Pulmonary Surfactant Protein A Interacts with Gel-Like Regions in Monolayers of Pulmonary Surfactant Lipid Extract

Lynn-Ann D. Worthman,* Kaushik Nag,* Nathan Rich,† Miguel L. F. Ruano,‡ Cristina Casals,‡ Jesus Pérez-Gil,‡ and Kevin M. W. Keough*§

Departments of *Biochemistry and †Physics, and §Discipline of Pediatrics, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3X9, Canada; and the ‡Departmento de Bioquimica y Biologia Molecular I, Facultad de Biologia, Universidad Complutense, 28040 Madrid, Spain

ABSTRACT Epifluorescence microscopy was used to investigate the interaction of pulmonary surfactant protein A (SP-A) with spread monolayers of porcine surfactant lipid extract (PSLE) containing 1 mol % fluorescent probe (NBD-PC) spread on a saline subphase (145 mM NaCl, 5 mM Tris-HCl, pH 6.9) containing 0, 0.13, or 0.16 μg/ml SP-A and 0, 1.64, or 5 mM $CaCl_2$. In the absence of SP-A, no differences were noted in PSLE monolayers in the absence or presence of Ca^{2+}. Circular probe-excluded (dark) domains were observed against a fluorescent background at low surface pressures ($\pi \sim 5$ mN/m) and the domains grew in size with increasing π. Above 25 mN/m, the domain size decreased with increasing π. The amount of observable dark phase was maximal at 18% of the total film area at $\pi \sim 25$ mN/m, then decreased to ~3% at $\pi \sim 40$ mN/m. The addition of 0.16 μg/ml SP-A with 0 or 1.64 mM Ca^{2+} in the subphase caused an aggregation of dark domains into a loose network, and the total amount of dark phase was increased to ~25% between π of 10–28 mN/m. Monolayer features in the presence of 5 mM Ca^{2+} and SP-A were not substantially different from those spread in the absence of SP-A, likely due to a self-association and aggregation of SP-A in the presence of higher concentrations of Ca^{2+}. PSLE films were spread on a subphase containing 0.16 μg/ml SP-A with covalently bound Texas Red (TR-SP-A). In the absence of Ca^{2+}, TR-SP-A associated with the reorganized dark phase (as seen with the lipid probe). The presence of 5 mM Ca^{2+} resulted in an appearance of TR-SP-A in the fluid phase and of aggregates at the fluid/gel phase boundaries of the monolayers. This study suggests that SP-A associates with PSLE monolayers, particularly with condensed or solid phase lipid, and results in some reorganization of rigid phase lipid in surfactant monolayers.

INTRODUCTION

Pulmonary surfactant (PS) is a heterogeneous lipid-protein material found in the aqueous lining layer of the lung, the main function of which is to reduce surface tension (γ) in the lung. This effect reduces the work involved in breathing and maintains the patency of terminal airways by preventing alveolar collapse at low transpulmonary pressures. PS reduces the surface tension at the alveolar-air interface by forming what has conventionally been considered to be a monomolecular film at the alveolar fluid surface. Indirect evidence indicates that the film is rich in dipalmitoylphosphatidylcholine (DPPC), and it has been suggested that anionic and unsaturated lipids of PS, as well as surfactant proteins A, B, and C, aid in surfactant adsorption and respreading at the surface (Fleming and Keough, 1988; King and Clements, 1972; Pérez-Gil et al., 1991; Takahashi and Fujiwara, 1986; Wang et al., 1995).

SP-A is a large glycoprotein consisting of eighteen 28–36-kDa monomers (Hawgood, 1989). SP-A monomers contain a collagen-like domain, allowing the formation of a triple helix from peptide trimers. Each monomer contains a globular carbohydrate recognition domain (CRD), a carbohydrate binding site and two high affinity Ca^{2+} binding sites, with one Ca^{2+} binding site considered to be located inside the carbohydrate binding site (Haagsman et al., 1989; Johansson and Curstedt, 1997; Kuroki and Voelker, 1994). The complex oligomeric structure of SP-A as isolated in its native form is reminiscent of a floral bouquet, and the overall structural organization is very similar to that of the C1q molecule of the complement system (King et al., 1989; Voss et al., 1988).

In vitro studies have shown that in liposomes SP-A interacts with gel-phase lipids, especially DPPC, and this interaction is dependent upon the sn-2 positioned acyl chain of the phospholipid (PL) (Casals et al., 1993; King et al., 1983; Kuroki and Akino, 1991). SP-A, in the presence of Ca^{2+}, is crucial in the conversion of PS lamellar bodies into tubular myelin (TM) (Benson et al., 1984; Suzuki et al., 1989; Williams et al., 1991). It has also been shown that PL aggregation in the presence of Ca^{2+} is enhanced by SP-A in a reversible manner (Hawgood et al., 1985), a phenomenon that may be relevant to TM formation (Haagsman et al., 1990; Meybloom et al., 1997). Recent studies have indicated that the mechanism of SP-A-mediated lipid aggregation differs from that of PL binding (McCormack et al., 1994). It has been proposed that occupancy of the high-affinity Ca^{2+} binding site in the CRD of the SP-A molecule results in a conformational change in SP-A structure. SP-A self-association and aggregation may require the occupation of both Ca^{2+} binding sites (Haagsman et al., 1990). Others have proposed that PL aggregation, as mediated by SP-A, is

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Address reprint requests to Dr. Kevin M. W. Keough, Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3X9, Canada. Tel.: 709-737-2530; Fax: 709-737-2552; E-mail: kevin.keough@mun.ca.

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not related to the self-association of SP-A molecules in the presence of Ca^{2+} (Ruano et al., 1996).

The role of SP-A in the physicochemical properties of PS is not fully determined. SP-A may accelerate the transfer of PS lipid to the air-hypophase interface in the presence of the hydrophobic surfactant proteins B and C (Chung et al., 1989; Schürch et al., 1992; Yu and Possmayer, 1993). The anionic nature of certain PS lipids, especially phosphatidylglycerol (PG), does not promote their mixing with acidic SP-A in spread monolayers at physiological pH, unless they are in the presence of Ca^{2+} (Taneva et al., 1995). Studies have indicated that SP-A and PS neutral lipids may reorganize DPPC in adsorbed films of pulmonary surfactant lipid extract, and may assist in the formation of a DPPC-rich reservoir in association with the monolayer (Yu and Possmayer, 1996). However, studies involving genetically altered mice deficient in SP-A have suggested that SP-A is not vital for normal respiratory functioning (Korfhagen et al., 1996).

Using epifluorescence microscopy (Discher et al., 1996; Grainger et al., 1990; Nag et al., 1991, 1998; Ruano et al., 1998), we have studied the association of SP-A with, and its distribution in, spread monolayers of porcine surfactant lipid extract (PSLE); PSLE contains all of the lipid associated with surfactant plus the hydrophobic surfactant proteins SP-B and SP-C (Takahashi and Fujiwara, 1986). Our aim was to determine whether SP-A modified the arrangement of lipids in such monolayers. Information on the interaction of SP-A with PSLE monolayers will provide insight into the role of SP-A in surfactant monolayer formation, both in terms of its interaction with components of the monolayer and with the lipids and proteins that ultimately result in the formation of tubular myelin.

MATERIALS AND METHODS

Materials

The fluorescent lipid probe, 1-palmitoyl-2{12[7-nitro-2,1,3-benzoxadiazole-4-yl)amino]dodecanoyl}-*sn*-glycero-3-phosphocholine (NBD-PC) was purchased from Avanti Polar Lipids (Pelham, AL). The fluorescent molecule used to chemically label SP-A, sulforhodamine 101 sulfonyl chloride or Texas Red (TR), was obtained from Molecular Probes Inc. (Eugene, OR). Reagent grade sodium chloride, calcium chloride, and the HPLC grade solvents, chloroform and methanol, were purchased from Fisher Scientific (Ottawa, ON).

