Filaments of surfactant protein A specifically interact with corrugated surfaces of phospholipid membranes

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Palaniyar, Nades, Ross A. Ridsdale, Stephen A. Hearn, Yew Meng Heng, F. Peter Ottensmeyer, Fred Possmayer, and George Harauz. Filaments of surfactant protein A specifically interact with corrugated surfaces of phospholipid membranes. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L631–L641, 1999.—Pulmonary surfactant, a mixture of lipids and surfactant proteins (SPs), plays an important role in respiration and gas exchange. SP-A, the major SP, exists as an octadecamer that can self-associate to form elongated protein filaments in vitro. We have studied here the association of purified bovine SP-A with lipid vesicle bilayers in vitro with negative staining with uranyl acetate and transmission electron microscopy. Native bovine surfactant was also examined by transmission electron microscopy of thinly sectioned embedded material. Lipid vesicles made from dipalmitoylphosphatidylcholine and egg phosphatidylcholine (1:1 wt/wt) generally showed a smooth surface morphology, but some large vesicles showed a corrugated one. On the smooth-surfaced vesicles, SP-A primarily interacted in the form of separate octadecamers or as multidirectional protein networks. On the surfaces of the striated vesicles, SP-A primarily formed regularly spaced unidirectional filaments. The mean spacing between adjacent striations and between adjacent filaments was 49 nm. The striated surfaces were not essential for the formation of filaments but appeared to stabilize them. In native surfactant preparations, SP-A was detected in the dense layers. This latter arrangement of the lipid bilayer-associated SP-A supported the potential relevance of the in vitro structures to the in vivo situation.

pulmonary surfactant; tubular myelin; lipid-protein interaction; membrane structure; ripple phase

SURFACTANT PROTEIN (SP) A is the major protein component in pulmonary surfactant and is also detected in other regions of the body (5, 12, 24). In addition to its role in the induction of phagocytosis, SP-A participates in surfactant processing (12, 20). It has been suggested that SP-A plays a role in the regulation of lipid secretion from alveolar type II cells, the formation of tubular myelin (TM) in the alveolar hypophase, adsorption of lipids to the air-liquid interface, and lipid recycling (20, 24). The surfactant lipids are secreted in the form of lamellar bodies (LBs), which consist of multiple layers of lipid bilayers (29, 30), unusually high concentrations of calcium (6), SPs, and other proteins (1, 7).

The structure of human and dog SP-As has been probed by biochemical and electron-microscopic methods (reviewed in Refs. 12, 20). Recently, Palaniyar et al. (22) and Ridsdale et al. (25) have elucidated the quaternary structure of bovine SP-A. The clearly identifiable quaternary structure of bovine and dog SP-As is that of an octadecamer, a hexamer of basic trimers. These SP-A octadecamers show a characteristic bouquet-shaped structure with six globular domains and a common stem composed of six triple-helical collagen-like regions. Calcium and other ions affect SP-A structure (8, 22, 25) and many other processes related to surfactant processing (9, 10).

The octadecameric arrangement assigned to SP-A suggests that the function of SP-A could be related to this complex structure. In addition to the individual octadecamers (22), SP-A also exists in vitro in one-dimensional arrangements that Palaniyar et al. (23) previously referred to as “fibers.” However, this word implies a type of regular arrangement (e.g., helical coiling) that has not yet been proved. Henceforth, we shall refer to these structures simply as “filaments.” Palaniyar et al. (22) also showed that SP-A octadecamers interact with the lipid bilayer surfaces via the head and/or globular domain. Furthermore, a subpopulation of SP-A was found in depressions in the lipid bilayers. Such a location indicated that most, if not all, of the trimeric headgroups of the SP-A octadecamer could interact with bilayers made of dipalmitoylphosphatidylcholine (DPPC) and egg phosphatidylcholine (egg PC). In the alveolus of the lung, SP-A is detected in different locations. LBs contain a limited amount of SP-A at the time of secretion from type II cells (1, 7). SP-A is secreted constitutively and independently of LBs, and some of this free SP-A appears to reach the LBs and other lipid structures (1, 14). The LBs that are connected to other membrane structures appear to contain increased amounts of SP-A (7), reflecting the involvement of SP-A in the structural conversion processes. Although a transgenic animal study (17) clearly showed that SP-A is essential for the conversion of LBs to the TM intermediate, the manner in which SP-A is involved in this conversion remains unknown.

