Effect of Hydrophobic Surfactant Peptides SP-B and SP-C on Binary Phospholipid Monolayers. I. Fluorescence and Dark-Field Microscopy

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ABSTRACT The influence of the hydrophobic proteins SP-B and SP-C, isolated from pulmonary surfactant, on the morphology of binary monomolecular lipid films containing phosphocholine and phosphoglycerol (DPPC and DPPG) at the air-water interface has been studied using epifluorescence and dark-field microscopy. In contrast to previously published studies, the monolayer experiments used the entire hydrophobic surfactant protein fraction (containing both the SP-B and SP-C peptides) at physiologically relevant concentrations (~1 wt %). Even at such low levels, the SP-B/C peptides induce the formation of a new phase in the surface monolayer that is of lower intrinsic order than the liquid condensed (LC) phase that forms in the pure lipid mixture. This presumably leads to a higher structural flexibility of the surface monolayer at high lateral pressure. Variation of the subphase pH indicates that electrostatic interaction dominates the association of the SP-B/C peptides with the lipid monolayer. As evidenced from dark-field microscopy, monolayer material is excluded from the DPPC/DPPG surface film on compression and forms three-dimensional, surface-associated structures of micron dimensions. Such exclusion bodies formed only with SP-B/C peptides. This observation provides the first direct optical evidence for the squeeze-out of pulmonary surfactant material in situ at the air-water interface upon increasing monolayer surface pressures.

INTRODUCTION

To facilitate breathing, the surface tension at the pulmonary air-alveolar interface in mammals must be reduced from 70 mN m^{-1} (the value at a pure A/W interface at 37°C) to near zero at low lung volumes (Schürch, 1982). In vivo, surface tension is regulated by pulmonary surfactant, a complex mixture of lipids and proteins produced by type II pneumocytes in the alveolar epithelium. Lung surfactant deficiency causes the RDS in premature infants (Avery and Mead, 1959), and lung surfactant inactivation has been implicated in the ARDS that can result from lung injury from a variety of causes (Bernard et al., 1994).

Lung surfactant secreted into the aqueous alveolar hypophase contains all the phospholipids and specific proteins associated with the surfactant system, from which a continuous film can rapidly adsorb at the air-alveolar interface (Haagsman and van Golde, 1991). As reviewed by a number of authors (e.g., van Golde et al., 1988; Notter and Wang, 1997; Goerke, 1998), the gross composition of typical mammalian surfactant isolates is ~85% phospholipid, 10% protein, and 5% neutral lipid. By far the major phospholipid class present is the phosphocholines (PCs), which include DPPC ~40% by weight plus a spectrum of PCs with mixed fatty chains (Kahn et al., 1995; Holm et al., 1996). Anionic phospholipids, including phosphoglycerols (PGs), comprise 10–15% of lung surfactant phospholipids. The protein fraction contains three biophysically active components designated as SP-A, SP-B, and SP-C, and a fourth surfactant protein of probable metabolic rather than biophysical significance has also been identified (Hawgood and Schiffer, 1991; Johansson et al., 1994). The biophysical interactions of SP-B and SP-C with phospholipids in films at the A/W interface form the emphasis of the current study.

SP-B and SP-C are of low molecular weight (~9 and 4 kDa of the monomers; see Note 1 at end of text) and hydrophobic in character (Johansson et al., 1994; Hawgood et al., 1998; Johansson, 1998); they are thought to be membrane-associated and are known to enhance the rate of adsorption of phospholipids from vesicles into the A/W interface (Oosterlaken-Dijksterhuis et al., 1991a, b). In addition, a number of studies have indicated that model phospholipid mixtures reconstituted with these hydrophobic surfactant proteins have excellent adsorption and dynamic surface tension-lowering ability that approaches that of natural pulmonary surfactant (Venkitaraman et al., 1990, 1991; Perez-Gil et al., 1992b; Wang et al., 1995; Pérez-Gil and Keough, 1998). Although significant research has addressed the biophysical interactions of lung surfactant phospholipids and hydrophobic proteins, the effects of SP-B/C on film structure, phase behavior, and refinement during cycling is still not fully understood.

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Abbreviations used: A/W, air-water; (A)RDS, (acute) respiratory distress syndrome; chl/meth, chloroform/methanol; (C)LSE, (calf) lung surfactant extract; 2-D, 3-D, two-, three-dimensional; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; LE, LC, liquid-expanded, liquid condensed monolayer phase; NBD-DMPE, nitrobenzoxadiazol-1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamin; PA, palmitic acid; PL-A_2, phospholipase A_2; SP-B/C, hydrophobic peptide fraction isolated from pulmonary surfactant, composed of surfactant peptides B and C.

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Because pulmonary surfactant lowers surface tension in a 2-D dynamically compressed monomolecular film at the alveolar A/W interface in vivo, Langmuir monolayers provide a highly relevant experimental system for investigating the roles of various system components and their interaction (Notter, 1984; Goerke and Clements, 1986). The power of film balance studies of cycled interfacial films has more recently been aided by the development of new, highly sensitive analytical instrumental methods capable of sophisticated and detailed analysis of monolayers. A variety of these surface-sensitive methods have now been applied to the study of surfactant monolayer model systems, including infrared spectroscopy (Dluhy et al., 1989; Pastrana-Rios et al., 1991, 1994, 1995; Gericke et al., 1997), fluorescence and Brewster angle microscopy (Perez-Gil et al., 1992a; Nag and Keough, 1993; Nag et al., 1996a, b; Discher et al., 1996; Lipp et al., 1996, 1997; Amrein et al., 1997), and scanning probe microscopy (Panaiotov et al., 1996; von Nahmen et al., 1997; Amrein et al., 1997).

In particular, fluorescence microscopy has proven useful in the study of surfactant monolayer model systems. For example, the mixing behavior of a series of binary and ternary model lipid systems composed of PC/PG mixtures in the presence and absence of calcium has been studied using monolayer fluorescence microscopy (Nag and Keough, 1993; Nag et al., 1994). In another series of experiments, fluorescein-labeled SP-C has been used to study the lipid packing rearrangements by the peptide in spread monolayers (Perez-Gil et al., 1992a; Nag et al., 1996a). The other major surfactant hydrophobic peptide, SP-B, has also been studied for its role in inducing morphological changes in lipid monolayers (Lipp et al., 1996, 1997). In addition, fluorescence and Brewster angle microscopy have recently been used to determine lateral phase separation in spread films of CLSE, a refined natural mixture containing the complete set of phospholipids, neutral lipids, and hydrophobic peptides (Discher et al., 1996).

As is well-documented by fluorescence (Lösche et al., 1983; Weis and McConnell, 1984) and Brewster angle (Hénon and Meunier, 1991; Hönig and Möbius, 1991) microscopy, the first-order phase transition in phospholipid monolayers is associated with mesoscopic (micron scale) phase separation between an isotropic fluid and an anisotropic hexatic phase (Lösche et al., 1988; Fischer et al., 1984; Kjaer et al., 1987), denoted as the LE and LC phases (Cadenhead et al., 1980). The inhomogeneities observed upon phase separation in lipid surface layers occur in a strictly monomolecular system (Helm et al., 1987; Vaknin et al., 1991), and the morphologies of the domains formed during the transition reflect a competition among the line tension between the phases, the electrostatic interaction, and chirality effects (Fischer et al., 1994; McConnell et al., 1986; Heckl et al., 1986; Mayer and Vanderlick, 1995).