All water was double-distilled, the second distillation being from dilute potassium permanganate. Glassware was chromic acid-washed, rinsed thoroughly, and baked at 180°C for 8 h before use.

Isolation of pulmonary surfactant and PSLE

Pulmonary surfactant was isolated from porcine lungs by bronchoalveolar lavage and purified by centrifugation procedures at 4°C (King and Clements, 1972; Keough et al., 1988). Lipids and hydrophobic surfactant proteins were extracted from pulmonary surfactant using mixtures of chloroform/methanol/water according to the method of Bligh and Dyer (1959). The resulting organic phases, PSLE, were pooled. The concentration of phosphorus in pulmonary surfactant and PSLE was obtained by an analysis of organic phosphorus (Keough and Kariel, 1987; Bartlett, 1959).

Isolation, purification, and labeling of SP-A

SP-A was isolated by extraction of the surfactant suspension with 1-butanol (Haagsman et al., 1987; Taneva et al., 1995). The purity of SP-A was determined by SDS-polyacrylamide gel electrophoresis (12% gels) under reducing conditions according to the method of Laemmli (1970). The concentration of SP-A was determined by the fluorescamine assay (Udenfriend et al., 1972) using bovine serum albumin as the standard. SP-A was labeled with TR using previously established procedures (Ruano et al., 1998; Nag et al., 1998).

Epifluorescence microscopy of spread monolayers of PSLE

Epifluorescence microscopy and surface pressure (π)-area measurements of solvent-spread monolayers of PSLE were performed on a surface balance whose construction and operation have been described previously (Nag et al., 1990, 1991). Monolayers of PSLE were spread from chloroform/methanol 3:1 (v/v) onto a buffered saline subphase (145 mM NaCl, 5 mM Tris, pH 6.9) containing 0, 0.13, or 0.16 μg/ml SP-A or 0.16 μg/ml TR-SP-A. All experiments were performed at 21 $\pm$ 2°C in the absence or presence of 1.64 or 5 mM Ca^{2+} in the subphase. The monolayer was initially compressed to a surface pressure of 10 mN/m, then expanded to the initial spreading pressure ($\sim$0 mN/m) and allowed to stand for 1 h. By initially compressing the monolayer, the transition of the lipids to gel phase is initiated and it has been suggested that the creation of fluid/gel phase boundaries may be needed to initiate the interaction of SP-A with the monolayer (Ruano et al., 1998). To obtain pressure-area isotherms for the purpose of comparisons between these and previously studied systems, monolayers were compressed at a rate of 4 $Å^2$/molecule/s in 30 increments. For the purpose of visualization of monolayer surface, pressure-area isotherms were obtained by compressing monolayers at a rate of 0.0089 $Å^2$/molecule/s in 30 increments. These rates are calculated from the total time required for full compression of the film, which includes periodic compression followed by short periods of no compression, during which visual images of the monolayer are acquired. Monolayer surface features were observed through a 40$\times$ objective lens, and images were videorecorded for 1 min after each incremental compression. By switching light filters, NBD fluorescence and TR fluorescence observation could be alternated. Image analysis was performed using JAVA 1.3 software (Jandel Scientific, Corte Madera, CA). Ten images were randomly selected from the videotape for each related pressure, and they were digitized. The number and size of dark domains for each image was determined from a set area of interest (AOI). The AOI encompassed $\sim$80% of the television screen size and was held constant for each analysis. The percentage of the monolayer region that excluded the probe in the images (percent dark) was calculated. This methodology has been used previously to observe lipid monolayers and has been discussed in detail elsewhere (Nag and Keough, 1993; Nag et al., 1991).

RESULTS

Fig. 1 shows the surface pressure (π)-area per phospholipid molecule isotherms of PSLE spread over subphases containing 0, 0.13, and 0.16 μg/ml SP-A in the absence (*panel A*) or presence (*panel B*) of 5 mM Ca^{2+}. The isotherms of PSLE monolayers had a lift-off (detectable surface pressure) at $\sim$90 $Å^2$/molecule. At π of 45 mN/m, the isotherm inflected into a short region with high compressibility,

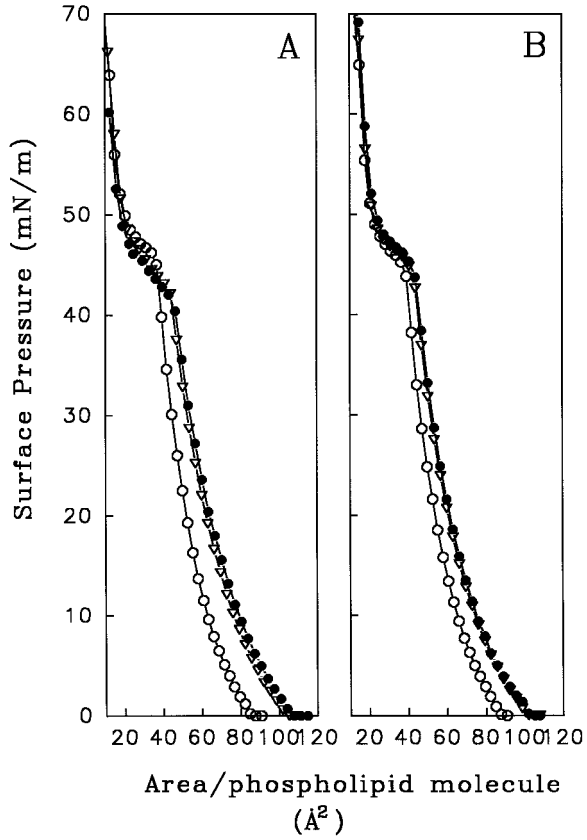

FIGURE 1 Pressure-area isotherms of PSLE monolayers spread over subphases containing 0 (○), 0.13 (●), and 0.16 μg/ml SP-A (▽) in the absence (*A*) or presence (*B*) of 5 mM Ca^{2+}. The monolayers were compressed in 30 steps at an initial rate of 4 $Å^2$/molecule/s on a subphase of 145 mM NaCl, 5 mM Tris (pH 6.9). The temperature of the subphase was 21 ± 2°C.

which was followed again by a sharp rise to ~70 mN/m. The presence of SP-A in the subphase shifted the lift-off to ~110 $Å^2$/phospholipid molecule. With incremental compression, the isotherms of PSLE in the presence of SP-A shifted toward the PSLE isotherm and at π ~45 mN/m adopted a π-area profile similar to that of PSLE in the absence of protein. The presence of 5 mM Ca^{2+} (*panel B*) did not significantly affect these properties, although the extent of the expansion caused by SP-A appears to be slightly reduced.

Images of monolayers of PSLE on subphases containing 0, 0.13, or 0.16 μg/ml SP-A at selected π are shown in Fig. 2. For the purpose of monolayer visualization, the PSLE films were compressed at a slow rate (0.0089 $Å^2$/molecule/s), which resulted in monolayer collapse at ~45 mN/m. *Panel A* presents typical images observed in spread monolayers of PSLE at various π. Nearly circular, probe-excluded (dark) domains were observed amid a fluorescent green background at low π (2–5 mN/m) despite the absence of a traditional liquid-expanded (LE) to liquid-condensed (LC) phase transition in the isotherm, as is seen in monolayers of pure lipid such as DPPC. These probe-excluded domains decreased in size with increased pressure (~5–7 mN/m); however, at such pressures there was an appearance of other small circular probe-excluded domains that we suspect comprise a different phase lipid. With further increases in π, the small dark domains grew larger until π of ~25 mN/m, when subsequent pressure increase resulted in a decrease in domain size. The appearance of dark domains in monolayers of PSLE has been reported previously (Discher et al., 1996; Nag et al., 1998). The addition of 0.13 μg/ml SP-A (*panel B*) in the subphase had a minimal effect on PSLE monolayer appearances. Dark domains were observed at low π, and grew in size with increased π up to 25 mN/m; thereafter a decrease in domain size was observed.