In this report, we describe, using transmission electron microscopy (TEM), how SP-A filaments interact...
with the surfaces of lipid vesicles made in vitro. Our results show that the surface environment created on lipid membranes can potentially stabilize spontaneous protein-protein interactions. These protein-lipid arrangements thereby form structures similar in appearance to those found in native pulmonary surfactant.

**MATERIALS AND METHODS**

**Protein purification.** SP-A was purified from bovine lung lavage as previously described (3). Briefly, natural surfactant obtained through lavage was delipidated with butanol, ether, ethanol, and ether. The dried precipitate was solubilized in 5 mM HEPES-NaOH (pH 7.4)-0.1 mM Na2EDTA and centrifuged to remove insoluble material. After the calcium concentration was adjusted to 1.0 mM, the protein was affinity bound on an immobilized D-mannose column (Pierce Chemical, Rockford, IL) and washed. The bound SP-A was eluted with 2.0 mM Na2EDTA in 5 mM HEPES-NaOH buffer (pH 7.4). The purified protein (100–250 µg/ml) was stored at 4°C in 5 mM Tris·HCl (pH 7.4)-1 mM Na2EDTA buffer until used. SP-A preparations were diluted to various concentrations (10–100 µg/ml) in 5 mM HEPES-NaOH buffer (pH 7.4) with and without 5 mM CaCl2 and spread for electron microscopy (see **Electron microscopy**).

**Preparation of lipid vesicles.** Lipids [synthetic DPPC as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine sodium salt and natural egg PC as L-α-lecithin-(phosphatidylcholine); Avanti Polar Lipids, Alabaster, AL] were dissolved in chloroform-hexane (1:1), and vesicles of heterogeneous sizes were made as previously described (22). Briefly, 5 µg of lipid or lipid mixture in a 20-µl volume were placed at the bottom of 5-ml borosilicate glass tubes (Fisher Scientific, Whitby, ON). A thin layer of lipid was left on the bottom of the tube after removal of the solvents by evaporation under a stream of nitrogen (BOC Gases Canada, Mississauga, ON) followed by 30 min of freeze-drying under vacuum. A 20-µl volume of 5 mM HEPES-NaOH buffer (pH 7.4) was added to the bottom of the tube, and the vesicles were made by sonication with a bath sonicator at 37°C (4 bursts of 5-min sonication, with temperature adjustment between bursts with ice or by heating in a water bath; Branson-R and Branson-1200, Branson Ultrasonics, Danbury, CT). The extensive sonication was necessary to facilitate hydration of DPPC vesicles, especially, because DPPC has a transition temperature of 42°C.

**Formation of SP-A-lipid vesicle complexes.** Freshly prepared lipid vesicles (5 µl) were mixed with 5 µl of 5 mM HEPES-NaOH buffer (pH 7.4) containing SP-A (20–50 µg/ml) in 500-µl Eppendorf tubes and incubated at 37°C for 10–20 min. Control reactions contained 5 µl of 5 mM HEPES-NaOH buffer. Preparation of lipid vesicles. Lipids [synthetic DPPC as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine sodium salt and natural egg PC as L-α-lecithin-(phosphatidylcholine); Avanti Polar Lipids, Alabaster, AL] were dissolved in chloroform-hexane (1:1), and vesicles of heterogeneous sizes were made as previously described (22). Briefly, 5 µg of lipid or lipid mixture in a 20-µl volume were placed at the bottom of 5-ml borosilicate glass tubes (Fisher Scientific, Whitby, ON). A thin layer of lipid was left on the bottom of the tube after removal of the solvents by evaporation under a stream of nitrogen (BOC Gases Canada, Mississauga, ON) followed by 30 min of freeze-drying under vacuum. A 20-µl volume of 5 mM HEPES-NaOH buffer (pH 7.4) was added to the bottom of the tube, and the vesicles were made by sonication with a bath sonicator at 37°C (4 bursts of 5-min sonication, with temperature adjustment between bursts with ice or by heating in a water bath; Branson-R and Branson-1200, Branson Ultrasonics, Danbury, CT). The extensive sonication was necessary to facilitate hydration of DPPC vesicles, especially, because DPPC has a transition temperature of 42°C.

