The lung lectin surfactant protein A aggregates phospholipid vesicles via a novel mechanism

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Surfactant protein A (SP-A), a lung-specific glycoprotein, consists of an *N*-terminal collagen-like domain and a *C*-terminal domain with a sequence similar to that of several Ca^{2+} -dependent lectins. SP-A induces a rapid Ca^{2+} -dependent aggregation of phospholipid vesicles. We report here that vesicle aggregation is mediated by Ca^{2+} -induced interactions between carbohydrate-binding domains and oligosaccharide moieties of SP-A. This novel mechanism of membrane interactions may be relevant to the formation of the membrane lattice of tubular myelin, an extracellular form of surfactant.

INTRODUCTION

Pulmonary surfactant consists of macromolecular complexes of lipids and specific proteins [1,2]. The surfactant lipids, particularly dipalmitoyl phosphatidylcholine, modify the surface forces at the air/fluid interface of the lung, promoting lung stability. Surfactant protein A (SP-A) is the most abundant of the lung-specific surfactant proteins [3,4]. The functional form of the protein is assembled through interactions in the collagen-like domain into a complex oligomer of approx. 18 subunits [5–7]. Each subunit of SP-A consists of an *N*-terminal domain containing a collagen-like sequence, and a *C*-terminal domain with a sequence similar to several Ca^{2+} -dependent lectins. The carbohydrate-binding domain of SP-A contains asparagine-linked oligosaccharide units. The structure of these units has been determined by ^1H -n.m.r. spectroscopy [8,9]. SP-A induces a rapid Ca^{2+} -dependent aggregation of phospholipid vesicles [10–12]. Binding of SP-A to immobilized monosaccharides shows a similar Ca^{2+} -dependency [13].

Several important roles have been attributed to SP-A, such as regulation of surfactant homeostasis [14], regulation of macrophage function [15] and formation of tubular myelin [16,17]. Tubular myelin is a unique extracellular lattice of membranes from which lipids in bilayers are thought to form a monomolecular film of phospholipids at the air/fluid interface. SP-A has been localized by immuno-electron microscopy in tubular myelin [16], and proteinaceous material with the dimensions of SP-A has been observed between tubular myelin membranes by post-fixation with OsO_4 /ferrocyanide [18]. In lungs of infants dying from respiratory distress syndrome, the lack of tubular myelin correlated with SP-A deficiency [19]. These and other studies suggest that SP-A–SP-A interactions provide the framework of tubular myelin. However, it must be emphasized that, in order to create the tubular myelin lattice, membrane fusions are required that are dependent on the presence of hydrophobic surfactant proteins [12], notably surfactant protein B (SP-B) [17].

In the present study we report a novel mechanism of membrane–membrane interactions induced by self-recognition of SP-A. This mechanism may be relevant to the formation of tubular myelin.

EXPERIMENTAL

Isolation of SP-A

Pulmonary surfactant was isolated from bronchoalveolar

lavage of patients with alveolar proteinosis [11]. SP-A was purified as described previously [13]. A collagenase-resistant fragment (CRF) of SP-A was purified by mannose-affinity chromatography [20]. SP-A was deglycosylated by *N*-glycosidase F treatment. The protein (400 μg) was transferred to deglycosylation buffer [20 mM-sodium phosphate buffer (pH 7.4)/10 mM-EDTA/0.2% Triton X-100] and digested with 16 units of *N*-glycosidase F (Boehringer 913782) at 37 °C. During overnight incubations 0.02% NaN_3 was included. Control incubations, containing all components except for the enzyme, were always included. Triton X-100 and low-molecular-mass compounds were routinely removed by Extracti-Gel D chromatography (Pierce Chemical Co.). In deglycosylation experiments, done as a function of time (Fig. 1), Extracti-Gel D chromatography was not performed. The presence of Triton X-100 (final concn. 0.002%) did not affect the aggregation assays. Electrophoretic analyses under reduced conditions were performed as described by Laemmli [21].

Vesicle aggregation

Measurements of SP-A-induced vesicle (dipalmitoyl phosphatidylcholine/phosphatidylglycerol, 7:3, w/w) aggregation were carried out at 37 °C on an Aminco DW-2a spectrophotometer as described previously [20]. Both the sample and reference cuvette contained all reactants. After 10 min pre-incubation at 37 °C, aggregation was initiated by the addition of CaCl_2 (final concentration 5 mM) to the sample cuvette.

Binding of SP-A to vesicles was determined as follows. Vesicles (500 μg), with a trace (0.05 μCi) of [^{14}C]dipalmitoyl phosphatidylcholine were incubated for 20 min at 37 °C with 50 μg of SP-A in 5 mM-Tris/HCl (pH 7.4)/150 mM-NaCl in a volume of 1 ml. After the incubation, vesicles were recovered by flotation (SW 40 rotor; 35 000 rev./min; 180 min; 37 °C) in the same buffer but including NaBr (1.64 M) and MgCl_2 (2 mM).

