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Surfactant Protein A (SP-A) Forms a Novel Supraquaternary Structure in the Form of Fibers

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We have found by transmission electron microscopy that bovine surfactant protein A (SP-A) formed extended fibers in the presence of calcium. On phosphatidylcholine or especially dipalmitoylphosphatidylcholine monolayers, SP-A at roughly 0.005 mg/ml formed large numbers of fibers and elaborate fibrous networks. This observation suggested that the weak protein:protein interactions amongst free SP-As could be stabilized by phospholipids. In the presence of glycolipid G_{M1}-ganglioside, SP-A's globular headgroup regions appeared enlarged and only small non-fibrous clusters were observed.

Surfactant protein A (SP-A[1]) is the major protein component of pulmonary surfactant [reviewed in 1-4], and has been implicated in the formation of tubular myelin [5,6], lipid adsorption to the air-liquid interface [7-9], the innate immune response [10,11] and possibly the regulation of secretion from type II cells [12,13]. Alterations of SP-A oligomerization or structure occur in some human disease conditions such as alveolar proteinosis [14]. Since SP-A improves the surface adsorption of surfactant lipids [7,8], SP-A analogues could prove useful in treatment of human conditions such as acute respiratory distress syndrome [15-17].

The primary structures of SP-As from various animals are similar [18,19], and the quaternary structures of human, dog, and bovine SP-A have been elucidated by computational secondary structure prediction [17], circular dichroism [20], and transmission electron microscopy (TEM) [18,21,22]. SP-A contains four distinct domains: a short N-terminal region, a collagen-like domain with a kink, a neck region with an amphipathic α-helix, and a globular head with a carbohydrate recognition domain (CRD),[3] which overlaps with calcium binding domains. The quaternary structure of an SP-A octadecamer resembles a "bouquet of tulips" in appearance [2,3]. Cations affect both the self-aggregation (or self-association) of SP-A, and SP-A-mediated lipid vesicle aggregation [13,23-26]. However, the natures of these phenomena are not clearly understood. Here, we report a hitherto unknown supraquaternary structural arrangement of bovine SP-A, that of fibers of various lengths formed in the presence of calcium at moderate protein concentrations.

EXPERIMENTAL PROCEDURES

Native SP-A was purified from bovine lung lavage as described previously [27]. The purified protein (125-200 μg/ml) was stored at 4°C in 5 mM Tris-HCl (pH 7.4) buffer until use. These SP-A preparations were diluted to various concentrations (5-50 μg/ml) in 5 mM HEPES-NaOH (pH 7.4), 0-5 mM $CaCl_2$, or 5 mM EDTA, and spread on a thin carbon support for TEM after various times of incubation at 37°C. SP-A samples (~5-10 μl) were pipetted onto Cu grids (200 or 400 mesh, Gilder, Marivac, Halifax, Canada) coated by a holey plastic film supporting a thin layer of carbon. After allowing 1-2 min for sample adhesion, excess liquid was removed by blotting on a torn filter paper. The specimen grid was then placed on a drop of 2-4% uranyl formate or uranyl acetate stain (J.B. EM, Montréal, Canada) for 30-60 s [21,22]. SP-As were also analyzed in association either with phospholipid (DPPC, egg PC, egg PC:PS (9:1, w/w)) monolayers or glycolipid G_{M1} overlays. In such experiments, a volume of 13 μl of SP-A (5-50 μg/ml) was placed in a 3 mm (diameter) × 1 mm (depth) teflon well, and a lipid monolayer generated at the air-liquid interface by carefully placing 0.8-1 μl of lipids (0.1-0.4 mg/ml) dissolved in chloro-

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[3] The abbreviations used are: CRD, carbohydrate recognition domain; DPPC, dipalmitoylphosphatidylcholine; G_{M1}, *Gal*β1→3*GalNAc*β1→4*Gal*(3←2α*NeuAc*)β1→4*Glc*β1→1*ceramide;* PC, unsaturated phosphatidylcholine from egg; PS, phosphatidylserine; SP-A, surfactant protein A; TEM, transmission electron microscope/y.