Optical microscopy on phase-separated surface monolayers has also been used to extract information on peptide interactions with lipids in a specific phase state (Haas and Möhwald, 1989; Grainger et al., 1990; Reichert et al., 1992; Subirade et al., 1995; Diederich et al., 1996; Mi et al., 1997). In such work, the disintegration of LC domains within an LE matrix through PL-A_2 has been studied in detail (Grainger et al., 1990). It has been observed that PL-A_2, which hydrolyzes the *sn*-2 acyl ester bond of glycerophospholipids organized in membranes or surface monolayers, attacks the LC domains in a monolayer at points on their perimeter. Since this example of the phase-specific lipolytic action of PL-A_2 has so far been the only case study of the molecular details of LC domain disintegration by a protein, and since this disintegration occurs in a peculiar and characteristic mode, it has since been regarded as an epitome for such processes.

The work described in this communication utilizes epifluorescence and dark-field microscopy to study the effect of hydrophobic SP-B and SP-C peptides, isolated in the naturally occurring ratio from endogenous calf lung surfactant, on spread monolayers of phosphocholine and phosphoglycerol. Epifluorescence results indicate that at low levels (~1 wt %) the presence of the hydrophobic peptides induces the formation of a new monolayer phase that may be of lower intrinsic order than the lipid's LC phase formed in pure lipid monolayers upon increasing the monolayer pressure. In effect, this induces a softening of the lipid material that makes the monolayer more amenable to topological deformations. Dark-field microscopy results demonstrate that material is excluded from the surface layer and forms 3-D structures, preferentially at those loci where domains have been dissolved in the course of the phase transition.

MATERIALS AND METHODS

The synthetic phospholipids DPPC and DPPG were obtained from Sigma (Deisenhofen, Germany) at 99%+ purity, and used as received. Headgroup-labeled NBD-PE were from Molecular Probes (Leiden, The Netherlands). Chloroform was from Merck (Darmstadt, Germany) and methanol from Sigma. All organic solvents were of p.a. grade. Measurements were performed on aqueous subphases (water: 18.2 MΩcm, Millipore Milli-Q, Eschborn, Germany) that contained 120 mM NaCl (Fluka, Neu-Ulm, Germany) at room temperature. Subphases at pH 7 or 6.2 were buffered using K_2HPO_4/KH_2PO_4. Subphases at pH ~5.5 were obtained without buffering due to the equilibrium dissolution of atmospheric CO_2 in water, while those at pH ~3.5–3.8 and pH ~1.7–1.9 were adjusted using citric acid and HCl, respectively. All substances were of p.a. grade.

SP-B/C were co-purified by gel-permeation chromatography from CLSE. Whole lung surfactant was obtained by saline lavage of lungs from freshly killed calves, followed by centrifugation at low speed (250 × *g* for 5 min) to remove cells and debris and finally at higher speed (12,500 × *g* for 30 min) to pellet surface-active aggregates as described previously (Notter et al., 1983; Notter and Shapiro, 1987). Pelleted whole surfactant was then extracted in chl/meth (Bligh and Dyer, 1959) to obtain CLSE. Separation of SP-B/C from phospholipids in CLSE was performed by gel permeation column chromatography through LH-20 (Pharmacia-LKB Biotechnology, Piscataway, NJ) in 2:1 chl/meth as described (Hall et al., 1994). Final SP-B/C isolates had a minimal phospholipid content of 0.03 μg phospholipid per microgram of protein based on the phosphate assay of Ames (1966) and the protein assays of Lowry et al. (1951) and Kaplan and Pederson (1985). Both SP-B and SP-C were present as judged from SDS-PAGE, ELISA, and N-terminal amino acid analysis. Before use, the SP-B/C mixture was stored at low temperature in 1:1 chl/meth at a concentration of 455 mg/l. The weight ratio of 1% of the hydrophobic

peptide to lipid used in this study after reconstitution with DPPC and DPPG is of the same order as the content of the hydrophobic apoproteins in endogenous surfactant as measured by the same protein assay (Lowry et al., 1951).

Phospholipid samples were prepared by mixing of DPPC and DPPG in solutions of chl/meth (3:1) at the desired molar ratios; these mixed lipid solutions were doped with the fluorescent probe (0.5 wt % of the head-labeled NBD-DMPE with respect to total lipid) before spreading. Aliquots of SP-B/C in chl/meth were added to the lipids in 3:1 chl/meth and thoroughly mixed to obtain the desired final lipid/protein ratios. Lipid/peptide samples with lipid concentrations of typically 0.3 mg/ml were applied to the surface of the Langmuir trough using a microliter syringe, followed by at least 10 min equilibration for solvent evaporation.

The Langmuir film microscope consists of a commercial epifluorescence microscope (Axiotech^{vario}, Carl Zeiss Jena, Germany) arranged on top of a Langmuir trough of local design with a maximum area of ~250 cm^2. The trough may be moved relative to the position of the objective lens with an x-y stage (Märzhäuser, Wetzlar, Germany) to select the observed area and to compensate for drifts in the film. The entire optical setup and film balance were placed on an active vibration isolation unit (JRS, Affoltern, Switzerland). The film is illuminated using a 50 W mercury arc lamp and the wavelength is selected by an appropriate beam splitter/filter combination (Zeiss filter set 9). To detect mesoscopic imperfections associated with the surface monolayer we have illuminated the surface with a red He-Ne laser at grazing incidence. In this illumination mode, the light scattered from 3-D structures is collected through the objective lens together with the fluorescent image. Images were observed using a 50× long-distance objective lens (Epiplan 50 LD, Carl Zeiss Jena). To bring the fluorescence and scattered light signals to comparable levels, the laser has been attenuated to <3 mW. Micrographs were recorded with an SIT camera (C2400−08, Hamamatsu, Herrsching, Germany) connected to the microscope by a 1× camera adapter. Pictures are stored directly into computer memory via an online image processor (Argus-20, Hamamatsu). The resolution of the images is 928 × 680 pixels. All images presented show selected areas of the contrast-enhanced raw data.

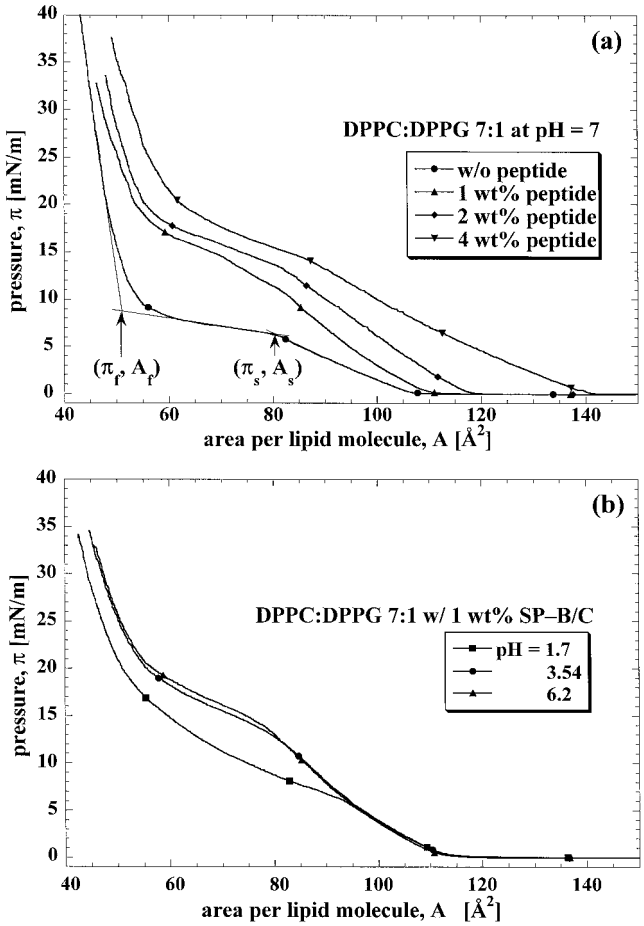

FIGURE 1 Pressure-area isotherms ($\vartheta = 22 \pm 2$°C) of surface monolayers of lipid mixtures (DPPC/DPPG 7:1, mol/mol) on 120 mM NaCl. (*a*) various concentrations of SP-B/C at pH 7; (●) binary lipid monolayer; (▲) DPPC/DPPG with 1 wt %; (◆) 2 wt %; (▼) 4 wt % SP-B/C. (*b*) DPPC/DPPG with 1 wt % SP-B/C on a subphase at (■) pH 1.7, (●) pH 3.54, and (▲) pH 6.2.