Ca^{2+} -dependent self-association of SP-A (10 μg) was investigated by turbidity measurements at 400 nm on an Aminco DW-2a spectrophotometer. The experiments were carried out at 37 °C in 5 mM-Tris/HCl, pH 7.4, in a total volume of 1 ml. Aggregation was initiated by the addition of Ca^{2+} (final concn. 5 mM).

Electron microscopy

Vesicle-aggregation assays were done as described above. Subsequently vesicles were rapidly frozen in liquid propane (–180 °C) from room temperature with a Reichert KF80 freezing

device. Neither cryoprotectants nor chemical fixatives were used. The fracturing and shadowing with Pt/C (45° angle) and C (90° angle) were performed in a Balzers BAF 300 freeze-etch unit at a vacuum of 66.7 μ Pa (5×10^{-7} Torr) and a temperature of -110°C . Replicas were cleaned in a sodium hypochlorite solution and distilled water before mounting on copper grids, and were observed in a JEOL 1200EX electron microscope at 80 kV.

RESULTS AND DISCUSSION

Vesicle aggregation is a convenient method to study the role of SP-A in membrane-membrane interactions as they occur in tubular myelin [10–12]. In the absence of lipid, Ca^{2+} induces self-

Fig. 1. Effect of deglycosylation of SP-A (a) on SP-A-induced vesicle aggregation (b) and self-association (c) of SP-A

SP-A was deglycosylated by *N*-glycosidase F at 37°C as a function of time (+ lanes, *N*-glycosidase F-treated; – lanes, control incubations). After 5 h incubation, no glycosylated SP-A could be detected by Coomassie Blue staining, whereas after 3 h incubation a faint band of glycosylated SP-A was always observed. Molecular-mass markers in kDa are indicated to the right of the gel. Vesicle aggregation by SP-A (b) and self-association of SP-A (c) are plotted as maximal turbidities measured as a function of incubation time of the deglycosylation reaction. Deglycosylation, SP-A-induced vesicle aggregation and self-association of SP-A were done as described in the Experimental section.

association of SP-A [20]. Self-association is a prerequisite for lipid aggregation and depends on a structurally intact collagen-like helix. Binding of SP-A to immobilized mannose shows a similar Ca^{2+} -dependency to vesicle aggregation [13].

A mild deglycosylation method was developed, using *N*-glycosidase F, to prepare SP-A devoid of asparagine-linked carbohydrate. After 5 h incubation, no glycosylated SP-A could be detected by Coomassie Blue staining (Fig. 1a). Deglycosylation of SP-A inhibits Ca^{2+} -induced vesicle aggregation. Fig. 1(b) shows the maximal aggregation as a function of incubation time of the deglycosylation reaction. Preparations of SP-A that were treated for 5 h or 7 h with *N*-glycosidase F had not completely lost the ability to aggregate vesicles. This may be explained by the presence of glycosylated SP-A that could not be detected by Coomassie Blue staining. The increase in turbidity after the addition of Ca^{2+} to vesicles and SP-A that had been treated for 24 h with enzyme was similar to that observed after addition of Ca^{2+} to SP-A alone (self-association). Self-association of SP-A was not affected by deglycosylation (Fig. 1c). Deglycosylation did also not affect the carbohydrate- and lipid-binding properties of SP-A. Deglycosylated SP-A was eluted from immobilized mannose in the presence of EDTA in exactly the same way as control SP-A. In the lipid-binding assay, both control and deglycosylated SP-A were recovered in the pellicle, together with the lipids. The addition of Ca^{2+} (5 mM) did not affect this distribution. SP-A-induced lipid aggregation can be reversed by EDTA, which indicates that vesicle are aggregated, but not fused.

Freeze-fracture electron microscopy of vesicles incubated in the presence of glycosylated SP-A and Ca^{2+} confirmed that the vesicles are aggregated (Fig. 2a). SP-A did not induce vesicle fusion, in line with the reversibility of this process after chelation of Ca^{2+} . Vesicles incubated in the presence of deglycosylated SP-A and Ca^{2+} were not aggregated. (Fig. 2b). If control SP-A was used and Ca^{2+} was omitted, the electron micrographs were similar to that shown in Fig. 2(b). These results suggest that the oligosaccharide moiety of SP-A is involved in vesicle-vesicle interactions.