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buffer (pH 7.4) without SP-A. After time was allowed for the protein to associate with lipids, 1.1 µl of 50 mM CaCl$_2$ were added to each tube, and the incubation was continued for various time periods at 37°C. (A parallel set of experiments was performed at 23°C, but the structures observed were effectively the same as those at 37°C, and these data are not reported here.) Control tubes received 1.1 µl of double-distilled H$_2$O in place of CaCl$_2$. Considering the time of CaCl$_2$ addition as the reference time point, 3- to 5-µl samples were withdrawn from the tubes at various times of incubation for TEM analysis. The reaction was monitored for 24 h. For addition as the reference time point, 3- to 5-µl samples were

Dehydration was achieved by a progressive lowering of the temperature. Without any further fixation, samples were fixed in this buffer with 4% glutaraldehyde for 1 h at room temperature. The lower temperature helped to stabilize the specimen considerably during imaging. Sections containing bovine surfactant were routinely examined with a Philips EM400T TEM aided with a Gatan cryoholder. The dark-field energy-loss images of unstained sections were obtained during imaging. Sections containing bovine surfactant were routinely examined with a Philips EM300 TEM. The dark-field energy-loss images of unstained sections were obtained with a Zeiss 902 TEM with an in-column energy filter.

Preparations of SP-A-lipid vesicles made in vitro were routinely examined and micrographed with a JEOL JEM-100CX TEM between ×20,000 and ×100,000 nominal magnification. Most of the images used here were taken at cryotemperatures (−171°C) with a Philips EM400T TEM aided with a Gatan cryoholder. Additional samples were dehydrated by the same method except that acetone was the initial solvent, followed by a rinse in 100% ethanol to remove the acetone, which is not compatible with LR White resin. Infiltration in LR White resin was done in two steps: first 50% resin in alcohol at −20°C for 18 h and then 100% resin at 23°C; the samples were polymerized at 60°C for 18 h. Sections made by these blocks were picked up on 300-mesh nickel grids. Some of these sections were probed with a polyclonal anti-SP-A antibody (gift from Dr. J. A. Whitsett, University of Cincinnati, Cincinnati, OH). Goat anti-rabbit IgG conjugated to 5-nm gold (Amersham, Oakville, ON) was used to tag the SP-A-specific antibody on the sections as previously described (11).

Electron microscopy. Preparations of SP-A-lipid vesicles with surfaces of lipid vesicles (circles). Most of isolated SP-A octadecamers interacted with surfaces of lipid vesicles (circles). Additional samples were dehydrated by the same method except that acetone was the initial solvent, followed by a rinse in 100% ethanol to remove the acetone, which is not compatible with LR White resin. Infiltration in LR White resin was done in two steps: first 50% resin in alcohol at −20°C for 18 h and then 100% resin at 23°C; the samples were polymerized at 60°C for 18 h. Sections made from these blocks were picked up on 300-mesh nickel grids. Some of these sections were probed with a polyclonal anti-SP-A antibody (gift from Dr. J. A. Whitsett, University of Cincinnati, Cincinnati, OH). Goat anti-rabbit IgG conjugated to 5-nm gold (Amersham, Oakville, ON) was used to tag the SP-A-specific antibody on the sections as previously described (11).

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RESULTS

Purification of SP-A. We consider our SP-A protein preparation to be pure as ascertained by SDS-PAGE with and without urea followed by silver staining (21). A butanol-ether isolation technique was followed to ensure that all of the lipid was removed. This exposure to organic solvents might potentially denature the protein. However, "gentle" purification techniques with detergents such as octylglucoside are inappropriate because residual detergent would disturb the SP-A-lipid membrane interactions that we have investigated here. Butanol-extracted SP-A has been the most widely studied, and so our present results are most relevant in the framework of the published literature. Indeed, the step of running SP-A through a mannose-binding column used a known biological property to purify it, and so we consider that our SP-A here is functional in this respect.