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form:hexane (1:1) solvent on the top of the droplet. A grid with a thin layer of carbon on a holey plastic support was touched onto the lipid surface after different incubation periods at 23°C or 37°C, and negatively stained with uranyl acetate. The stained grids were air dried for at least 1 h before examination by TEM. Specimens were examined and micrographed using a JEOL JEM-100CX TEM at 16,000-100,000X nominal magnification. SP-A molecules or fibers on the negatives were magnified to a final magnification of ~300,000 using a CCD camera (Princeton Instruments, 1400, EM-PIX, Rockwood, Canada) and IS1000 software (Alpha Innotech Co., San Leandro, California, USA) run on an IBM compatible personal computer for further examination.

RESULTS

SP-As Form Fibers of Various Lengths in the Absence of Lipids

Purified protein preparations were diluted to various concentrations, incubated in buffers with or without calcium ions, negatively stained and examined by TEM. In the absence of calcium ions, or after chelation of calcium by EDTA, SP-A existed primarily as individual octadecamers (Fig. 1a). In the presence of 5 mM $CaCl_2$, SP-A formed protein aggregates, or fibers of different lengths, or protein networks (Figs. 1b,c).

When low concentrations (<5 μg/ml) of protein were spread on a carbon support, most existed as individual SP-A octadecamers, whereas at high concentrations (>20-50 μg/ml) the proteins formed aggregates within 30 min of incubation at 37°C. When the SP-A concentration was moderate (5-20 μg/ml), SP-A formed a mixture of both fibers and aggregates (Fig. 1b) [21,22]. Clearly identifiable networks (Fig. 1c) were composed of thin ordered strands of SP-As, suggesting that they were not random precipitates. The orientations of SP-A octadecamers in some of the fibers were identifiable when the protein was in the calcium buffer for 30 min or more. In Fig. 1c, the stems of some of these SP-As are pointing away from the fiber; hence, we surmise that SP-As probably interacted with each other via their globular domains (or a nearby region), and less likely by the stems.

In summary, results from this experiment (Fig. 1) showed that calcium and a moderate protein concentration were essential and sufficient for the formation of SP-A fibers, and suggested that this supraquaternary arrangement was effected by headgroup:headgroup interactions.

SP-As Form Fibers and Extensive Protein Networks on Phospholipid Monolayers

Other SP-A preparations were diluted in 5 mM $CaCl_2$-containing buffer and incubated with a phospholipid monolayer. After various times, the monolayer was picked up on a grid, negatively stained and analyzed by TEM. In the absence of SP-A, the lipid monolayers were very uniform in appearance (Fig. 2a). In the presence of SP-A, occasional SP-A octadecamers, some linear arrays of proteins, frequent fibers or fibrous networks, and some large aggregates were all visible (Figs. 2b-d). Some SP-A stems were detectable in the fibers, and they appeared to be free (Fig. 2b; arrows). In short fibers, only a single octadecamer molecule contributed to the width of the fiber (Fig. 2b). Some of the fibers appeared to have a sinusoidal periodicity (Fig. 2d), perhaps reflecting a three-dimensional helical organization. Some fibrous regions appeared wider (Figs. 2c,d) compared to regions of the typical fibers (Fig. 2b), and resembled non-ordered SP-A aggregates.