The presence of 0.16 μg/ml SP-A in the subphase had a dramatic effect on the appearance of PSLE monolayer textures (Fig. 2 *C*). At π of ~10 mN/m, dark domains organized into a loose "network" of aggregated dark domains, with adjacent areas being highly fluorescent. With increased π, the "network" was not as intensely dark and the nucleation of tiny dark domains was observed. At π ~27 mN/m, the network disappeared, leaving some tiny dark domains that persisted at higher pressures amid a fluorescent background.

Fig. 3 shows typical images of PSLE on subphases containing 0, 0.13, and 0.16 μg/ml SP-A in the presence of 5 mM Ca^{2+}. The presence of 5 mM Ca^{2+} did not have a substantial effect on PSLE monolayer surface organization. The nucleation of dark domains was observed at low π (5 mN/m) and the domains grew in size until π ~25 mN/m. Beyond this π the visible amount of dark phase was decreased. When various concentrations of SP-A were added to the subphase (*panels B* and *C*), the appearance and behavior of the PSLE monolayers were not very different from films in the absence of SP-A.

Typical images of monolayers of PSLE spread on a buffered saline subphase containing 0.16 μg/ml TR-SP-A in the absence or presence of 5 mM Ca^{2+} are shown in Fig. 4. When observed with NBD-PC fluorescence (*right-hand panels*), PSLE monolayers spread over subphases containing protein in the absence of Ca^{2+} (Fig. 4 *A*) showed the appearance of a loosely organized "network" of dark regions amid a fluorescent background at π ~10 mN/m and higher, as described previously (Fig. 2 *C*). The "network" was no longer detectable at π beyond 30 mN/m. The images observed with the aide of TR-SP-A fluorescence (*left-hand panels*) were reversed to those observed with NBD-PC fluorescence. Organized "networks" of fluorescent domains were observed with compression up to $\pi \geq 30$ mN/m, above which only tiny aggregates of TR-SP-A fluorescence were observed randomly at the film surface. Although our equipment did not allow us to obtain data fast enough to record superimposable images, when the filters were switched it could be observed by eye that the red areas from

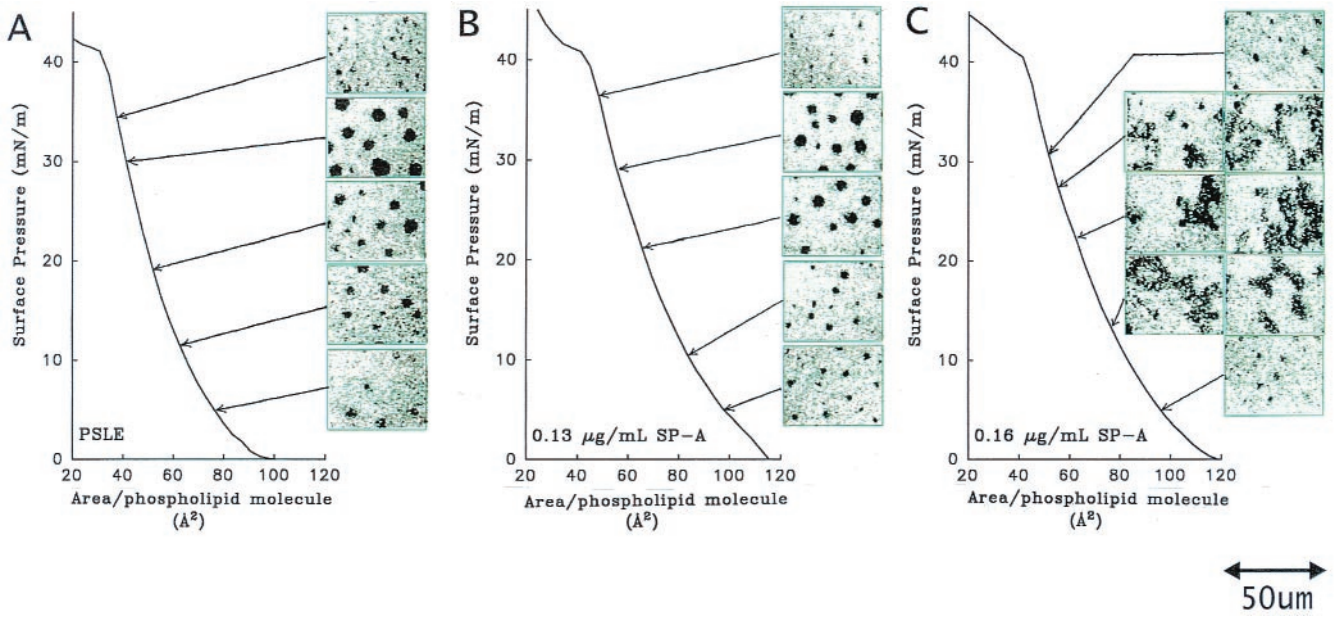

FIGURE 2 Isotherms and fluorescent images of monolayers of PSLE spread on buffered saline subphase containing 0 (*A*), 0.13 (*B*), and 0.16 μg/ml SP-A (*C*) in the absence of Ca^{2+}. The green background indicates the phase containing the fluorescent probe NBD-PC. The images were collected using a black and white CCD camera and are shown in false color to convey the impression given during direct observation. The scale bar is 50 μm.

TR-SP-A fluorescence corresponded to the dark areas seen with NBD-PC fluorescence.

Images obtained from PSLE monolayers spread onto a subphase containing 0.16 μg/ml TR-SP-A and 5 mM Ca^{2+} did not show a reorganization of dark domains as viewed through NBD-PC fluorescence (Fig. 4 *B*, *right panel*). The domains were circular, and they grew in size with compression until π of 35 mN/m were reached. Beyond this π, there was a decrease in the detectable size and number of dark domains. TR-SP-A fluorescence (*left panel*) was minimal at lower π ($\leq$10 mN/m) when small regions of fluorescence likely representing aggregates of labeled protein were ob-