DPPC-egg PC lipid vesicles form corrugated surfaces. To study the SP-A-lipid bilayer interactions, we used single phospholipids or combinations of phospholipids that are present in pulmonary surfactant (16, 24, 33). Vesicles were made with DPPC, egg PC, or DPPC-egg PC (1:1 wt/wt). These preparations primarily contained vesicles with smooth surfaces; however, their sizes were different depending on the type of lipid used (Fig. 1). DPPC generated exclusively large (~10-µm) unilamellar vesicles that often folded back onto themselves (Fig. 1A). Occasionally, these vesicles also showed striated surfaces (data not shown). Vesicles made of unsaturated egg PC alone generated heterogeneous "small"- to "medium"-sized (~300-nm) unilamellar vesicles (Fig. 1B). The DPPC-egg PC vesicles generally had an appearance typical of those formed by egg PC alone, as seen in Fig. 1A. Noticeably, however, this lipid mixture also generated some vesicles with corrugated surfaces (Fig. 1C). Formation of such striations was more frequent at 23°C compared with 37°C (data not shown). We interpret the dark edges of the vesicles to represent negative stain and the dark striations to indicate stain lying in the corrugation valleys. Uranium stain may be trapped under the corrugated vesicles, but we do not envisage this to be a common occurrence under these conditions.

The addition of 5 mM CaCl₂ to these vesicles did not significantly alter the surface morphology during short incubation periods (up to 2 h) except that the vesicles were larger, suggesting an occurrence of vesicle fusion in a time-dependent manner (data not shown). Adding 5 mM CaCl₂ and SP-A (5 µl of 20–50 µg/ml) to these lipid vesicle preparations (5 µl of 250 µg/ml) produced different types of associations. SP-A associated with all of these vesicles (Fig. 1, D–F). However, interaction of SP-A with DPPC alone in the presence of calcium led primarily to the formation of larger aggregates (Fig. 1D), whereas SP-A associated with egg PC vesicles without any apparent specificity (Fig. 1E). Interestingly, SP-A bound to the mixture of DPPC-egg PC by preferentially interacting with striations found on the vesicles (Fig. 1F; see SP-A filaments specifically interact with vesicles in corrugation valleys). Although larger vesicles contained striations, smaller vesicles were not devoid of such structures. This result implies that the striations are not merely staining or drying artifacts of larger vesicles.

These results (Fig. 1) revealed, first, that the lipid combination influenced the structures on the surfaces of the lipid bilayers and especially the DPPC-containing larger lipid vesicles generated striations. Second, SP-A interacted in a preferential manner with the striations on the vesicles made with this lipid mixture.

SP-A on the smooth bilayers form protein networks. There were different types of interactions of SP-A with smooth vesicles. Smooth vesicles contained SP-A octadecamers dispersed on their surfaces (Fig. 2A, circles). Some of the SP-A specifically interacted with the edges of small vesicles, whereas others could be identified on the surfaces of large vesicles (Fig. 2A, arrows and circles). Another subpopulation of smooth vesicles interacted with arranged or aggregated SP-As (Fig. 2, B–D). Regions of the grids where fewer lipid vesicles were found contained many self-associated SP-As or free SP-A octadecamers (Fig. 2B). Some vesicles and SP-A...
appeared to have specifically interacted with the edges and specific regions of the vesicles (Fig. 2C, arrows). The SP-As involved in such interactions showed linear filamentous arrangements involving a number of SP-As. The SP-A octadecamers in such situations appeared akin to links of a chain (see details in Individual SP-A octadecamers interact preferentially at the valleys of the corrugations). In another subpopulation of the smooth vesicles, SP-As formed short filaments or irregular protein networks (Fig. 2D). These networks often extended over the entire surface of the vesicle. Under the conditions tested, these networks were the most commonly observed type of SP-A-smooth vesicle interaction.