RESULTS

Isotherms

Both DPPC and DPPG show room temperature monolayer isotherms with characteristic first-order transitions from the disordered (with respect to conformations of the lipid's alkyl chains) LE phase to the ordered LC phase (Albrecht et al., 1978; Lösche et al., 1983). The phase transition onset pressure, π_s, at a given temperature is a characteristic quantity for a given lipid in a monolayer on a particular subphase. For DPPC on pure water, π_s ~4 mN m^{-1} at ϑ = 20.5°C and shifts by $\partial\pi_s/\partial\vartheta \sim +1.5$ mN m^{-1} K^{-1} at room temperature until at ϑ_t ~43°C a tricritical point is reached (Albrecht et al., 1978). On 120 mM NaCl, the ionic strength used in this study, π_s is shifted to ~9.5 mN m^{-1} and 12.5 mN m^{-1} at 20°C for DPPC and DPPG, respectively. Qualitatively, the binary mixture DPPC/DPPG (7:1 mol/mol), which was investigated as a model lipid matrix in this study, shows a room temperature isotherm that closely resembles the isotherms of the pure compounds (Fig. 1). This figure also illustrates that quite small amounts of SP-B/C exert a pronounced influence upon these isotherms. Fig. 1 *a* shows that just 1 wt % of the peptide mixture broadens the phase transition considerably and shifts the transition pressure by ~5 mN m^{-1}. The addition of 2 wt % of the SP-B/C peptide leads to a further broadening and a further shift by ~2 mN m^{-1}. Further increase of the SP-B/C concentration (e.g., 4 wt % and beyond) leads to an expansion of the monolayer, possibly indicative of incorporation of peptide assemblies into the lipid. However, the monolayer pressure increase does not keep abreast with the peptide concentration, sloping off beyond ~4 wt %. After the completion of the phase transition, at π_f ~18 mN m^{-1} in the lipid monolayers with peptide, the compressibility of the surface films is largely enhanced by the peptide at 1 wt % and even more so at 2 and 4 wt % over that of the pure lipid monolayer.

The isotherms indicate a strong interaction of the peptide with the phospholipid that saturates at rather low concentrations. This interaction is at least partially electrostatic in its nature. Fig. 1 *b* shows the pH dependence, between pH ~1.75 and 7, of room temperature isotherms of the phospholipid mixture with 1 wt % of SP-B/C incorporated. Whereas above pH 3.5 isotherms coincide essentially, the isotherm at the lowest pH (below the pK of the PG headgroup) indicates a significant condensation of the mixed monolayer. Since a similar pH dependence is, however, also

observed with the phospholipid mixture without surfactant peptide, it remains unclear from the isotherms whether or not the peptide-lipid interaction is affected by the protonation of the PG headgroup.

Fluorescence images of DPPC/DPPG (7:1) + 1 wt % SP-B/C

As shown in Fig. 2, mixed phospholipid monolayers (DPPC/DPPG, 7:1 mol/mol) on 120 mM NaCl solution, pH 6.2, develop phase-separated LC domain structures in a qualitatively similar fashion as reported for pure phospholipids (Lösche et al., 1983, 1988; Nag and Keough, 1993). These domain structures appear first near the kink in the isotherm at $\pi_s \sim 7$ mN m^{-1}. As π increases, electrostatic repulsion between the charged DPPG headgroups, shielded by counterions to form molecular dipoles perpendicular to the surface whose length is determined by image charges and Debye screening, leads to the elongation of the boundary lines between the coexisting phases. As a result, pointed protrusions form at the apices of the domain's lobes (see Fig. 2, image at 17.6 mN m^{-1}). Starting at $\sim$16 mN m^{-1}, the images lose their crispness and the visual impression is a progressive blurring of the domain boundaries. Above 19 mN m^{-1}, only very little contrast remains in the images. The observed blurring may be caused by dissolution of dye into the dye-depleted regions in the monolayer after the termination of the phase transition, i.e., when both the dye-enriched and the dye-depleted regions have become the same phase (see Note 2).

Close to the onset of the phase transition in PC/PG monolayers with low concentrations of SP-B/C, domains of the ordered phase form in a fashion similar to those in pure binary lipid monolayers. For DPPC/DPPG (7:1) + 1 wt % SP-B/C on 120 mM NaCl solution at pH 6.2, Fig. 3 shows domains formed at π_s (which is, however, higher than in the pure lipid monolayer, cf. Fig. 1 *a*). They appear similar to those depicted in Fig. 2 (e.g., image at 7 mN m^{-1}). Significant differences in the fluorescent images of this monolayer, however, begin to appear at $\sim$14 mN m^{-1}, which is a surface pressure close to the midpoint of the phase transition. As shown in Fig. 3 (images taken above 14.0 mN m^{-1}), the solid domain dissolves under the influence of the SP-B/C peptides. Beginning at their periphery, solid (i.e., dye-depleted) lipid is dispersed from the compact cores of the domains and is extruded into the dye-enriched phase. This leads to the formation of branches that appear at an intermediate gray level and emanate outward preferentially from points on the perimeter where hexagonal domain faces meet at 60° angles (see insert in the upper middle image in