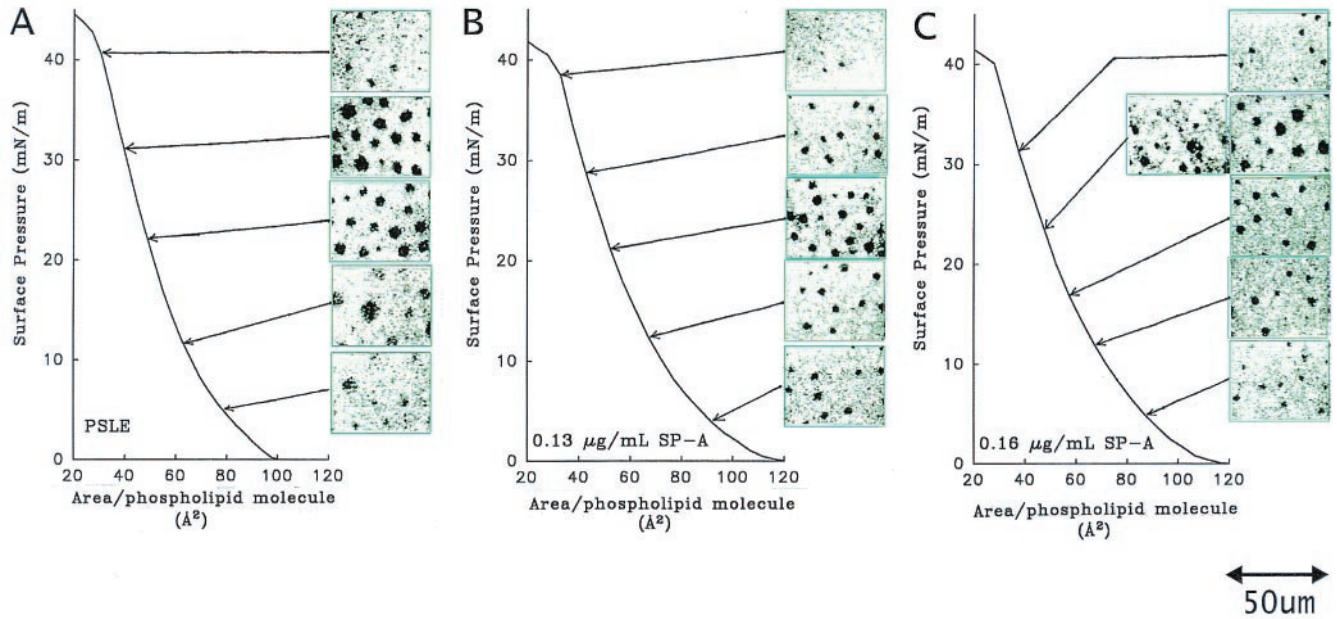

FIGURE 3 Isotherms and fluorescent images of monolayers of PSLE spread on buffered saline subphase containing 0 (*A*), 0.13 (*B*), and 0.16 μg/ml SP-A (*C*) in the presence of 5 mM Ca^{2+}. The scale bar is 50 μg.

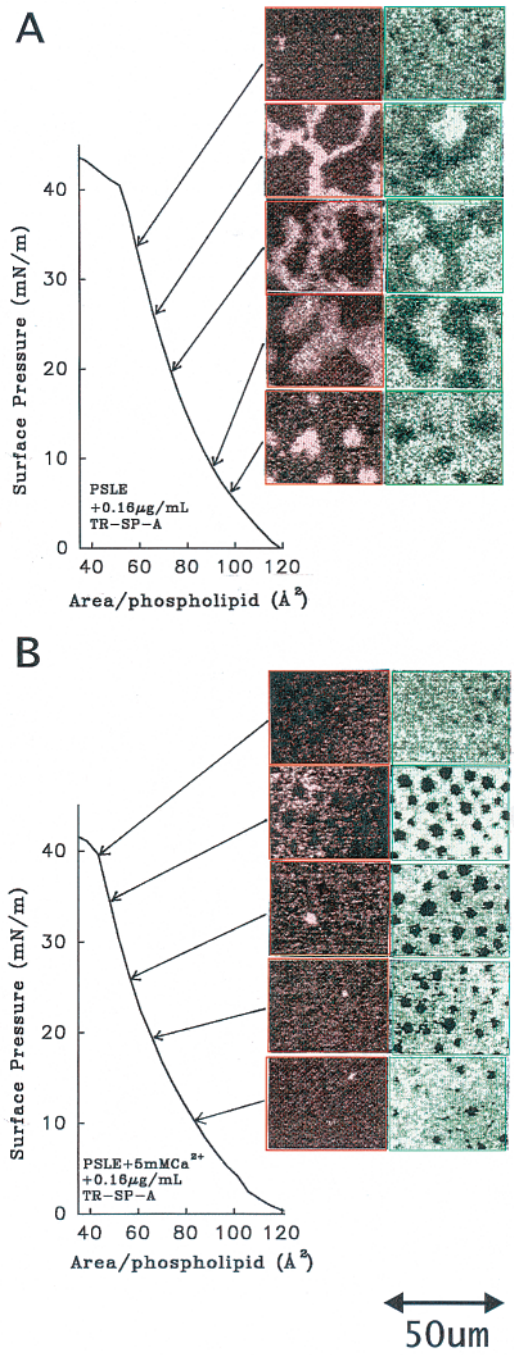

FIGURE 4 Isotherms and fluorescent images of PSLE spread on buffered saline subphase containing 0.16 μg/ml TR-SP-A in the absence (*A*) or presence (*B*) of 5 mM Ca^{2+}. The green background (*right panel*) indicates the phase containing the fluorescent probe NBD-PC, and the red background (*left panel*) indicates phase containing the fluorescently labeled SP-A. The scale bar is 50 μm.

served amid a dark background. Increasing π allowed for the visualization of dark domains (25–35 mN/m), but the red fluorescence of the background was not intense. Aggregations of TR-SP-A were observed in the fluid phase or near the fluid/gel phase boundaries. At π beyond 35 mN/m, the dark domains were no longer detectable, and the TR-SP-A fluorescence was seen in very small areas distributed apparently randomly across the fields of view.

The amount of dark area as a percentage of the total area in PSLE monolayers containing various concentrations of SP-A in the absence (*top*) and presence (*bottom*) of 5 mM Ca^{2+} is shown in Fig. 5. The percent dark area in monolayers of PSLE increased with rising π and reached 18% at $\pi \sim 25$ mN/m, then gradually decreased to 3% at 40 mN/m. Similar amounts of condensation in monolayers of lipid extracts of calf and pig lung surfactants have been reported (Discher et al., 1996; Nag et al., 1998) although the amount of dark phase observed by Discher et al. (1996) was somewhat higher than that observed in this study. Nag et al. (1998) did not observe the nucleation of dark domains until $\pi \sim 10$ mN/m, yet there was dark phase ($\sim$5%) seen in this study at π as low as 2 mN/m. Some recent work in our laboratory suggests that the dark domains seen at very low pressures may be protein-rich, probe-excluding regions, while the dark domains usually associated with condensed

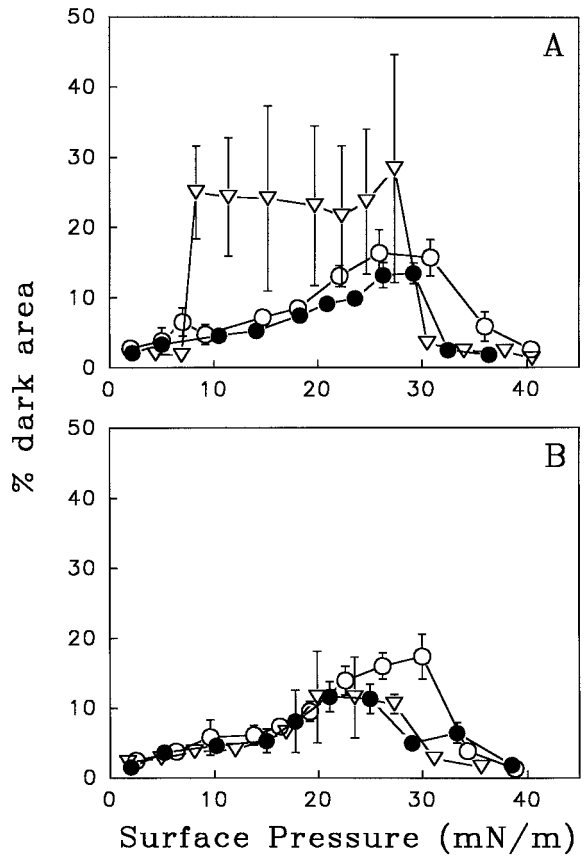

FIGURE 5 The percent dark (probe-excluded) area of PSLE monolayers spread on subphases containing 0 (○), 0.13 (●), 0.16 μg/ml (▽) SP-A in the subphase in the absence (*A*) and presence (*B*) of 5 mM Ca^{2+}. Error bars indicate $\pm$1 SD for 10 frames analyzed at each π. Error bars not shown are within the size of the symbol.

phase do not appear until pressures above 7 mN/m (Taneva and Keough, 1999). The presence of 0.13 μg/ml SP-A in the subphase did not have a substantial effect on the total proportion of dark area observed in PSLE monolayers. Subphase concentrations of 0.16 μg/ml SP-A increased the amount of dark phase to 25% at π between 10 and 27 mN/m, but beyond this π the amount of dark phase decreased to ~2%.