These observations (Fig. 2) suggest that SP-A interacts with smooth lipid bilayers either as individual octadecamers or as filaments that can form interconnected networks. These SP-A filaments appeared to interact with these vesicles in a nonorganized fashion.

Individual SP-A octadecamers interact preferentially at the valleys of the corrugations. The lipid combination DPPC-egg PC (1:1 wt/wt) generated corrugated vesicles, and the association of SP-A to these vesicles was studied in detail. In the presence of calcium ions, most SP-As were found in association with these vesicles and were detected in the corrugations on their surfaces (Fig. 3). Two primary types of SP-A arrangements were observed on these vesicles. In the first type, SP-As were arranged in the direction of the striations (Fig. 3A). The spacing between two adjacent SP-As in a single striation was variable. Some of the striated regions were devoid of SP-A octadecamers. In the second type, SP-As were arranged in the striations but appeared aligned in the perpendicular direction (Fig. 3B; compare with Ref. 28). However, there were no direct physical connections visible between any of the two adjacent SP-A molecules.

The overall arrangement of the SP-As (Fig. 3) clearly showed, first, that the corrugated surfaces were the favored location for the SP-A octadecamers and, second, that individual SP-A octadecamers were in the valleys rather than on the crests. Third, SP-As were arranged in both parallel and perpendicular directions to the striations, suggesting some specific SP-A-lipid interactions.

SP-A filaments specifically interact with vesicles in corrugation valleys. Many SP-A plus lipid vesicle preparations showed short filaments, especially at relatively high protein concentrations (20–50 µg/ml; Fig. 4, A and B). These short filaments were composed of only a few SP-A octadecamers and specifically interacted with the valleys of the corrugated vesicles (Fig. 4, C and D). The short filaments encompassed the entire length of some of the smaller vesicles or part of the length of larger vesicles.

Longer SP-A filaments were also seen extending from the edges of the lipid bilayers (Fig. 5). These extended SP-A filaments followed the directions of the striations but extended beyond the vesicles to a limited distance. Furthermore, these filament extensions were strikingly similar in length (Fig. 5A). Although most of the SP-A filaments interacted with lipid bilayers in the direction of the long axis of the vesicles, others interacted in a direction perpendicular to that axis (Fig. 5B) in which the striations on the vesicles also appeared to lie. In most instances, the filaments outside the vesicles appeared unstable and showed discontinuities along their length (Fig. 5B). Another type of SP-A filament extension was noted on the sides of vesicles. Some of
the filaments at the vesicle edges continued to extend and reach the vesicle surface. However, when there was no lipid under the extended filaments, they often terminated shortly beyond the edge (Fig. 5C, between the lines).

Interestingly, these filaments not only precisely followed the striations found on the surface of each vesicle, but they also bent with bends of the striations (Fig. 6A). Although the short, free filaments found outside these vesicles were normally straight (Fig. 6B), the long filaments could show a distinct overall periodicity (Fig. 6C). However, when these filaments were on the striated surfaces of the vesicles, they were confined to the valleys and lacked any noticeable periodicity (Fig. 6D). Noticeably, filaments found on the corrugated vesicles were not observed interacting with each other in lateral directions. The corrugations on the lipid vesicle surfaces showed differences in their nature (Fig. 7A and B). Some striations were clearly visible and appeared to be deep (Fig. 7A), whereas others appeared to be very shallow (Fig. 7B). Although most of the SP-A filaments interacted with the vesicles in parallel lines and bent with the bends in the corrugations (Figs. 6 and 7A), some filaments could be found at angles to each other (Fig. 7C). These filaments once again followed the direction of the striations and terminated at the edges of the vesicles.

These categories of images (Figs. 4–7) showed three important characteristics of the interaction between the SP-A filaments and the striated vesicles. First, SP-A could form free filaments but tended to interact specifically with valleys of the striated vesicles. Second, the SP-A filaments did not necessarily confine themselves to the surfaces of the vesicles; instead, they extended beyond the edges of the vesicles but only to a limited length. Third, the SP-A filaments associated with the side edges of vesicles often extended beyond the vesicle region for a short distance and sometimes reached other parts of the vesicle. Hence, the SP-A filaments correlated with the striations on the lipid surfaces.