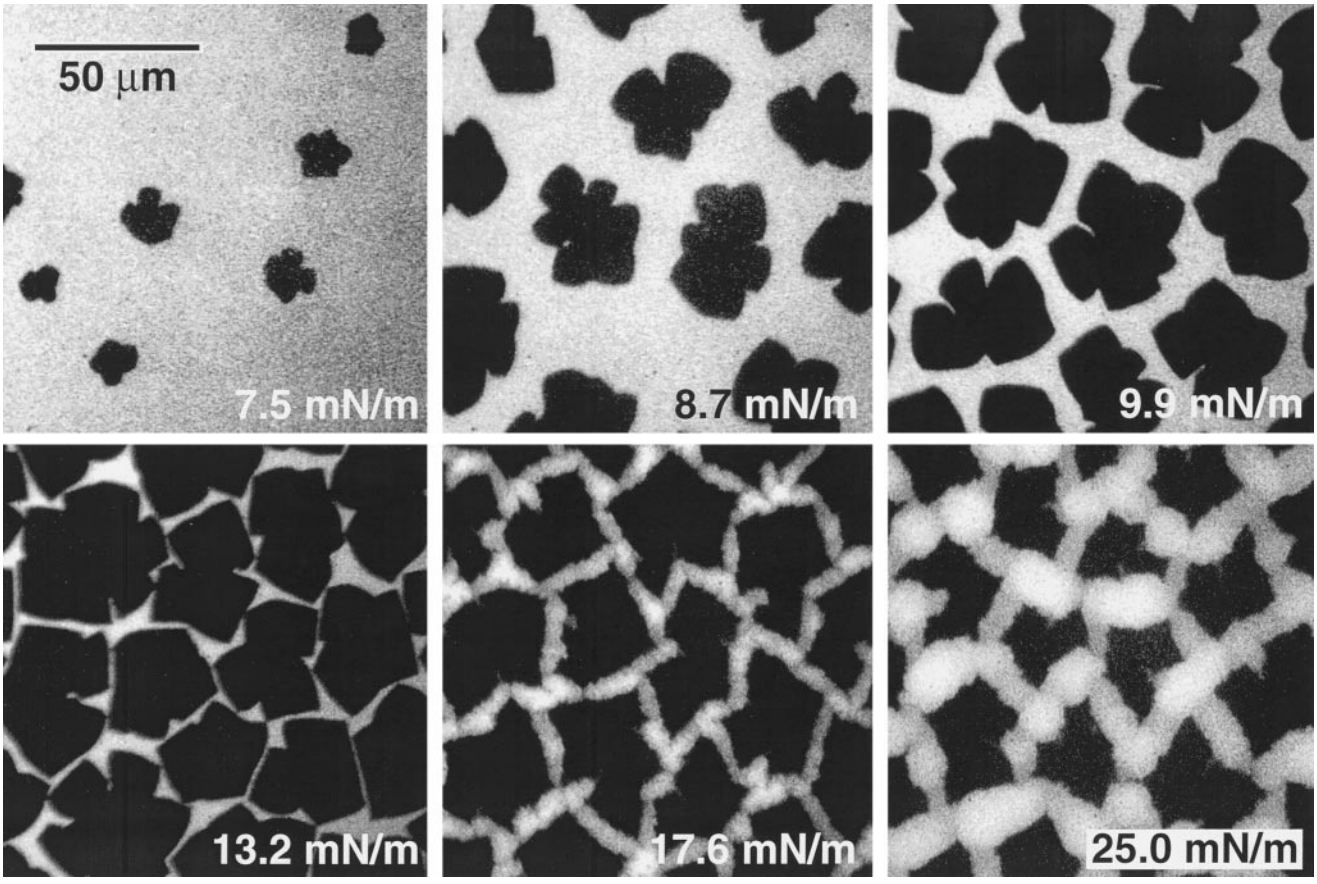

FIGURE 2 Fluorescence micrographs of a monomolecular film at the A/W interface containing a 7:1 (mol/mol) binary mixture of DPPC/DPPG. Images are shown at increasing surface pressures as indicated. The bar at the upper left is 50 μm.

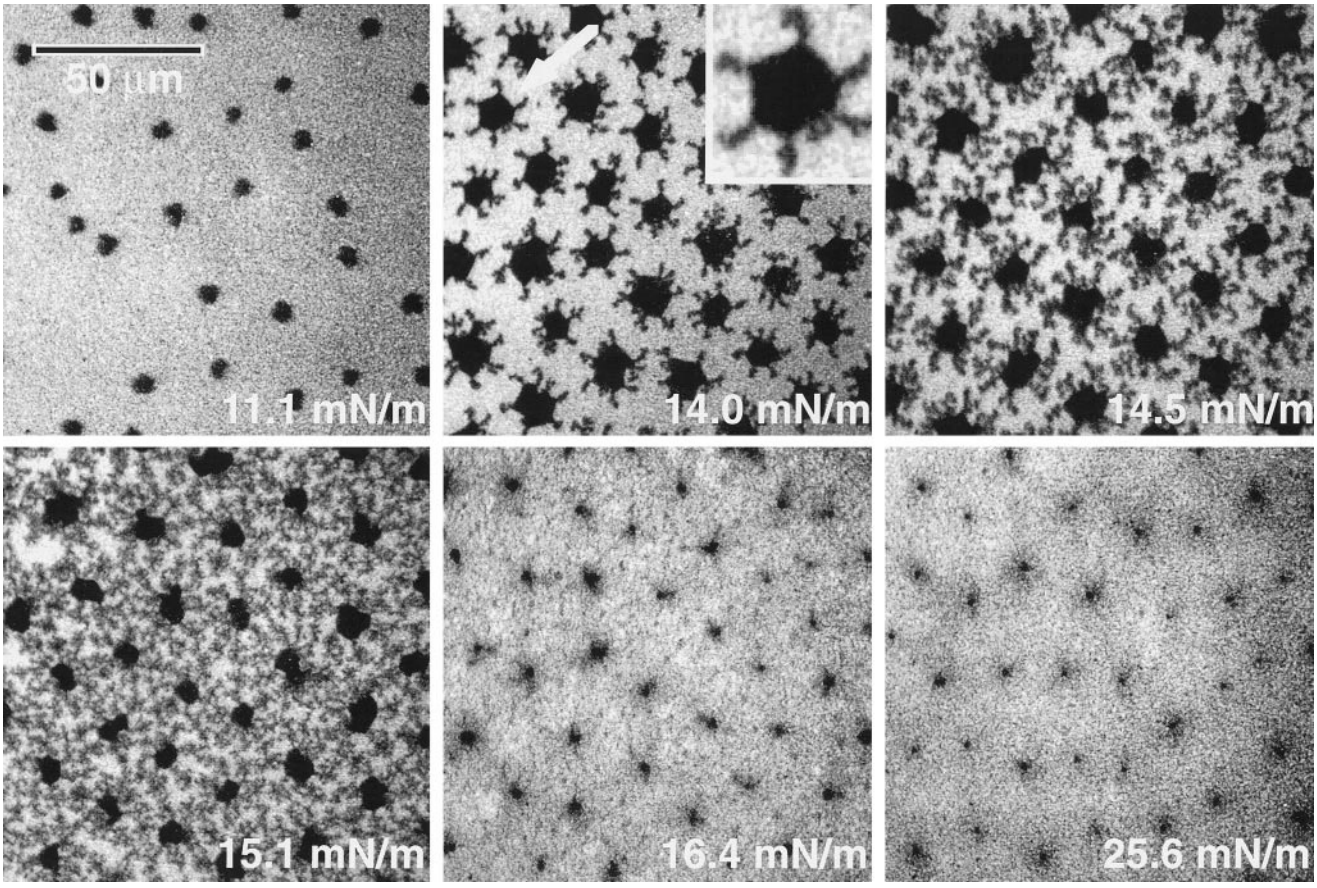

FIGURE 3 Fluorescence micrographs of a monomolecular film at the A/W interface containing a 7:1 (mol/mol) mixture of DPPC/DPPG with 1 wt % SP-B/C surfactant peptides. Images are shown at increasing surface pressures as indicated. The bar at the upper left is 50 μm.