The presence of 5 mM Ca^{2+} in the subphase (Fig. 5 *B*) did not substantially alter the amount of dark area observed on the surface of PSLE monolayers. Subphase concentrations of 0.13 and 0.16 μg/ml SP-A appeared to decrease the amount of dark area observed over the range pressures of 25–35 mN/m. Analysis of domain sizes (data not shown) indicated that the presence of both 5 mM Ca^{2+} and SP-A reduced sizes and dispersion of sizes of the lipid domains.

Isotherms of PSLE spread over subphases containing 0, 1.64, and 5 mM Ca^{2+} plus or minus 0.16 μg/ml SP-A in the subphase are shown in Fig. 6. The value of 1.64 mM Ca^{2+} most closely reflects the concentration in the hypophase of the lung (Nielson et al., 1981). The presence of Ca^{2+} at either concentration did not substantially affect the PSLE monolayer isotherm. The presence of 0.16 μg/ml SP-A at various concentrations of Ca^{2+} expanded the area occupied by the PSLE film. Isotherm profiles were similar to those previously recorded.

Monolayers of PSLE spread on subphases containing 1.64 mM Ca^{2+} plus or minus 0.16 μg/ml SP-A are shown in Fig. 7. The appearance of PSLE films in the presence of

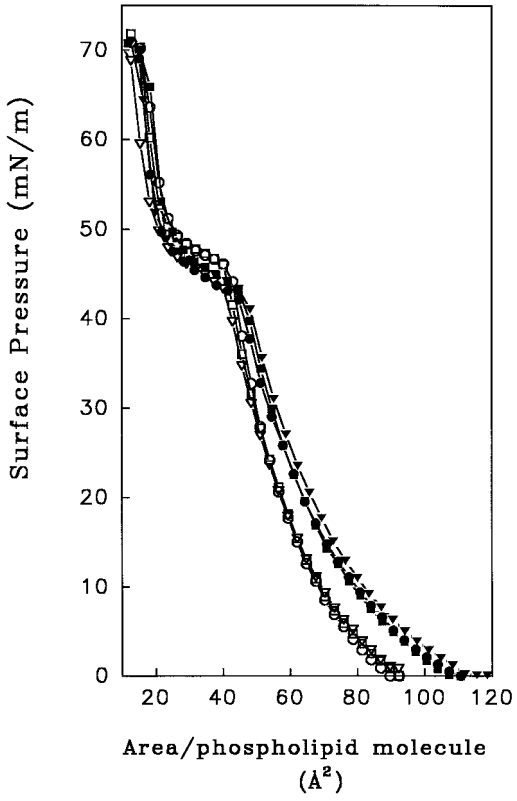

FIGURE 6 Pressure-area isotherms of PSLE (○), PSLE with 0.16 μg/ml SP-A (●), PSLE with 1.64 mM Ca^{2+} (▽), PSLE with 0.16 μg/ml SP-A and 1.64 mM Ca^{2+} (▼), PSLE with 5 mM Ca^{2+} (□), and PSLE with 0.16 μg/ml SP-A and 5 mM Ca^{2+} (■). The monolayers were compressed in steps at a rate of 4 Å^2/molecule/s on a subphase of 145 mM NaCl, 5 mM Tris (pH 6.9). The temperature of the subphase was 21 ± 2°C.

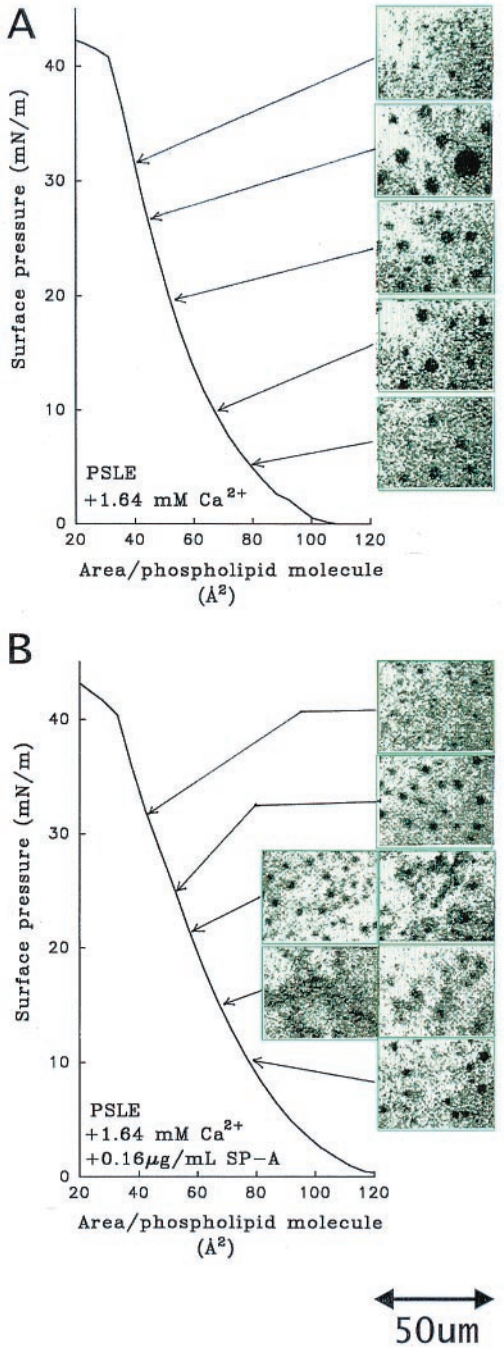

FIGURE 7 Isotherms and fluorescent images of monolayers of PSLE spread over buffered saline subphases containing 1.64 mM Ca^{2+} (*A*) or 1.64 mM Ca^{2+} plus 0.16 μg/ml SP-A (*B*). The scale bar is 50 μm.

1.64 mM Ca^{2+} (*A*) had characteristics intermediate between films of PSLE found over either 0 or 5 mM Ca^{2+}. The presence of 0.16 μg/ml SP-A and 1.64 mM Ca^{2+} in the subphase resulted in a reorganization of PSLE films, but higher π was required to initiate this reorganization compared to PSLE films spread over protein in the absence of Ca^{2+}. Domains aggregated into a network at ~13 mN/m, and the reorganization of domains was apparent up to $\pi >$ 20 mN/m. With increased π, small dark domains were observed amid a fluorescent green background. Further compression resulted in a decrease in domain size.

The percent dark area of PSLE with or without 0.16 μg/ml SP-A in the subphase in the presence of various concentrations of Ca^{2+} is summarized in Fig. 8. In the absence of SP-A the percent dark area of PSLE films containing various subphase concentrations of Ca^{2+} was very similar. The area of dark phase reached ~18% with increased π for each of the films. The presence of 0.16 μg/ml SP-A with 0 or 1.64 mM Ca^{2+} caused an increase in the amount of dark area in PSLE monolayers (~25%). Higher π was required in PSLE films spread over 1.64 mM Ca^{2+} plus SP-A before they showed a large increase in dark phase in comparison to films with no calcium present. With 5 mM Ca^{2+} and 0.16 μg/ml SP-A, the amount of dark area was slightly reduced over the range of 25–35 mN/m compared to that in PSLE films in the absence of SP-A.