To study whether the ability of SP-A to bind carbohydrates is involved in vesicle aggregation, the following study was designed. Aggregation experiments were performed in the presence of yeast mannan. This mannose homopolysaccharide was able to block SP-A-induced vesicle aggregation (Fig. 3). In addition, vesicles that are aggregated could be dissociated by the addition of excess mannan (Fig. 3). These experiments show that, if the carbohydrate-binding sites of SP-A are occupied, Ca^{2+} -induced vesicle aggregation is inhibited. Taken together with the observation that deglycosylated SP-A does not induce vesicle aggregation, this strongly suggests that SP-A self-recognition via its carbohydrate-binding domain is involved in membrane-membrane interactions.

Further support for this conclusion was obtained by experiments using a CRF of SP-A. This fragment is produced by degradation of the collagenous domain of SP-A by bacterial collagenase. The resulting fragment contains both the carbohydrate-binding domain and the oligosaccharide moiety. CRF is a trimer under non-denaturing conditions [7] and is able to bind to immobilized mannose in the presence of Ca^{2+} , although a higher concentration of Ca^{2+} is required than for the intact protein [20]. This property was used to purify the fragment. In contrast with SP-A, CRF does not bind to lipids and does not induce vesicle aggregation. CRF was used in vesicle aggregation assays to compete with SP-A-SP-A interactions by both acting as a ligand for SP-A and as a lectin that blocks the oligosaccharide moiety of SP-A. A 10-fold excess (by weight) of CRF indeed prevents the formation of stable aggregates (Fig. 4). Initial

Fig. 2. Analysis of vesicles, incubated in the presence of Ca^{2+} (5 mM) and SP-A, by freeze-fracture electron microscopy
(a) Control SP-A; (b) deglycosylated SP-A. The bar represents 200 nm.

Fig. 3. Mannan prevents and reverses SP-A-induced vesicle aggregation

The presence of yeast mannan prevented vesicle aggregation in a dose-dependent way. At a concentration of 1% (w/v), only self-association was observed (a). Vesicle aggregation induced by the addition of vesicles to self-associated SP-A could be reversed by the addition of 1% (w/v) mannan (b).

Fig. 4. Vesicle aggregation is reversed by the purified C-terminal carbohydrate-binding domain of SP-A

Addition of a collagenase-resistant fragment of SP-A (CRF, 100 μg) to vesicles aggregated in the presence of 25 mM- Ca^{2+} and 10 μg of SP-A resulted in a slow dissociation of vesicles (a). Inclusion of CRF in the aggregation reaction also resulted in vesicle dissociation, but did not prevent initial aggregation (b).

aggregation was not prevented by CRF. This may be explained by the difference of binding valency between CRF and SP-A. One molecule of SP-A contains six domains similar to oligomeric CRF. Binding of one molecule SP-A to another molecule of SP-A on a different vesicle will result in vesicle aggregation, whereas binding of several molecules of CRF to one molecule of SP-A may be required to prevent SP-A-SP-A interactions. Consequently, aggregates will dissociate slowly until equilibrium is attained. CRF-induced dissociation was observed at 25 mM- Ca^{2+} , but not at 5 mM- Ca^{2+} . A concentration of 25 mM- Ca^{2+} was chosen because at that concentration CRF binds to immobilized mannose [20]. Vesicle aggregation, induced by SP-A and 25 mM- Ca^{2+} , could also be reversed by the addition of CRF (Fig. 4).

These observations suggest that the ability to bind carbohydrate may be required for the formation of the unique membrane lattice of tubular myelin. Several investigators ob-

served 'drumstick-like' particles with the dimensions of SP-A in tubular myelin. These particles either contacted each other in the centre of tubules with a diameter of less than 40 nm and formed a continuous filamentous central core, or were separated from one another in tubules with a diameter of more than 40 nm [18]. The morphological data and our present results suggest the possibility that two kinds of interactions occur between SP-A molecules in tubular myelin: (i) lateral interactions that are independent of oligosaccharide and (ii) radial interactions that are dependent on the carbohydrate moiety. Both type of interactions may be required for tubular myelin formation and SP-A-induced vesicle aggregation. It is attractive to speculate that the Ca^{2+} -induced self-recognition of SP-A in the alveolar subphase leading to the formation of tubular myelin may be important in the regulation of the formation of the phospholipid monolayer at the air/liquid interface of the lung.

Recently some evidence was provided for a structural role of lectins. Interactions of the cerebellar soluble lectin with glycoproteins may be involved in myelin compaction [22], and interactions of lectins with oligosaccharides may lead to the formation of highly ordered cross-linked lattices [23]. Lectin-carbohydrate interactions could be a universal mechanism to induce rapid, specific and reversible changes, not only in cell-cell and cell-matrix interactions [24], but also in a number of different interactions between membranes, both extracellular and intracellular.

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