Fig. 3, taken at 14.0 mN m^{-1}, where the domain indicated by the arrow in the main panel is shown at 3× magnification). The dissolved material is thus not uniformly redistributed into the liquid, but rather forms a new phase that grows at the expense of both the LE and LC phases. This new phase appears at a gray level intermediate to those of LC and LE. Within a narrow pressure range (14–15 mN m^{-1}), the branches that constitute the new phase elongate and at the same time contract in their lateral dimension, while tip splitting occurs upon growth into the liquid phase. These phenomena, tip splitting (Fischer et al., 1984; Miller et al., 1986; Buka and Palffy-Muhoray, 1988) and lateral contraction upon growth (Gaub et al., 1986; Heckl et al., 1986), are frequently observed in directional structure formation processes in organic solids, both in bulk (Langer, 1980) and in molecular films (Möhwald, 1986). Above 15 mN m^{-1}, the dissolved lipid forms a delicate meshwork that covers the entire area between the cores of the solid domains, while the ordered phase is dissolved until only micron-sized grains remain. These are stable up to high lateral pressures (see images taken at 25.6 mN m^{-1} in Fig. 3; above 25 mN m^{-1}, the morphologies do not visibly change their appearance up to at least 50 mN m^{-1}; see Note 3). One effect of the SP-B/C mixture is thus to dissolve the ordered phase of the lipid and induce the formation of a distinct new phase that dissolves the lipophilic dye better, and is hence presumably of lower intrinsic order.

We have analyzed the gray level distribution images shown in Figs. 2 and 3 in the form of histograms and have thus followed the evolution of the coexisting phases identified in these images as a function of the progress of the phase transition. For this purpose, we have defined a reduced transition pressure that takes on values between 0 and 1 during the progress of the phase transition:

$$\pi_n = \frac{\pi - \pi_s}{\pi_f - \pi_s}.$$

In this equation, π is the measured surface pressure, π_s is the surface pressure at the start of the transition, and π_f is the surface pressure at the end of the transition, as graphically determined from the intersection of the extrapolated slopes of the linear portions in the isotherm during and beyond a phase transition, cf. Fig. 1 *a*. Fig. 4 shows the area fraction of the bright, dark and, in the case of peptide interaction with the lipid, gray phases plotted against π_n. These area fractions were obtained from the background-corrected micrographs by integration over Gaussians fitted to the histograms. Fig. 4 *a* shows area fractions calculated from images of a lipid-only DPPC/DPPG binary mixture,

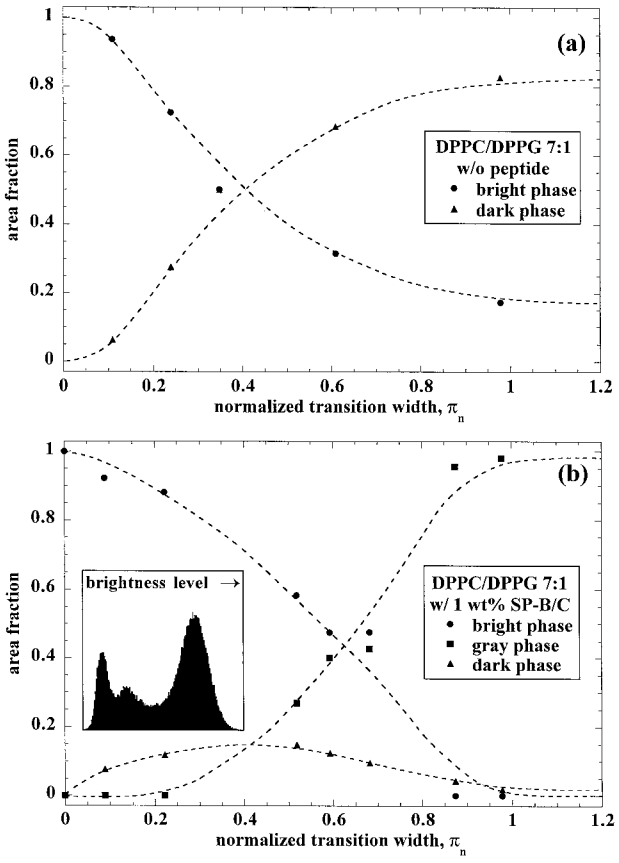

FIGURE 4 (*a*) Area fraction of the bright and dark phases as taken from histograms of the fluorescence micrograph images of a 7:1 (mol/mol) binary mixture of DPPC/DPPG. Representative images of the mixture from which these area fractions have been calculated are shown in Fig. 2. The area fractions of the bright (liquid) phase (●) and the dark (solid) phase (▲) are plotted against π_n, the reduced transition pressure. (*b*) Area fraction of the bright, dark, and intermediate phases as taken from histograms of the fluorescence micrograph images of a 7:1 (mol/mol) mixture of DPPC/DPPG with 1 wt % SP-B/C surfactant peptide. Representative images of the mixture from which these area fractions have been calculated are shown in Fig. 3. The area fractions of the bright (liquid) phase (●), gray ("intermediate") phase (■), and the dark (solid) phase (▲) are plotted against π_n.

some of which are shown in Fig. 2. In this binary lipid mixture, only two phases, bright and dark, are observed, corresponding to the LE matrix and LC domains. As the phase transition proceeds, it is evident that the bright (LE) phase decreases in proportion to the growth of the dark (LC) phase, as expected.

In a similar vein, Fig. 4 *b* shows area fractions calculated from images of a lipid-peptide complex of DPPC/DPPG (7:1) with 1 wt % SP-B/C incorporated, some of which are shown in Fig. 3. In this lipid-peptide complex, three gray levels (bright, dark, and medium, see sample histogram in the inset deduced from the image, Fig. 3 at $\pi = 14.5$ mN m^{-1}) are distinguished, corresponding to the liquid, solid, and "intermediate" phases. The difference in the evolution of the phases between the pure lipid film (Fig. 4 *a*) and the film incorporating the peptide (Fig. 4 *b*) is striking. Although the area fraction of the liquid phase decreases through the transition, very little solid phase is formed. Rather, the predominant product of the transformation is the "intermediate" phase. It is also evident from Fig. 4 *b* that the evolution of the gray areas in the monolayer has features similar to that of a phase transition.

We also studied under the fluorescence microscope model surfactant monolayers with larger SP-B/C concentrations, e.g., 4 wt % of the peptide mixture in DPPC/DPPG (7:1) (data not shown). In such experiments, we observed a qualitatively similar development of the phase morphologies as shown in Fig. 3. However, image contrast was invariably very faint, so that a quantitative comparison is difficult to obtain. Generally, dark LC domains were at best observed only in a narrow area interval of the π-A curve, cf. Fig. 1. After the formation of small area fractions of the LC domains, these were observed to dissolve quickly. Thereafter, the monolayer retained a fuzzy, nondescript appearance, roughly comparable to the morphology shown in Fig. 2 B, panel C of Nag et al. (1996a), however, with much less contrast.

The pH dependence of the SP-B/C impact on the phase behavior of the DPPC/DPPG monolayer is illustrated in Fig. 5. This figure shows fluorescence micrographs as a function of pH of lipid-only binary monolayers (*upper three panels*) obtained at comparable surface pressures ($\pi = 15 \pm 2$ mN m^{-1}). In addition, fluorescence micrographs of a mixed lipid monolayer with 1 wt % SP-B/C incorporated, also obtained at $\pi = 15 \pm 2$ mN m^{-1}, are shown in the three bottom panels. Whereas the formation and persistence of ordered domains in monolayers without peptide does not depend on the pH, it is clear that the presence of ordered phase areas is sensitive to pH in the presence of the peptide. At pH 1.9, domain morphologies are essentially independent of the presence of the peptide, as it is seen that solid domains do persist in either case, with or without peptide. At pH 6.2, however, the solid domains are almost entirely dissolved at 15 mN m^{-1} in the presence of SP-B/C. At intermediate pH values, the dissolution of the solid domains in the presence of the peptide is still incomplete (*lower middle panel*, pH 3.6). Thus, the action of the peptide depends critically on pH, and may be related to either the deprotonation of the acidic amino acid residues or of the acidic lipid headgroups. In this context it is worth noting that the pK of PG in bilayer membranes is close to 3 (Cevc and Marsh, 1987).