DISCUSSION

The surface characteristics of PS have been studied extensively, as they are directly applicable to the ability of the lung to function properly during respiration (Schürch et al., 1992; Wang et al., 1995; Yu and Possmayer, 1993). When compressed, PSLE monolayers do not undergo a classical LE/LC phase transition, although regions of condensed DPPC in rich domains are found at intermediate pressures (Discher et al., 1996). A highly compressible region is observed at π ~45 mN/m, and it is possible that non-DPPC components are excluded from the monolayer surface at this pressure and under these operating conditions. In surface balances of the type used here, PSLE monolayers, when compressed rapidly at room temperature, reach π of ~72 mN/m. Previous studies suggest that of components present in surfactant in significant amounts, only those forming phases rich in DPPC attain such high π in balances such as these (Goerke and Gonzales, 1981; Hawco et al., 1981; Hildebran et al., 1979). In recent work with monolayers in captive bubbles under very rapid compression it has been found that unsaturated PC can reach high π (Crane and Hall, 2000).

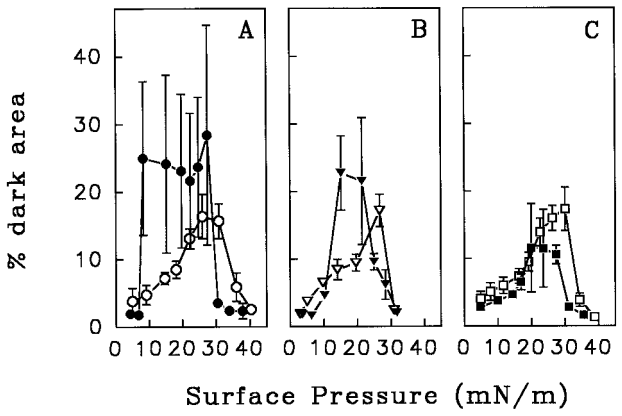

FIGURE 8 The percent dark (probe-excluded) area of PSLE (*open symbols*) and PSLE spread over buffered saline subphases containing 0.16 μg/ml SP-A (*filled symbols*) with 0 (*A*), 1.64 (*B*), and 5 mM Ca^{2+} (*C*). Error bars indicate $\pm$1 SD.

The presence of the water-soluble SP-A expanded the PSLE monolayer, indicative of SP-A association with the monolayer surface. It is possible that SP-A, or a part of it, may be inserting into the monolayer, or that it indirectly alters PSLE lipid packing by associating with, but not penetrating, the monolayer surface, or both (Heckl et al., 1985; Pérez-Gil et al., 1992; Ruano et al., 1998). At higher π, pressure-area isotherms of monolayers of PSLE spread over SP-A followed profiles similar to those of PSLE in the absence of protein. This may be a consequence of the pressure-induced exclusion of SP-A from the monolayer (Ruano et al., 1998; Taneva et al., 1995). In the absence of SP-A, characteristics of PSLE monolayers spread over subphases containing 5 mM Ca^{2+} were not substantially different from monolayers spread over subphases devoid of Ca^{2+}. This is likely due to the relatively small proportions of negatively charged lipid found in surfactants.

Monolayers of PSLE with the NBD-PC probe show a phase partitioning in the range of 2–40 mN/m into fluid, probe-containing regions plus dark, probe-excluding domains. At low π (2–7 mN/m), the dark domains appeared to be different from the domains that appeared at π ~5–7 mN/m and higher. Other studies in our laboratory suggest that the dark domains observed at very low π may be rich in hydrophoric proteins. These potentially protein-rich domains dissolve into the monolayer lipid with increased π, and this process overlaps with the nucleation of traditional condensed phase (Taneva and Keough, 1999). The appearance of the dark domains, including those associated with condensed-phase lipids, is not accompanied by a region of high compressibility, as is seen for the traditional LE/LC transition in monolayers (Discher et al., 1996; Nag et al., 1998). It has been noted that the maximum amount of condensed probe-excluded phase seen in monolayers of calf lung surfactant lipid extract corresponded to the amount of DPPC in this surfactant extract, and it was suggested that the dark domains at $\pi >$ 7–8 mN/m were pure or nearly pure condensed-phase DPPC (Discher et al., 1996). At $\pi >$ 35 mN/m the dark domains decrease in size, an occurrence also recorded in monolayers of calf lung lipid extract visualized by both fluorescence and Brewster angle microscopy

(BAM). This latter technique argues against the possibility that unusual interactions with the fluorescent probe cause this change in monolayer appearance (Discher et al., 1996). It has been suggested that higher π results in an increase in packing density of the molecules in the fluid phase (probe-rich region) such that it approaches a state similar to that of the gel-like dark domains. Probe redistribution occurs in the film (Discher et al., 1996) likely accompanied by a decrease in line tension at the domain edges, and as a consequence, the two phases are nearly indistinguishable with the fluorescent lipid probe. It has also been suggested that the transformation of appearance at $\pi > 35$ mN/m is related to a redistribution of dark and probe-containing regions, and that the homogeneous distribution of fluorescence is caused by a limit in optical resolution (Nag et al., 1998).

In the absence of added Ca^{2+}, 0.13 μg/ml SP-A did not substantially change the general appearance of PSLE monolayers, but the presence of 0.16 μg/ml SP-A did. Although the lack of apparent change up to 0.13 μg/ml might represent a limitation in detectability, it may imply that a threshold SP-A concentration may be necessary to initiate lipid reorganization. The reorganization and increase in amount of dark domains induced by SP-A may be a consequence of specific interactions of SP-A with gel-phase lipids, particularly DPPC (Casals et al., 1993; Kuroki and Akino, 1991), accompanied by some domain aggregation promoted by SP-A self-association. Because SP-A might bind to ordered-phase lipid, it could expand the lipid molecular packing somewhat in this region. Thus, the apparent increase in total dark area in PSLE films spread over SP-A may be a consequence of lipid molecular packing of PSLE in the condensed phase being slightly less dense than pure DPPC films, and not due to an increase in the total number of condensed-phase lipids. This might occur especially if the DPPC-rich condensed phase contained small amounts of other lipids or proteins, such as SP-B. The binding of SP-A to lipids (DPPC or DPPC/egg-PG 7:3) requires micromolar amounts of Ca^{2+}. Such small amounts of Ca^{2+} might be present in the nominally Ca^{2+}-free buffer given the absence of a Ca^{2+} chelator in the buffer. SP-A aggregation requires Ca^{2+} concentrations in the millimolar range, but it is induced also by physiological concentrations of saline (Haagsman et al., 1990). It is possible that the SP-A dependent domain redistribution seen here is caused by SP-A self-interaction, and it could be modulated by the interaction between it and the hydrophobic proteins SP-B and SP-C in the PSLE. The appearance of the network, including less contrast between the darkened probe-excluded and fluorescent regions seen at pressures $\sim$25 mN/m, might result from a new phase with SP-A as a component (Discher et al., 1996).