SP-A filament formation and striations on the vesicles were interdependent, but these events were not always mutually inclusive. To establish the relationship between the formation of filaments and the presence of striations on the surfaces of the vesicles, we measured the dimensions of the filaments and of the striations under different situations. The average length of all SP-A filaments was $145 \pm 610$ nm (n = 127; Fig. 8A), and these filaments were significantly shorter than the length of the vesicle striations ($330 \pm 27$ nm; P < 0.01; n = 42; Fig. 8A). When the lengths of continuous SP-A filaments found within the vesicles ($189 \pm 23$ nm; n = 23) were plotted against the respective striation lengths ($286 \pm 28$ nm; n = 23), they showed a strong linear relationship (correlation coefficient 0.81; Fig. 8B). To ascertain the number of SP-A octadecamers that contributed to the width of the filaments, we measured this dimension at the widest point of the filaments. The mean value was $19.1 \pm 0.8$ nm (n = 37), roughly the dimension of a single octadecamer.
The average spacing between two adjacent filaments (from center to center) was $49 \pm 1.1$ nm ($n = 63$), reflecting the average spacing (from trough to trough) between the striations ($49 \pm 1.0$ nm; $n = 63$) found on the surfaces of the vesicles (correlation coefficient 0.74; Fig. 8C). Hence SP-A filaments on the striated vesicles were related to the corrugations, as already indicated by the micrograph. In addition, the extensions of the protein filaments beyond the vesicle surface ($90 \pm 7$ nm; $n = 44$) were shorter than the entire filament ($145 \pm 10$ nm; $P < 0.01; n = 127$). Furthermore, free SP-A filaments found independent of the striated vesicle surfaces were also much smaller ($87 \pm 32$ nm; $n = 22$), and this average length was not significantly different from that of the filament segments that extended outside the vesicles ($P < 0.77$).

These results (Fig. 8) indicated that the striations found on the vesicle surfaces presented suitable locations for SP-A-lipid bilayer interaction and for either the formation or extension of these filaments. Because the results in SP-A filaments specifically interact with vesicles in corrugation valleys showed that free SP-A filaments can exist on nonlipid regions (Fig. 4), we conclude that both of these processes, namely the formation of filaments and the striations of the vesicles, were highly related but were not completely dependent on one another.

Multilayered vesicles or common myelin from native surfactant also contain arrays of SP-A. After examining the nature of SP-A interactions with lipid vesicles made in vitro, we analyzed the morphology of native surfactant to investigate the arrangement of SP-A on the lipid bilayer surface. Surfactant from bovine lung lavage fluid was fixed, treated with acetone, embedded in resin, and sectioned and analyzed by TEM with and without negative staining. Some sections were probed with SP-A antibody, and the location of SP-A was detected by TEM with tagged gold particles. Analysis of unstained specimens by dark-field TEM showed that the existence of regularly arranged electron-dense ma-
materiaux (Fig. 9A) and their organization appeared similar in the stained preparations (7, 29). Analysis of antibody-labeled sections by bright-field TEM showed that the antibodies detected SP-As in many layers of the loosely packed common myelin (Fig. 9B). Several layers of electron-dense material found in these native preparations existed as continuous stretches. Measurements of distances of gold particles from the electron-dense edges of these proteinaceous layers showed that SP-A antibodies closely interacted with such layers (7.3 ± 0.3 nm; n = 116). The dimensions of several of these electron-dense layers were measured in regularly arranged areas. These SP-A-containing layers showed a periodicity of 40.5 ± 0.8 nm (n = 39), and adjacent proteinaceous layers were separated by a 24.7 ± 0.7-nm protein-free space (n = 39). The width of these proteinaceous layers was 18.8 ± 0.4 nm (n = 39), and this value was not significantly different from the maximum thickness of the SP-A filaments detected on the DPPC-egg PC vesicles (19.1 ± 0.8 nm; P < 0.71; n = 37).

