 $\mu\text{g/ml}$ of DPPC/DPPG (7:3, w/w) vesicles in 150 mM NaCl, 1 mM Ca^{2+} , 25 mM acetate buffer, pH 4.0 with or without 10 $\mu\text{g}/\mu\text{l}$ mannan (a) or 100 mM *N*-acetylmannosamine (b). After 10 min equilibration at 37 $^{\circ}\text{C}$, vesicle aggregation was started by addition of dog SP-A (8 $\mu\text{g/ml}$). The results shown are from a representative one of three experiments. Similar plots were obtained with pig SP-A.

effect of mannan increases when phospholipid/protein and phospholipid/mannan weight ratios were decreased (Figure 6b). The addition of excess mannan to lipid/protein aggregates previously formed does not dissociate those aggregates (results not shown). On the other hand, the presence of an excess of *N*-acetylmannosamine (100 mM), which binds to the carbohydrate-binding domain of SP-A very efficiently [32], does not influence vesicle aggregation induced by dog or pig SP-A at pH 7.4 (Figure 6c). Higher concentrations of that monosaccharide (200 mM) are needed to cause a 20% decrease of vesicle aggregation at pH 7.4.

The possibility that the inhibitory effect of mannan on vesicle aggregation is due to the interaction of the polysaccharide with the membrane cannot be excluded. Therefore, we studied the effect of the presence of mannan and *N*-acetylmannosamine on vesicle aggregation at pH 4.0. At this pH, mannan does not bind to either SP-A or other collectins such as mannan-binding protein (MBP) or conglutinin [32]. Figure 7 shows that the presence of mannan (10 $\mu\text{g}/\mu\text{l}$) or *N*-acetylmannosamine (100 mM) decreases vesicle aggregation induced by dog or pig SP-A at pH 4.0. The inhibitory effect of mannan is higher at pH 4.0 than at pH 7.4. From these data we conclude that the effect of sugars on vesicle aggregation is independent of the binding of carbohydrates to the carbohydrate recognition domain (CRD) of SP-A. The effect of mannan on vesicle aggregation might be due to the interaction of carbohydrates with lipid vesicles that could lead to alterations in the physical properties of membranes [33]. Goodrich et al. [34] reported that the intercalation of carbohydrates into the interfacial region of membranes resulted in a lowering of the phase-transition temperature via expansion of the lattice and modification of the whole lipid interfacial region. We previously showed [11] that the interaction of SP-A with phospholipid vesicles requires the lipids to be in the gel phase and that SP-A-induced vesicle aggregation was strongly dependent on the physical state of the vesicles. Therefore, it is conceivable that the process of lipid aggregation would be influenced by the presence of excess mannan if the interaction between carbohydrates and phospholipid vesicles resulted in a lowering of the gel-to-liquid-crystalline phase-transition tem-

perature. Alternatively, the intercalation of carbohydrates into the interfacial region of membranes could impede the proximity between vesicles and consequently the aggregation between them.

DISCUSSION

The ability of SP-A to aggregate phospholipid vesicles has been widely studied [11,13,18–22]. It was recently reported that the CRD domain of SP-A was directly implicated in that process [25,35,36]. However, the mechanism involved in the vesicle aggregation phenomenon is poorly understood. It has been suggested that vesicle aggregation could be mediated by Ca^{2+} -dependent self-association of SP-A [19]. The results presented here prove that the process of lipid aggregation induced by SP-A cannot be correlated with that of self-association of the protein. These two processes have very different requirements of Ca^{2+} . The Ca^{2+} activation constant ($K_a^{\text{Ca}^{2+}}$) for SP-A-dependent vesicle aggregation was $0.74 \pm 0.29 \mu\text{M}$ for pig SP-A and $98 \pm 5 \mu\text{M}$ for dog SP-A. In contrast, $K_a^{\text{Ca}^{2+}}$ for self-aggregation of SP-A was $2.36 \pm 0.15 \text{ mM}$ and $0.70 \pm 0.06 \text{ mM}$ for pig and dog SP-A respectively. In addition, deglycosylation of the protein caused a 30–40% decrease in vesicle aggregation without influencing self-association of SP-A. Reduction of the lipid aggregation activity of deglycosylated SP-A could be due to structural modifications in the C-terminal region of SP-A caused by removing the carbohydrate moiety. It was recently shown that the C-terminal region of SP-A is critical for lipid vesicle aggregation [25,35,36]. Nothing is known about the structural domain of SP-A directly involved in the process of self-association.