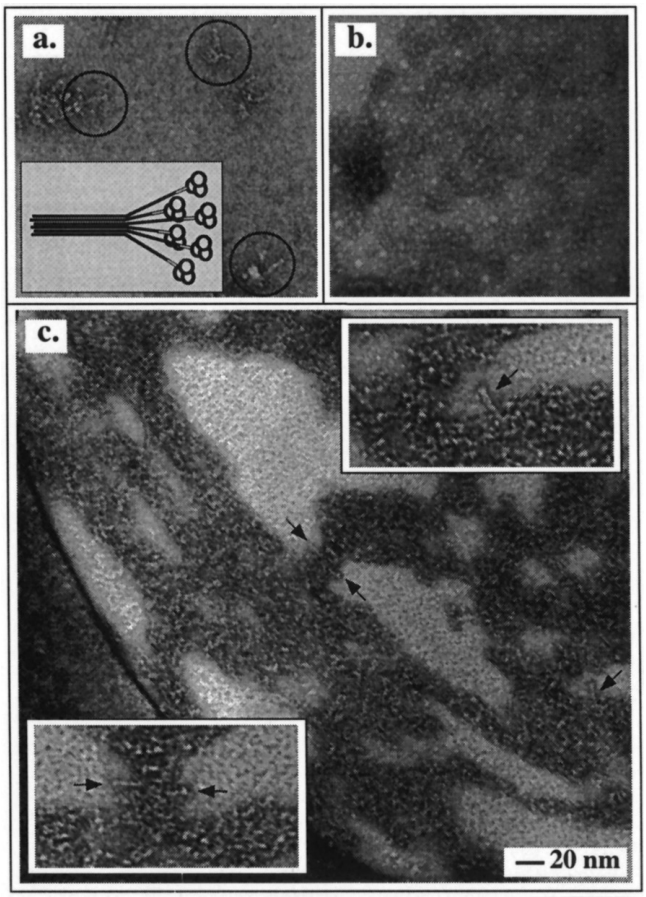

FIG. 1. SP-A forms fibers of various lengths without any added lipid. (a) In 5 mM EDTA, most of the SP-As exist as individual octadecamers. Diagrammatic representation of an SP-A octadecamer is shown in inset. (b,c) After incubation with 5 mM $CaCl_2$ for 30 min, many SP-A fibers are (b) short, but some are (c) long and interconnected. Clearly identifiable stems are marked by arrows, and 2-fold magnified views are shown in insets.

Moreover, SP-A fibers were more commonly found on DPPC or egg PC:PS (9:1, w/w) monolayers compared to unsaturated PC (egg PC) monolayers. This finding is biologically relevant because DPPC is the major surfactant phospholipid [7] and SP-A interacts preferentially and specifically with DPPC in a calcium-dependent manner [9,28].