Results from these native structures showed that, first, several layers of multilayered vesicles contained SP-A. Second, even the removal of most of the lipids from this preparation by alcohol and acetone did not disrupt the continuity of the layers. Hence the SP-As in these layers could be interconnected. Third, the width of the proteinaceous layers is the same as the width of the SP-A filaments formed in vitro.

DISCUSSION

Early studies on pulmonary surfactant in the 1950s revealed phospholipids to be its major constituents, with a high content of PC and, in particular, DPPC (reviewed in Refs. 12, 24). Electron-microscopic, biochemical, and biophysical studies demonstrated the presence of distinct structural forms of pulmonary surfactant, all of identical lipid composition. This observation suggested that the surfactant apoproteins were primarily responsible for determining structural characteristics. This view was consistent with the fluid mosaic model for biological membranes in which the lipid bilayer was considered to be a relatively inert matrix for membrane proteins. The studies presented here support the concept that the lipid constituents of surfactant possess specific properties that allow for cooperative lipid-protein interactions that could initiate surfactant structural organization.

It was clear from a previous study by Palaniyar et al. (22) that SP-A could form various arrangements depending on the experimental conditions. Our experiments with various lipids and lipid combinations showed that certain phospholipid combinations yielded modified surface structures (Fig. 1). The striated surfaces on the bilayers were formed only on the DPPC-containing

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Fig. 8. Dimensions of SP-A filaments and corrugations under different conditions. A: frequency distribution of lengths of SP-A filaments and lipid vesicle striations. Filaments detected with and without association of lipid vesicles were included in analysis (n = 127 measurements). Average length of filaments was 145 ± 10 nm, and average length of striations was 330 ± 27 nm. B: relationship between lengths of striations and lengths of SP-A filaments found within such lipid vesicles (n = 23 measurements). These 2 parameters showed a linear relationship, with a correlation coefficient of 0.81. C: relationship between striation spacings and SP-A filament spacings. Spacings of SP-A filaments found on corrugated vesicles and corresponding spacings of striations were measured and plotted (n = 63 measurements). These 2 parameters showed a linear relationship, with a correlation coefficient of 0.74.
vesicles. The formation of such structures was not dependent on the presence of either calcium or SP-A (Fig. 1). An interesting fact about the DPPC-egg PC (1:1) mixture is that this composition is similar to the ratio of DPPC to unsaturated PCs found in pulmonary surfactant (13, 16, 24, 33). Furthermore, saturated plus unsaturated PCs constitute the bulk (>80%) of the surfactant lipids (24). DPPC is the only major lipid constituent required to attain low surface tension during monolayer compression, and its properties greatly influence surfactant phase characteristics (15, 24). Although the mechanism by which the striations on DPPC-egg PC vesicles are formed is unknown, it is likely that these striations could be related to the conversion of multivesicular lipid membranes into TM. Among SPs, hydrophobic SP-B and SP-C could facilitate surface tension reduction, SP-B could facilitate membrane fusion, and SP-A could act to enhance such effects (24).

A previous investigation by Palaniyar et al. (23) revealed that SP-A octadecamers could form novel supraquaternary structures, most easily described as filaments, even in the absence of lipids. However, SP-A also formed extensive filamentous networks when interacting with lipid monolayers. Our TEM studies agreed with other recent biochemical and biophysical reports (20, 22, 32) that showed that the SP-A head region was responsible for the interaction with lipid. Hence the surrounding environment could influence SP-A head-group interactions. Interestingly, SP-A network arrangements similar to those seen on lipid monolayers were detected here on the surfaces of smooth lipid bilayers (Fig. 2). This observation emphasizes the notion that SP-A-SP-A interactions predominate on smooth lipid surfaces.

A special feature of the SP-A filaments seen on the striated vesicles is that they follow only one direction and do not form multidirectional networks. This observation can be explained if the trimer headgroups of SP-A interact with the lipids in a lateral direction in the corrugations (Figs. 4–9). These SP-A filaments uniquely interact with the striated DPPC-egg PC vesicles. Both SP-A filaments and individual octadecamers are found exclusively in the valleys of the striations (Figs. 3 and 4). This result suggests that the lipid regions interacting with the SP-A octadecamers or SP-A filaments provide certain conditions favorable for the SP-A-lipid bilayer interaction. Such an interaction may be explained in many ways. Either 1) the curved surfaces of the striations provide proper dimensions within which the SP-A heads can fit, 2) the striations of the bilayers contain certain lipids that interact with SP-A with high affinity, i.e., gel-phase DPPC, or 3) both situations exist.