To resolve the question whether or not squeeze-out of monolayer material occurs at high lateral pressure, we have investigated surface monolayers, with and without SP-B/C, using dark-field microscopy with side-illumination as described in the Materials and Methods section. Fig. 6 shows representative fluorescent and scattered light images of a lipid-peptide monolayer film of DPPC/DPPG (7:1) with 1 wt % SP-B/C at two pH values; all images were obtained at comparable surface pressures ($\pi \sim 25$ mN m^{-1}). On the left side, the figure presents images of the lipid-peptide monolayers collected in fluorescence mode only. In addition, the

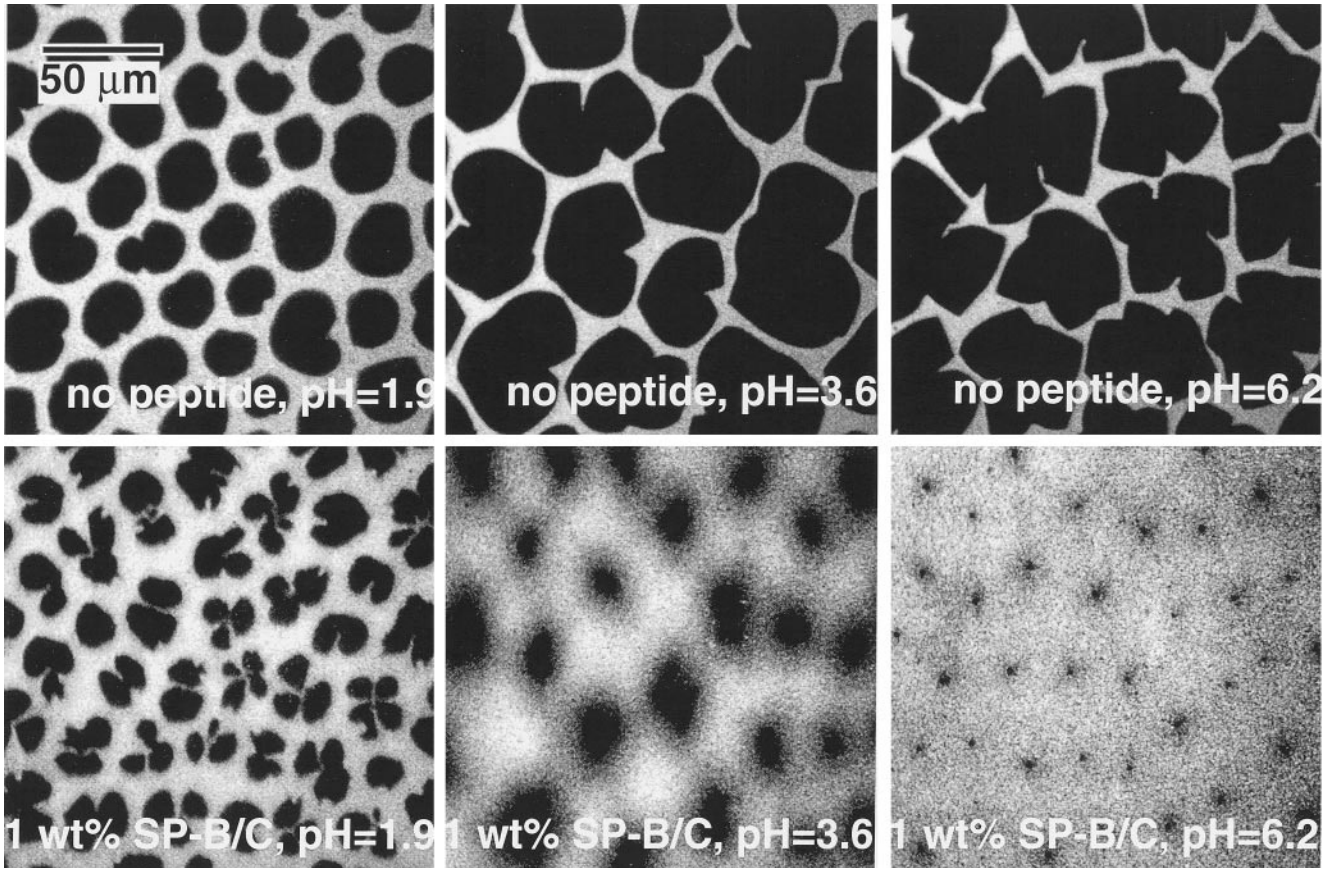

FIGURE 5 Fluorescence micrographs illustrating the effect of pH and SP-B/C surfactant peptide on the evolution of the solid lipid phase. The upper three images are of a 7:1 (mol/mol) binary mixture of DPPC/DPPG (without SP-B/C peptide), with pH values as indicated. The bottom three images are of a 7:1 (mol/mol) mixture of DPPC/DPPG with 1 wt % SP-B/C at the same pH values as with the pure lipid monolayers. Each of the six images presented in this figure was acquired at a comparable lateral surface pressure (15 ± 2 mN m^{-1}). The bar at the upper left is 50 μm.

right side shows images in which both fluorescent light and scattered light from mesoscopic 3-D exclusions from the lipid-peptide monolayer are collected simultaneously. Particularly at pH 6.2 it is obvious that the whole monolayer surface is studded rather homogeneously with small, interface-associated objects that scatter the grazingly incident light. The exclusion particles form upon compression of the lipid/peptide surface layer beyond $\sim$20 mN m^{-1} after the solid phase has been largely dissolved. In comparison, if side illumination is applied to pure lipid monolayers, no scattered light intensity is observed at any pressure. Similarly, we have not observed such exclusions in peptide/lipid surface layers at pH 1.9.

The size of the objects giving rise to the scattered light is 1 μm in diameter or smaller and is presumably below the optical resolution of the instrument. It is interesting to note that the bright exclusions are precisely in focus only if the image area is slightly elevated, by $\sim$1 μm, above the level of the monolayer. This indicates that the exclusions form elevations on top of the monolayer rather than depressions into the subphase. These exclusions are stable and do not visibly change their morphology up to high surface pressures (50 mN m^{-1}).

DISCUSSION

One of the paradigms of pulmonary surfactant research over the last few decades has been the hypothesis that successive inhalation-exhalation cycles of the alveoli causes the surfactant to undergo refining such that the surface film is enriched in one component (Hildebran et al., 1979; Goerke and Clements, 1986). The so-called "squeeze-out" hypothesis suggests that, in order to achieve low surface interfacial surface tension, the monomolecular film becomes enriched in DPPC. Since the surface tension at the air/alveolar interface approaches values near 0 mN m^{-1} (Schürch et al., 1978), corresponding to a surface pressure $\pi \sim$70 mN m^{-1}, and since DPPC is capable of withstanding high surface pressure values without collapse in vitro in Langmuir film balance experiments, it has been argued that a DPPC-rich interface, produced in vivo upon alveolar compression (Clements, 1977), is a prerequisite for proper functioning of the pulmonary system. Although widely accepted, this hypothesis has proven very difficult to verify. Dynamic cycling experiments have provided some indirect evidence for the selective elimination of unsaturated lipids and the enrichment of the DPPC component (Nag and Keough, 1993;