On the other hand, the binding of phospholipids to either dog or pig SP-A at very low concentrations of Ca^{2+} (1 μM) caused a marked protection of SP-A from trypsin degradation. At this Ca^{2+} concentration lipid aggregation did not occur (dog SP-A) or is half-maximal (pig SP-A). The protection was only slightly higher at 1 mM Ca^{2+} when the extent of lipid/SP-A aggregates was maximal. The binding of phospholipids to SP-A would lead to a reduced accessibility of the trypsin cleavage targets located in the domains in which phospholipids are bound. Both the hydrophobic region of SP-A (neck domain) and the region of the small disulphide loop in the CRD have been proved to be involved in the lipid binding properties of SP-A [35,36]. We have previously shown [11] that the interaction of phospholipids with either pig or human SP-A caused a conformational change in the protein molecule affecting tryptophan residues of SP-A, which are located in the C-terminal 38 amino acids (at positions 191 and 213) in all species studied until now. Therefore, it is conceivable that this conformational change caused by phospholipid binding, and the subsequent aggregation at higher concentrations of Ca^{2+} , led to a reduced exposure of trypsin cleavage targets located not only in the region in which phospholipids are bound but also in closed areas of the CRD. In contrast, the effect of self-association on the trypsin sensitivity of SP-A was variable depending on the SP-A species. Thus, the self-aggregated form of pig SP-A was much more sensitive to trypsin degradation than its non-aggregated form, whereas dog SP-A was less susceptible to proteolysis after self-aggregation.

Taken together, all these results show that the process of lipid aggregation is not dependent on the self-association of the protein which occurs at supramillimolar concentrations of Ca^{2+} . The question that remains is what is the mechanism involved in the vesicle aggregation phenomenon? Haagsman et al. [23] have proposed that vesicle aggregation could be mediated by Ca^{2+} -induced interactions between carbohydrate-binding domains and the oligosaccharide moieties of SP-A. This hypothesis could be

supported by two facts: (1) the low Ca^{2+} requirement for lectin activity [32], which is similar to that for vesicle aggregation; and (2) the critical requirement of the CRD for vesicle aggregation [25,35,36]. We found, however, that the carbohydrate moiety of SP-A was not critical for vesicle aggregation, in agreement with previous studies [24,37]. Furthermore, lipid/SP-A aggregates could not be dissociated by addition of excess mannan. The presence of that homopolysaccharide decreased vesicle aggregation by a mechanism that is independent of the binding of mannan to the carbohydrate-binding domain of SP-A. Reduction of vesicle aggregation occurred at both pH 7.4 and pH 4.0. However, at acidic pH the binding of mannan to SP-A or to other C-type lectins is abrogated [32]. These results exclude the involvement of interactions between oligosaccharide moieties and carbohydrate-binding sites of SP-A in vesicle aggregation.

Recent studies [25] suggested that the binding of SP-A to phospholipid vesicles and the subsequent aggregation event were separate processes, because residues Glu¹⁹⁵ and Arg¹⁹⁷ of the CRD of rat SP-A were critical determinants for vesicle aggregation but not for phospholipid binding. Therefore, it is possible that in the presence of Ca^{2+} and after lipid binding, additional protein-structure-related changes could occur that lead to aggregation. A possible model to explain that process would be the following: the binding of phospholipids to SP-A could induce a specific Ca^{2+} -dependent conformational change in the protein molecule which could trigger protein-protein interactions between the CRDs of SP-A. Therefore, SP-A/liposome complexes could aggregate by a mechanism of SP-A self-association that must be necessarily different from that which occurs without lipids and is triggered by supramillimolar concentrations of calcium.

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