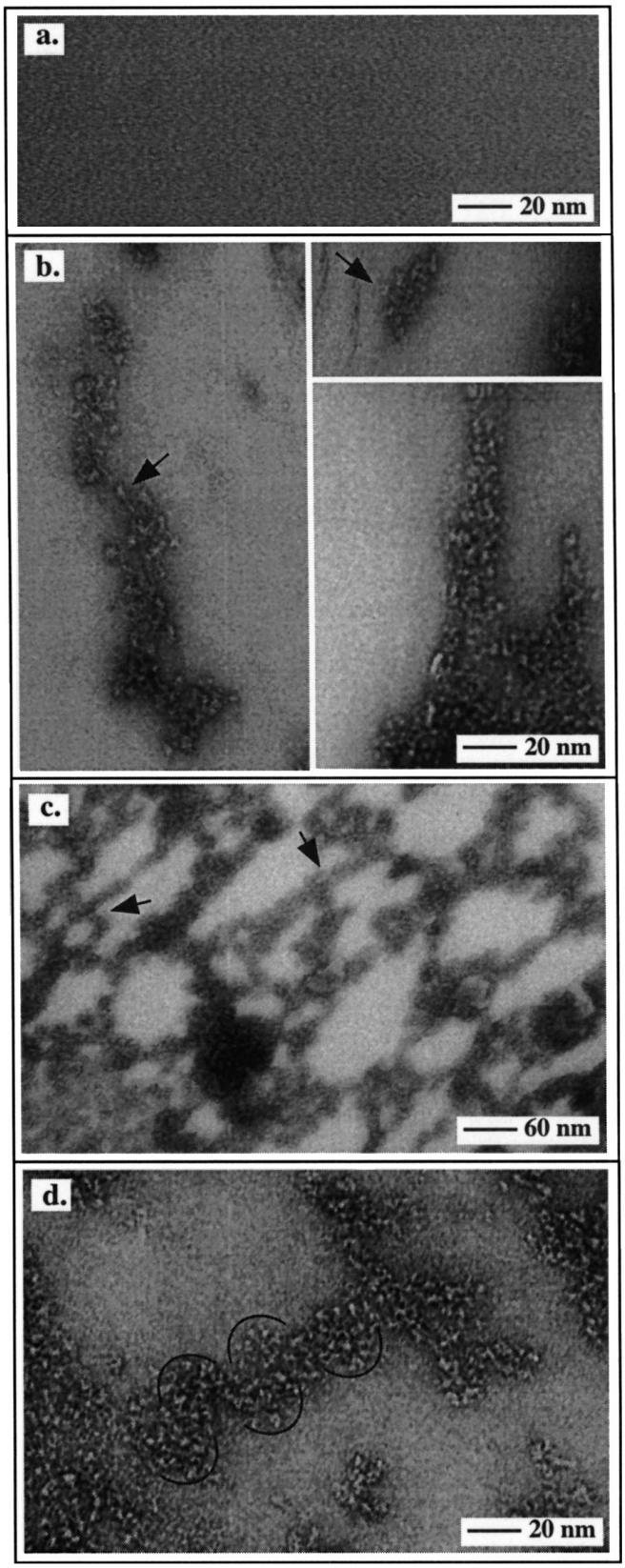

FIG. 2. SP-As form protein fibers and extensive networks on phospholipid monolayers. (a) A phospholipid monolayer in the absence of SP-A appears smooth. (b) Short SP-A fibers contain a few SP-A molecules, and at places only a single SP-A octadecamer contributes to the width of the fiber. (c) Extensive fibrous networks of SP-A. Individual fibers are interconnected. Fibers with "X" or "Y" junctions are indicated by arrows. (d) A magnified view of the fibrous network shown in (c). Some of these fibers suggest a quasi-helical arrangement, indicated by semicircles.

Many regions on the phospholipid monolayers contained extensive networks of SP-A fibers (Fig. 2c). These networks contained clearly identifiable junctions where three to four fibrous branches united to form "Y" or "X" junctions (Fig 2c; arrows). This observation suggested that an individual octadecamer was capable of interacting with two or three other octadecamers on different complexes and in neighbouring fibers.

In summary, the results from this experiment showed that phospholipid (especially DPPC) monolayers enhanced fiber formation, perhaps by stabilizing protein-protein interactions, and suggested further that fibers and networks thereof were formed by interactions amongst headgroups of individual SP-A complexes.

Fiber Formation of SP-A Is Inhibited by Binding of G_{M1}

The head region of the SP-A contains a carbohydrate recognition domain and binds certain sugars [29-32]. Bovine SP-A was incubated with an overlay of a complex glycolipid, ganglioside G_{M1}, in calcium-containing buffer, and was prepared for and analyzed by TEM as before. In the absence of SP-A, the specimen support appeared primarily smooth (data not shown). With SP-A, isolated protein octadecamers and small aggregates, but no long fibers, were detected (Fig. 3). The globular headgroup domains of the SP-A appeared enlarged in the presence of G_{M1} (Fig. 3a versus Fig. 1a). This observation can be explained by the cooperative binding of many G_{M1} moieties, even though such binding is weak [29,32,33].

The results from this experiment (Fig. 3) showed that glycolipid binding altered both the quaternary and supraquaternary structures of SP-A, perhaps because the binding of glycolipids to SP-A headgroups limited their interactions with one another, and hence the formation of long fibers.

DISCUSSION

It is important to study SP-A because of its many roles in lung surfactant activity and processing [4,7,34-36], and in innate immune response [10,11]. We have recently shown that various cations, especially calcium, converted the SP-A octadecamers from an "opened-bouquet" structure to a "closed-bouquet" one [21,22]. The role of such conformational

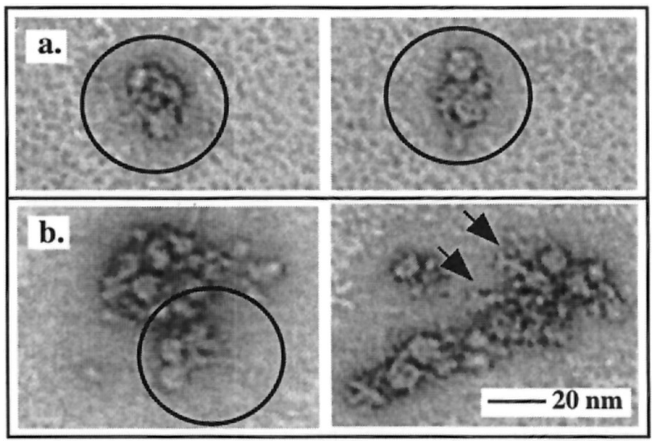

FIG. 3. SP-A does not form fibers on G_{M1} ganglioside overlays but its head appearance is altered. (a) In the presence of G_{M1}, individual SP-A octadecamers with larger heads are seen (circled). (b) Other SP-As aggregate into small groups or larger aggregates (data not shown).

alterations in SP-A function *in vivo* is not known. In those studies, individual SP-A octadecamers were primarily obtained at a low protein concentration. The present study has shown that SP-A at relatively higher, yet still moderate, concentrations (>5-20 μg/ml) formed fibers of various lengths, and extensive networks in a calcium-dependent and phospholipid-stabilized manner.