Fluorescently labeled SP-A colocalized with the dark domains observed using the fluorescent lipid probe, suggesting that SP-A preferentially absorbed into, or near to, the ordered-phase regions. Epifluorescence studies of the interaction of TR-SP-A with monolayers of DPPC alone have shown that TR-SP-A is found in the LE phase and at the LC/LE boundaries, possibly as SP-A aggregates (Ruano et al., 1998). Dark domains in PSLE monolayers may not be quite pure DPPC, and that might allow greater ease of their interaction with SP-A. Discher et al. (1999) noted that neutral lipids of surfactant added to the phospholipid components cause an increase in the amount of condensed phase in monolayers, a finding consistent with the view that the condensed domains are not pure DPPC. The fluid phase of PSLE likely contains negatively charged lipids like PG, and this could act to "repel" the negatively charged SP-A from this region and promote its interaction with the condensed phase. Charge repulsion cannot completely explain the association with the condensed domains, however, since the distribution of TR-SP-A in DPPC/DPPG (7:3) (Ruano et al., 1998) was different from that seen with TR-SP-A and PSLE. In monolayers of PSLE, the possible decreased order of lipids, or small amounts of other components, in the condensed regions may allow the partial insertion of SP-A into the domain interior, or easier association than that observed with tightly packed condensed domains in pure DPPC films. At pressures of $\sim$30 mN/m and higher, the decrease in protein fluorescence offers evidence of the exclusion of SP-A from the film surface, as seen from studies of SP-A in DPPC as well (Ruano et al., 1998; Taneva et al., 1995).

Calcium may interact with both negatively charged lipids and the SP-A. The results here are likely due to calcium-SP-A effects. SP-A is soluble in its native octadecameric form at low ionic strength and physiological pH, but it aggregates and undergoes a slight conformational change at physiological concentrations of Ca^{2+} and saline solutions (Haagsman et al., 1990; Ruano et al., 1996). In the system containing 5 mM Ca^{2+} the SP-A is likely to be substantially aggregated, but PSLE isotherms obtained in the presence of SP-A and Ca^{2+} indicate that the monolayer is expanded by the SP-A, although somewhat less so than in the absence of Ca^{2+}. The dark domains in monolayers of PSLE were smaller in the presence of both SP-A and 5 mM Ca^{2+} than in the presence of Ca^{2+} alone, and there was a decrease in the amount of dark area recorded in such films. These observations are consistent with some insertion into or perturbation of packing of the monolayer by SP-A. In monolayers of DPPG and DPPC/DPPG in the absence of Ca^{2+}, SP-A caused minimal perturbation because of mutual repulsion between negative lipids and negative proteins (Ruano et al., 1998; Taneva et al., 1995). In DPPC/DPPG systems the addition of Ca^{2+} allowed greater perturbation of the monolayer by SP-A, presumably by reducing the charge in the negative lipid (Taneva et al., 1995). The pattern of interaction between SP-A and monolayers of PSLE in the presence or absence of Ca^{2+} is amenable to similar interpretation.

The presence of Ca^{2+} at a concentration of 1.64 mM used during these experiments reflects the hypophase environment that bathes the epithelial lining of the lung (Nielson et al., 1981). Previous studies have shown that the half-maximal self-association of porcine SP-A requires 2.36 mM Ca^{2+} (Ruano et al., 1996) and the presence of near physiological levels of saline may be a factor contributing to SP-A self-association (Haagsman et al., 1990). In this study, properties intermediate between those systems containing no or 5 mM Ca^{2+} were observed, as might be expected from the intermediate Ca^{2+} concentration.

This study indicates that SP-A can induce a rearrangement of solid domains in the surfactant monolayer. Such a change, especially the production of a "network" of solid phase, could contribute to surfactant film formation at the air-water interface. This could mean that SP-A could sort PS material and it could aid in stabilizing PS monolayers. The sorting induced by SP-A may allow for selective exclusion of non-DPPC components from the surface. If the same sorting also occurred in the bulk phases of surfactant, such as tubular myelin and lamellar bodies, then it could predispose the system for selective insertion of DPPC into the monolayer during film formation.

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REFERENCES

Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466–478.

Benson, B. J., M. C. Williams, K. Sueishi, J. Goerke, and T. Sargeant. 1984. Role of calcium ions in the structure and function of pulmonary surfactant. *Biochim. Biophys. Acta.* 793:18–27.

Bligh, E., and W. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917.

Casals, C., E. Miguel, and J. Pérez-Gil. 1993. Tryptophan fluorescence study on the interaction of pulmonary surfactant protein A with phospholipid vesicles. *Biochem. J.* 296:585–593.

Chung, J., S-H. Yu, J. A. Whitsett, P. G. R. Harding, and F. Possmayer. 1989. Effect of surfactant-associated protein A (SP-A) on the activity of lipid extract surfactant. *Biochim. Biophys. Acta.* 1002:348–358.

Crane, J. M., and S. B. Hall. 2000. Stability of pulmonary surfactant monolayers depends on rate of compression. *Biophys. J.* 78:487*a*. (Abstr.).

Discher, B. M., K. M. Maloney, D. W. Grainger, C. A. Sousa, and S. B. Hall. 1999. Neutral lipids induce critical behavior in interfacial monolayers of pulmonary surfactant. *Biochemistry.* 38:374–383.

Discher, B. M., K. M. Maloney, W. R. Schief, Jr., D. W. Grainger, V. Vogel, and S. B. Hall. 1996. Lateral phase separation in interfacial films of pulmonary surfactant. *Biophys. J.* 71:2583–2590.

Fleming, B. D., and K. M. W. Keough. 1988. Surface respreading after collapse of monolayers containing major lipids of pulmonary surfactant. *Chem. Phys. Lipids.* 49:81–86.

Goerke, J., and J. Gonzales. 1981. Temperature dependence of dipalmitoylphosphatidylcholine monolayer stability. *J. Appl. Physiol.: Respirat. Environ. Exercise Physiol.* 51:1108–1114.

Grainger, D. W., A. Reichert, H. Ringsdorf, and C. Salesse. 1990. Hydrolytic action of phospholipase A2 in monolayers in the phase transition region: direct observation of enzyme domain formation using fluorescence microscopy. *Biochim. Biophys. Acta.* 1023:365–379.

Haagsman, H. P., S. Hawgood, T. Sargeant, D. Buckley, R. T. White, K. Drickamer, and B. J. Benson. 1987. The major lung surfactant protein, SP 28–36, is a calcium-dependent, carbohydrate-binding protein. *J. Biol. Chem.* 262:13877–13880.

Haagsman, H. P., T. Sargeant, P. V. Hauschka, B. J. Benson, and S. Hawgood. 1990. Binding of calcium to SP-A, a surfactant-associated protein. *Biochemistry.* 29:8894–8900.

Haagsman, H. P., R. T. White, J. Schilling, K. Lau, B. J. Benson, J. Golden, S. Hawgood, and J. A. Clements. 1989. Studies of the structure of lung surfactant protein SP-A. *Am. J. Physiol. Lung Cell Mol. Physiol.* 257: L421–L429.

Hawco, M. J., P. J. Davis, K. P. Coolbear, and K. M. W. Keough. 1981. Exclusion of fluid lipid during compression of monolayers of mixtures of dipalmitoylphosphatidylcholine with some other phosphatidylcholines. *Biochim. Biophys. Acta.* 646:185–197.

Hawgood, S. 1989. Pulmonary surfactant apoproteins: a review of protein and genomic structure. *Am. J. Physiol. Lung Cell Mol. Physiol.* 257: L13–L22.

Hawgood, S., B. J. Benson, and R. L. Hamilton. 1985. Effects of a surfactant-associated protein and calcium ions on the structure and surface activity of lung surfactant lipid. *Biochemistry.* 24:184–190.

Heckl, W. M., M. Lösche, H. Scheer, and H. Möhwald. 1985. Protein/lipid interactions in phospholipid monolayers containing the bacterial antenna protein B800–850. *Biochim. Biophys. Acta.* 810:73–83.

Hildebran, J. N., J. Goerke, and J. A. Clements. 1979. Pulmonary surface film stability and composition. *J. Appl. Physiol.: Respirat. Environ. Exercise Physiol.* 47:604–611.