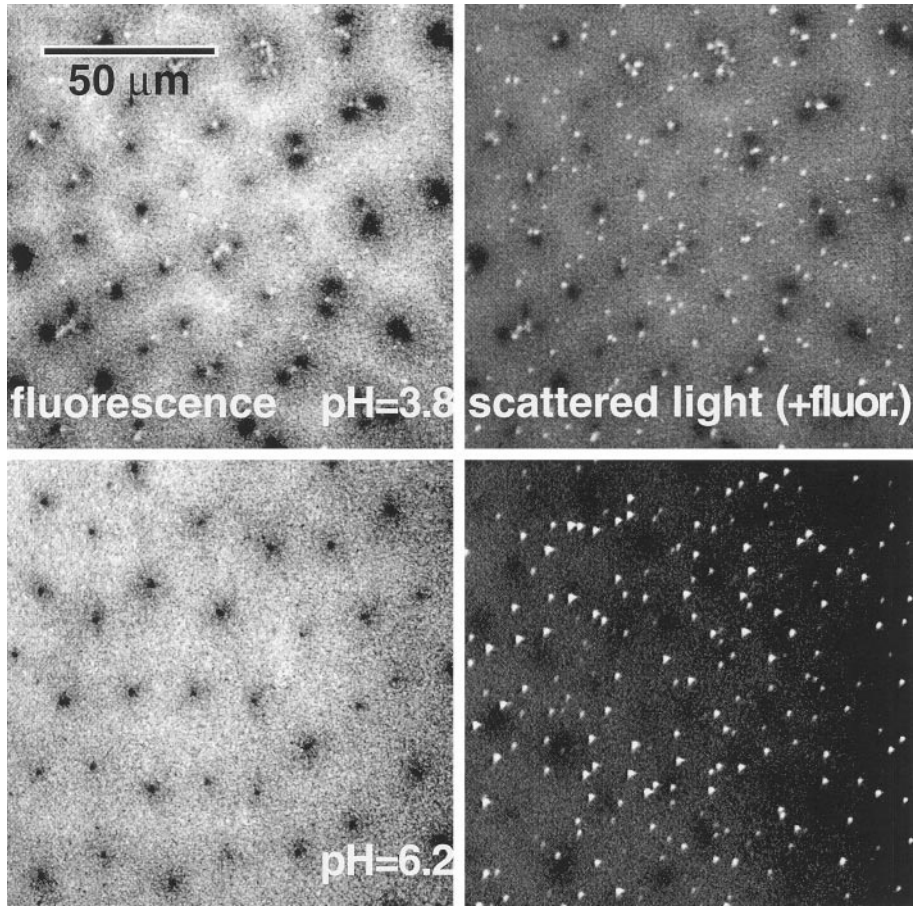

FIGURE 6 Fluorescence micrographs and optical micrographs visualizing scattered light from 3-D exclusion bodies adhering to a mixed monolayer (DPPC/DPPG 7:1, mol/mol) with 1 wt % SP-B/C peptide at pH~3.8 (*upper images*) and pH~6.2 (*lower images*). The images on the left were acquired in fluorescence mode while in the images on the right scattered light and fluorescence emission were simultaneously collected. The white dots apparent in the images on the right arise from interface-associated structures that are 1 μm in size or less. Each of the four images presented in this figure was acquired at a comparable lateral surface pressure (π ~25 mN m^{-1}). The bar at the upper left is 50 μm.

Schürch et al., 1994). In addition, it has been suggested that the hydrophobic surfactant peptides may play a pivotal role in this surface refinement process, with SP-B and SP-C being differently excluded from monolayer films (Nag et al., 1997). Another reason for the formation of 3-D exclusion bodies adhering to the surface monolayer may be a reduction of the alveolar/air interface area upon exhalation below the physical limit imposed by the amount of material deposited in the surface monolayer during inhalation.

A variety of fluorescence monolayer studies previously appearing in the literature have attempted to define the roles of the hydrophobic proteins SP-B and SP-C in pulmonary surfactant function. The current fluorescence and grazing-incidence optical microscopic study of monolayer films differs from these previously published articles in several important respects. First, the current work uses the mixed hydrophobic peptide fraction containing SP-B and SP-C co-purified in chl/meth from whole surfactant in lung lavage. Second, the film studies here utilize low protein contents of 1%, the same order as the hydrophobic protein content found in natural surfactant (Hawgood and Schiffer, 1991; Johansson et al., 1994; Wang and McGuire, 1997) and substantially lower than used in many prior model studies in vitro (Perez-Gil et al., 1992a; Nag et al., 1996a, b, 1997; Lipp et al., 1996, 1997). Our optical observations at 4 wt % protein suggest that one may miss basic features of the peptides' impact on the lipid phases by using unphysiologically high concentrations. The approach of determining the effects of mixed SP-B/C on phospholipid films with the proteins in their endogenous ratio and content enhances the relevance of our results for pulmonary surfactant films in vivo. Focusing on the specific effects of either hydrophobic surfactant protein separately, while important, may miss critical interactions present in the native material due to the combined biophysical effects of the two peptides together.

Several previous reports (Oosterlaken-Dijksterhuis et al., 1991a, b, 1992) have demonstrated that SP-B and SP-C act together to mediate both lipid mixing and protein insertion into monolayers. In addition, films of lung surfactant extracts that incorporate all the lipids and hydrophobic proteins in native surfactant have been studied by both fluorescence and Brewster angle microscopy (Discher et al., 1996; Nag et al., 1998). These studies have determined that a lateral phase separation occurs in surfactant extract films with the formation of condensed domains upon monolayer compression. The present study extends these findings and quantitates the effects of mixed SP-B/C on phospholipid phase transitions and film morphology as determined both from fluorescence and dark-field microscopy (Figs. 3–6).

Several previous reports have investigated the role of SP-B or SP-C in monolayer surface refinement. One fluorescence study observed collapse structures when 20 wt %

of the positively charged synthetic 25-amino acid N-terminal peptide fragment of SP-B was added to negatively charged PA monolayers (Lipp et al., 1996). However, PA itself nucleates and forms collapse structures, unlike the DPPC/DPPG monolayers that are physiologically more relevant. The effect of the synthetic SP-B peptide fragment on PA was to stabilize monolayers against collapse up to a higher pressure value than observed with the pure PA and to induce a more uniform distribution of the collapse structures. Recent work that studied the impact of fluorescently labeled SP-B and SP-C on phospholipid monolayers also implicated the peptides in monolayer surface refinement (Nag et al., 1997), although no direct evidence of 3-D surface-associated exclusion structures was presented. The exact composition of the monolayer at high pressure was not identified from the images; however, it was estimated that some fraction of both the SP-B and SP-C remained in the monolayer.

Similarly, a variety of studies have attempted to define the impact of SP-B and SP-C on lipid monolayers in the low-pressure regime, i.e., before monolayer collapse. Several of these studies have specifically looked at the role of SP-C on the formation and morphology of solid domains in the monolayer (Perez-Gil et al., 1992a; Nag et al., 1996a, 1997). It was reported that SP-C altered the lipid packing in the monolayer, resulting in both a reduced size and increased number density of condensed-phase lipid domains. For monolayers containing SP-B it was shown that the surfactant peptide inhibits the formation of condensed domains, both in phospholipid and fatty acid monolayers, and produces a distinct morphology at high surface pressures (Lipp et al., 1996; Nag et al., 1997). Finally, whole LSE (incorporating all the native surfactant lipids and hydrophobic proteins) has been studied with both fluorescence and Brewster angle microscopy (Discher et al., 1996; Nag et al., 1998). These studies have determined that lateral phase separation occurs in the native material with the formation of condensed domains upon monolayer compression. Furthermore, the latter study has also reported the appearance of "thin black filamentous regions" upon the dissolution of the solid phase at high pressures, above $\pi \sim 45$ mN/m, which obviously describes a transformation that may be similar to the one reported here. From the data presented in that publication, it remains, however, unclear whether or not the two morphologies are identical.