We argue that the fibrous SP-A arrangements that we have observed are not drying artefacts, because they exist under a variety of conditions but not all, are formed only within limited ranges of calcium and protein concentration, and so must represent a real physical entity. Furthermore, unidentified forms of high molecular weight SP-A assemblies have been noted by others in gel filtration experiments [37-39] and by TEM [18]. It is well established that SP-A self-associates to form larger, higher-order structures under various pH and ionic conditions [24,26,33,39]. Finally since SP-A forms long, linear arrangements *in vivo* in tubular myelin, it is worthwhile to consider critically whether our *in vitro* structures might have biological significance.

Several biochemical experiments have shown that the addition of calcium ions to SP-A or SP-A:phospholipid vesicle mixtures increased the absorbance at 400 nm within a few minutes [13,23-26,40,41]. These conditions are referred to as SP-A "self-aggregation" and "vesicle aggregation", respectively. However, the mechanisms involved in these processes are not clearly understood. For example, Ruano *et al.* [26] and others [25] have shown that the lipid association of SP-A and subsequent vesicle aggregation required only μM concentrations of calcium, whereas self-association of SP-As required mM concentrations of calcium. Based on our findings, it is apparent that SP-A forms fibers on phospholipid surfaces in the absence of chelator or in the presence of calcium, in addition to any non-specific self-aggregation (Figs. 1, 2). Therefore, these supraquaternary structures potentially play a role in aggregating lipid vesicles, at least, at physiological calcium concentrations [38-43]. In interpreting the results of such experiments, whether the SP-As are in "opened-bouquet" or "closed-bouquet" form should also be considered because these two conformational forms of SP-A interact with phospholipids differently [21]. Furthermore, calcium-dependent phospholipid binding of SP-A is reversible *in vitro* [25], and a similar reversible lipid binding and SP-A structural alteration may also exist in *in vivo* [44,45].

The process of SP-A fiber formation is probably very complex. For example, SP-A quaternary structure, and interaction with phospholipid bilayers, is altered in the presence of calcium [21,22,23,33,38,39], and SP-A binds to DPPC in a calcium-dependent manner [9,28,35]. Although some biochemical evidence suggests that SP-A interacts with acyl moieties of the lipid via a hydrophobic interaction [28,35], many recent reports suggest that SP-A can bind to the phospholipid polar groups via ionic interactions [9] and via their globular headgroups [13,21,40,46,47]. Covalently-linked carbohydrate moieties of SP-A appear not be required for SP-A:SP-A interactions observed at mM concentrations of calcium [24,26]. Here, clearly identifiable stems of SP-A octadecamers extend out from the fibers (Figs. 1-3), suggesting that SP-As might interact with each other via their globular headgroups or a nearby region. The fact that SP-As form extensive protein networks on phospholipid monolayers (Fig. 2) suggest that different trimeric globular headgroups in a single SP-A octadecamer may be capable of binding to either lipids or other SP-As. Indeed, the multiple headgroups are the best means for an SP-A octadecamer to effect simultaneous interactions with lipids and with at least two other SP-A octadecamers. Since SP-A forms more frequent and extensive fibrous networks on lipid monolayers than on a carbon support, we suggest that SP-A:SP-A interaction is relatively weak, but the binding of SP-A to the lipid moieties on the phospholipid monolayers stabilizes the protein complexes with respect to one another.

The calcium-dependent interaction of SP-A with glycolipids is different than with phospholipids (Fig. 3), as has also been determined biochemically [30-32]. The globular domain of the SP-A contains two or more calcium binding domains which overlap with the CRD [29,33]. The CRD also contains a covalently linked carbohydrate moiety in all known SP-As [2,3]. When the SP-As were spread in association with a G_{M1} overlay, the globular headgroups of the SP-A appeared enlarged (Fig. 3). This weak interaction of G_{M1} carbohydrate moi-

eties with SP-A headgroups appeared to inhibit the formation of protein networks. Such an effect may explain why SP-A mediated lipid vesicle aggregation is reduced at high calcium concentrations by competing sugars also at high concentrations [24,26], and in the presence of different types of lipids [30-32].

CONCLUDING REMARKS

A complex relationship of SP-As and surfactant lipids exists *in vivo.* The lipid-rich lamellar bodies of pulmonary surfactant are converted into tubular myelin structures in the alveolar hypophase [5,6,45,48]. This unique protein-lipid structure shows a tubular lattice organization and contains arrays of SP-A on the walls [49,50], especially at the corners of the lattices [51-53]. Our finding that the SP-A forms fibers suggests the existence of an intermediate which could facilitate the construction of experimental models to elaborate the formation and the ultrastructural organization of tubular myelin.

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