That SP-A can preferentially interact with depressions on DPPC-containing lipid surfaces is shown from previous experiments by Palaniyar et al. (22). In addition, it has been established that SP-A preferentially interacts with DPPC gel-phase regions (2, 15). Our observations are consistent with ripple-phase formation of gel-phase disaturated phospholipids in which spacings of the ripples are specific for different lipids. DPPC alone forms ripples with a periodicity of 15–25 nm (19); however, the spacing measured in our experiments shows a periodicity of ~50 nm. This spacing may be unique for saturated DPPC-unsaturated PC (1:1 wt/wt) because the presence of unsaturated PC together with DPPC is known to increase the periodicity (4). Hence although the exact nature of the striated structures is uncertain, formation of these structures by lateral phase separation is most likely. These phenomena would occur more frequently at 23°C compared with 37°C as we have observed (data not shown); however, other factors such as cations are also likely to affect them in vivo. SP-A interacts with many lipids in the presence or absence of calcium but preferentially binds to gel-phase DPPC (2, 15, 18, 32). Consequently, we favor the scenario that the striations represent gel-phase DPPC that forms a surface arrangement that interacts specifically with SP-A trimeric headgroups within the octadecamers.

Many of the observations of the SP-A filaments show their close connection with the corrugated surfaces of the vesicles. For example, SP-A filaments are preferentially located in the valleys of the corrugations and also curve with vesicle corrugations (Figs. 5–7). Although some of the protein filaments extend beyond the vesicle edges, they are relatively short (Fig. 5). In contrast,
SP-A forms varying lengths of linear arrays in the presence of Ca$^{2+}$ with or without association to lipid vesicles (Fig. 4). The corrugations of the vesicles do not always contain SP-A filaments, and at times, only individual SP-A octadecamers are detected in the corrugations (Fig. 3). These results establish that the protein arrays can assemble independently of the striated surfaces on the lipid vesicles. Considering all of these observations, it appears that the corrugated surfaces of the lipid vesicles enhance but are not essential for the formation of arranged SP-A filaments.

How are these observations related to the situation in vivo? To address this question we studied SP-A in native surfactant preparations. SP-A were readily detected in the multimamellar vesicular structures (Fig. 9b). The SP-A arrangements on such lipid structures (Fig. 9) appeared to be similar to the SP-A filaments detected on the surfaces of the stripped vesicles (Figs. 4–8). This is in agreement with previous reports that showed the presence of continuous proteinaceous materials on the surfaces of the vesicles or LBs. For example, Williams (29) and Hasset et al. (9) have detected the presence of regularly arranged electron-dense particles of ~8–12 nm on one side of the membranes, especially on the one that connected LBs and TM structures, in the alveolar hypophase. Similar particles were also detected in sections made from mixtures of SP-A and lipid vesicles assembled in vitro (28, 31). Interestingly, these particles and the organized membranes could be collapsed and re-formed with alternate treatment of such complexes with a calcium chelator and calcium, respectively (10, 30). Fixed multimamellar structures remain intact when the lipid is removed (Fig. 9), and their spacing is comparable to the size of SP-A (22, 25). A similar rigid spacing has been reported by others in different systems (7, 31). Interestingly, Sanderson and Vatter (26) and others (27, 29) have observed the presence of corrugated surfaces on some thin sections containing lung surfactant. All of these results and TEM results by Palaniyar et al. (21) support the view that SP-A and surfactant lipids can play an important role in the conversion of LBs or other vesicular lipid structures into TM. Because SP-A null transgenic mice lack TM structures (17), it is apparent that SP-A is essential for formation of these fascinating lipid-protein structures. The studies described here strongly suggest that SP-A filament-DPPC-containing lipid vesicle interaction could play an important role in the TM conversion process.

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REFERENCES