The fluorescence images presented here demonstrate that, when studied in combination, the hydrophobic surfactant peptides SP-B/C have pronounced and marked effects on monolayer morphology already at physiological concentrations. In particular, Fig. 3 suggests the formation of a new phase upon SP-B/C interaction with the mixed phospholipid monolayer. The transition that leads to the formation of this new phase is not recognized in the isotherm, which may be due to the fact that it interferes with the well-known LE/LC phase transition of the phospholipid. However, the material that constitutes the gray areas within the monolayer is clearly distinct from both the disordered fluid phase, which dissolves the fluorescent label well and thus appears bright in the micrographs, and the ordered solid phase, which has a low solubility for the dye and thus appears dark. The new phase forms by dissolution of solid material from the LC phase and does not simply mix with the LE phase (cf. Fig. 3, images taken at 14.5 and 15.1 mN m^{-1}). Moreover, it exhibits growth patterns similar to those observed with other true phases in different monolayer systems (Miller et al., 1986; Gaub et al., 1986), such that the designation as a new phase seems appropriate.

It is easy to speculate, but difficult to assess experimentally, what the composition of this new phase is. Since it occurs only in the pseudo-ternary system DPPC/DPPG SP-B/C and is sensitive to pH changes it seems quite safe to speculate that the surfactant peptide is one component of this "intermediate" phase which interacts, presumably electrostatically, with the acidic PG lipid. Most remarkably, and most importantly in the context of the physiological action of pulmonary surfactant, is that the new phase is obviously more susceptible to plastic deformation than the peptide-free ordered LC monolayer phase. It seems thus that the interaction of the surfactant peptides with the lipids does indeed lead to a softening of the monolayer material, which has been postulated to be one major role of the peptide in the system. For example, a fluidizing effect of the peptide on the condensed phase in (similar but not identically composed) phospholipid monolayers has been observed with fluorescently labeled SP-B and SP-C peptides (Nag et al., 1996a, 1997), as well as with SP-B and its synthetic N-terminal peptide fragment in PA monolayers (Lipp et al., 1996).

One has to keep in mind, though, that the experiments reported here and in earlier related studies have generally not been performed at physiological temperature. The tricritical point in DPPC monolayers for the separation of the LE and LC phases, located at $\vartheta_t \sim 43°C$ (Albrecht et al., 1978), is, however, well above the physiological temperature. Consequently, nucleation and growth of the rather brittle LC phase will also occur in monolayers with a high DPPC content under physiological conditions. The presence of solid LC domains could then potentially pose a serious problem for respiratory function, which depends on a dynamic response of the alveolar lining to inhalation-exhalation cycles. Therefore, the observation of a distinct new phase with high plasticity is direct experimental evidence that confirms one of the putative roles of the hydrophobic pulmonary surfactant peptides, namely softening of the monolayer material lining the alveolar surface. This may be crucial for the functioning of the system.

Previous work has indicated the possibility of ionic interaction between the SP-B peptide and anionic lipids (Longo et al., 1993; Lipp et al., 1996). Fluorescence micrographs and monolayer isotherms as a function of pH reported in this study clearly show that electrostatic interaction is critical for the functioning of the surfactant system. Fig. 5 in this work clearly shows the impact of the SP-B/C peptides on the DPPC/DPPG binary monolayer morphology

as a function of pH. At pH 1.9 the LC development and the phase morphology across the LE/LC phase transition is essentially identical, regardless of whether SP-B/C peptides are present or not. Only when the subphase pH is raised to 6.2, which is a value well above the pK of the PG headgroup, does the monolayer exhibit the distinct new monolayer phase. This pH dependence strongly suggests that the interaction of the surfactant hydrophobic peptides is only partially a hydrophobic effect. The data demonstrate that an electrostatic component is also required for the formation of a physiologically relevant supramolecular complex.

The scattered light microscopy results reported in Fig. 6 provide the first direct optical evidence in situ for the presence of 3-D surface-associated particles resulting from "squeeze-out" of pulmonary surfactant from the monolayer. Similarly to the formation of the new monolayer phase, these structures appear only upon addition of SP-B/C peptides, in physiologically relevant concentrations, to the DPPC/DPPG surface monofilm; no such structures appear in the absence of the peptide. In addition, these structures appear only as the surface pressure is raised beyond $\pi \sim 20$ mN m^{-1}. The formation of these micron-sized exclusion bodies does again require electrostatic interactions at the molecular level, as no exclusions have been observed at pH 1.9, while the maximum area density of exclusions was observed at pH 6.2. These observations correlate with the putative electrostatic interaction between the SP-B/C peptides and the acidic phospholipid components, as described above and visualized in Fig. 5.

Although the surface-associated 3-D structures can be detected by visualizing the scattered light, this technique is incapable of identifying the composition of such structures. We have, however, performed a series of complementary experiments using external reflection infrared spectroscopy of DPPC/DPPG monolayer films containing SP-B/C peptides. This infrared method has allowed us to differentiate the effects of the hydrophobic peptides on the individual lipid monolayer components. The results from these experiments will be presented in a related article.

CONCLUSIONS

The current study of an in vitro model for the lining at the alveolar surfaces in mammalian lungs has revealed compelling evidence for two of the putative functions of this surfactant system that have been speculated upon in the literature for some time: a softening of the monolayer material, presumably essential for a high structural flexibility of the lining that the compressed surface monolayer constitutes, and surface refinement by means of squeeze-out of monolayer material upon compression. Fluorescence microscopy on mixed phospholipid monolayers showed that SP-B/C disrupts the ordered solid lipid domains that form upon compression across the lipid's main (monolayer) phase transition. This indicated that the peptide fraction acts in softening the lipid, which may be crucial for the performance of the material during the dimensional changes of the monolayer imposed during inhalation-exhalation cycles. Dark-field microscopy has for the first time provided direct optical evidence for the formation of 3-D exclusion bodies adhering to the monolayer at higher surface pressure. These particles are smaller than 1 μm in size (smaller than the optical resolution of the experiment) and are presumably located $\sim$1 μm above the monolayer in the air compartment of the sample chamber. The observation that both of these phenomena inferred by the hydrophobic surfactant peptide SP-B/C mixture in the phospholipid monolayers is strictly pH-sensitive has demonstrated that electrostatic interactions between the peptides and the lipids play an important role in determining the physiological properties of the complex surfactant mixture.

NOTES

1. The association state of, particularly, SP-B in its active state is still a matter of debate.

2. Note that accumulation of dye in the LE phase upon driving the monolayer through the phase transition leads to an increase of the phase transition temperature, equivalent to an increased local value of the transition pressure at which the dye-enriched areas transform into the ordered phase. After the global monolayer pressure has reached this local transition pressure value, both dye-enriched and dye-depleted areas have been transformed into the ordered phase and diffusion against the concentration gradient will redistribute the probe.

3. A surface pressure $\pi \sim 50$ mN m^{-1} is the maximum pressure sustained in the film balance used for this series of experiments.

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