Johansson, J., and T. Curstedt. 1997. Molecular structures and interactions of pulmonary surfactant components. *Eur. J. Biochem.* 244:675–693.

Keough, K. M. W., and N. Kariel. 1987. Differential scanning calorimetric studies of aqueous dispersions of phosphatidylcholines containing two polyenic chains. *Biochim. Biophys. Acta.* 902:11–18.

Keough, K. M. W., C. S. Parsons, P. T. Phang, and M. G. Tweeddale. 1988. Interactions between plasma proteins and pulmonary surfactant: surface balance studies. *Can. J. Physiol. Pharmacol.* 66:1166–1173.

King, R. J., M. C. Carmichael, and P. M. Horowitz. 1983. Reassembly of lipid-protein complexes of pulmonary surfactant. Proposed mechanism of interaction. *J. Biol. Chem.* 258:10672–10680.

King, R. J., and J. A. Clements. 1972. Surface active material from dog lung. II. Composition and physiological correlations. *Am. J. Physiol.* 223:715–726.

King, R. J., D. Simon, and P. M. Horowitz. 1989. Aspects of secondary and quaternary structure of surfactant protein A from canine lung. *Biochim. Biophys. Acta.* 1001:294–301.

Korfhagen, T. R., M. D. Bruno, G. F. Ross, K. M. Huelsman, M. Ikegami, A. H. Jobe, S. E. Wert, B. R. Stripp, R. E. Jorris, S. W. Glasser, C. J. Bachurski, H. S. Iwamoto, and J. A. Whitsett. 1996. Altered surfactant function and structure in SP-A gene targeted mice. *Proc. Natl. Acad. Sci. U.S.A.* 93:9594–9599.

Kuroki, Y., and T. Akino. 1991. Pulmonary surfactant protein A (SP-A) specifically binds dipalmitoylphosphatidylcholine. *J. Biol. Chem.* 266: 3068–3073.

Kuroki, Y., and D. R. Voelker. 1994. Pulmonary surfactant proteins. *J. Biol. Chem.* 269:25943–25946.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 277:680–685.

McCormack, F. X., Y. Kuroki, J. J. Stewart, R. J. Mason, and D. R. Voelker. 1994. Surfactant protein A amino acids Glu^{195} and Arg^{197} are essential for receptor binding, phospholipid aggregation, regulation of secretion, and the facilitated uptake of phospholipid by type II cells. *J. Biol. Chem.* 269:29801–29807.

Meybloom, A., D. Maretzki, P. A. Stevens, and K. P. Hofmann. 1997. Reversible calcium-dependent interaction of liposomes with pulmonary surfactant protein A. *J. Biol. Chem.* 272:14600–14605.

Nag, K., C. Boland, N. Rich, and K. M. W. Keough. 1990. Design and construction of an epifluorescence microscopic surface balance for the study of lipid monolayer phase transitions. *Rev. Sci. Instrum.* 61: 3425–3430.

Nag, K., C. Boland, N. Rich, and K. M. W. Keough. 1991. Epifluorescence microscopic observation of monolayers of dipalmitoylphosphatidylcholine: dependence of domain size on compression rates. *Biochim. Biophys. Acta.* 1068:157–160.

Nag, K., and K. M. W. Keough. 1993. Epifluorescence microscopic studies on monolayers containing mixtures of dioleoyl and dipalmitoylphosphatidylcholine. *Biophys. J.* 65:1019–1026.

Nag, K., J. Pérez-Gil, M. L. F. Ruano, L. A. Worthman, J. Stewart, C. Casals, and K. M. W. Keough. 1998. Phase transitions in films of lung surfactant at the air-water interface. *Biophys. J.* 74:2983–2995.

Nielson, D. W., J. Goerke, and J. A. Clements. 1981. Alveolar subphase pH in the lungs of anesthetized rabbits. *Proc. Natl. Acad. Sci. U.S.A.* 78:7119–7123.

Pérez-Gil, J., K. Nag, S. Taneva, and K. M. W. Keough. 1992. Pulmonary surfactant protein SP-C causes packing rearrangements of dipalmitoylphosphatidylcholine in spread monolayers. *Biophys. J.* 63:197–204.

Pérez-Gil, J., J. Tucker, G. Simatos, and K. M. W. Keough. 1991. Interfacial adsorption of simple lipid mixtures combined with hydrophobic surfactant protein from pig lung. *Biochem. Cell Biol.* 70:332–338.

Ruano, M. L. F., E. Miguel, J. Pérez-Gil, and C. Casals. 1996. Comparison of lipid aggregation and self-aggregation activities of pulmonary surfactant-associated protein A. *Biochem. J.* 313:683–689.

Ruano, M. L. F., K. Nag, L-A. Worthman, C. Casals, J. Pérez-Gil, and K. M. W. Keough. 1998. Differential partitioning of pulmonary surfactant protein SP-A into regions of monolayers of dipalmitoylphosphatidylcholine (DPPC) and DPPC/dipalmitoylphosphatidylglycerol. *Biophys. J.* 74:1101–1109.

Schürch, S., F. Possmayer, S. Cheng, and A. Cockshutt. 1992. Pulmonary SP-A enhances adsorption and appears to induce surface sorting of lipid extract surfactant. *Am. J. Physiol. Lung Cell Mol. Physiol.* 263: L210–L218.

Suzuki, Y., Y. Fujita, and K. Kogishi. 1989. Reconstitution of tubular myelin from synthetic lipids and protein associated with pig pulmonary surfactant. *Am. Rev. Respir. Dis.* 140:75–81.

Takahashi, A., and T. Fujiwara. 1986. Proteolipid in bovine lung surfactant: its role in surfactant function. *Biochim. Biophys. Acta.* 135: 527–532.

Taneva, S. T., and K. M. W. Keough. 1999. Effects of surfactant associated protein SP-A on phase properties of phospholipid monolayers containing SP-B or SP-C. *Biophys. J.* 76:212*a*. (Abstr.).

Taneva, S., T. McEachren, J. Stewart, and K. M. W. Keough. 1995. Pulmonary surfactant protein SP-A with phospholipids in spread monolayers at the air-water interface. *Biochemistry.* 34:10279–10289.

Udenfriend, S., S. Stein, P. Bohlen, W. Dairman, W. Loimgrukes, and M. Weigele. 1972. Fluorescamine: a reagent for assay of amino acids, peptides and primary amines in the picomole range. *Science (Wash. DC).* 178:871–872.

Voss, T., H. Eistetter, K. P. Schäfer, and J. Engel. 1988. Macromolecular organization of natural and recombinant lung surfactant protein SP 28–36: structural homology with the complement factor C1q. *J. Mol. Biol.* 201:219–227.

Wang, Z., S. B. Hall, and R. H. Notter. 1995. Dynamic surface activity of films of lung surfactant phospholipids, hydrophobic proteins, and neutral lipids. *J. Lipid Res.* 36:1283–1293.

Williams, M. C., S. Hawgood, and R. L. Hamilton. 1991. Changes in lipid structure produced by surfactant proteins SP-A, SP-B, and SP-C. *Am. J. Respir. Cell Mol. Biol.* 5:41–50.

Yu, S-H., and F. Possmayer. 1993. Adsorption, compression and stability of surface films from natural, lipid extract and reconstituted pulmonary surfactant. *Biochim. Biophys. Acta.* 1167:264–271.

Yu, S-H., and F. Possmayer. 1996. Effect of pulmonary surfactant protein A and neutral lipid on accretion and organization of dipalmitoylphosphatidylcholine in surface films. *J. Lipid Res.* 37:1278–1288.