Combinations of Fluorescently Labeled Pulmonary Surfactant Proteins SP-B and SP-C in Phospholipid Films

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ABSTRACT Hydrophobic pulmonary surfactant (PS) proteins B (SP-B) and C (SP-C) modulate the surface properties of PS lipids. Epifluorescence microscopy was performed on solvent-spread monolayers of fluorescently labeled porcine SP-B (R-SP-B, labeled with Texas Red) and SP-C (F-SP-C, labeled with fluorescein) in dipalmitoylphosphatidylcholine (DPPC) (at protein concentrations of 10 and 20 wt%, and 10 wt% of both) under conditions of cyclic compression and expansion. Matrix-assisted laser desorption/ionization (MALDI) spectroscopy of R-SP-B and F-SP-C indicated that the proteins were intact and labeled with the appropriate fluorescent probe. The monolayers were compressed and expanded for four cycles at an initial rate of 0.64 $Å^2 \cdot mol^{-1} \cdot s^{-1}$ (333 $mm^2 \cdot s \cdot {}^{-1}$) up to a surface pressure $\pi \approx 65$ mN/m, and π-area per residue (π-A) isotherms at 22 ± 1°C were obtained. The monolayers were microscopically observed for the fluorescence emission of the individual proteins present in the film lipid matrix, and their visual features were video recorded for image analysis. The π-A isotherms of the DPPC/protein monolayers showed characteristic "squeeze out" effects at $\pi \approx 43$ mN/m for R-SP-B and 55 mN/m for F-SP-C, as had previously been observed for monolayers of the native proteins in DPPC. Both proteins associated with the expanded (fluid) phase of DPPC monolayers remained in or associated with the monolayers at high π (~65 mN/m) and redispersed in the monolayer upon its reexpansion. At comparable π and area/molecule of the lipid, the proteins reduced the amounts of condensed (gel-like) phase of DPPC monolayers, with F-SP-C having a greater effect on a weight basis than did R-SP-B. In any one of the lipid/protein monolayers the amounts of the DPPC in condensed phase were the same at equivalent π during compression and expansion and from cycle to cycle. This indicated that only minor loss of components from these systems occurred between compression-expansion cycles. This study indicates that hydrophobic PS proteins associate with the fluid phase of DPPC in films, some proteins remain at high surface pressures in the films, and such lipid-protein films can still attain high π during compression.

INTRODUCTION

Pulmonary surfactant (PS) is found in the aqueous lining layer of the lung alveoli. It maintains lung stability and prevents alveolar collapse at end-expiration. PS is secreted in bilayer structures in the alveolar fluid and undergoes transformations to form a surface active monolayer or film at the air-water interface (Goerke and Clements, 1986). Although the exact nature of the surface film is currently unresolved, the film reduces the surface tension (γ) of the interface by counteracting the contractile forces of lung (Clements, 1977; Goerke and Clements, 1986; Keough, 1992). PS is rich in dipalmitoylphosphatidylcholine (DPPC), among other lipids, and it contains specific proteins called surfactant protein A (SP-A), B (SP-B), C (SP-C), and D (SP-D) (Possmayer, 1990). Current models of PS dynamics in vivo suggest that through a process of surface refining the film becomes DPPC-rich, and it is this film that reduces the interfacial tension of the interface to very low values at end expiration (Goerke and Clements, 1986; Keough, 1992). Although DPPC monolayer films can withstand high surface pressure (π) and allow low surface tension (γ), the rate of surface adsorption to form monolayers and the spreadability of phospholipids, especially those including high amounts of DPPC, at an air-water interface are low compared to those of natural PS (Notter et al., 1980; Snik et al., 1978). The hydrophobic PS proteins SP-B and SP-C enhance the surface-seeking properties of DPPC (Curstedt et al., 1987; Takahasi et al., 1990; Yu and Possmayer, 1990). Some lipid/protein systems can reproduce many of the surface properties of native PS in vitro (Notter et al., 1987; Sarin et al., 1990; Suzuki et al., 1986; Smith et al., 1988; Tanaka et al., 1986). Such lipid-protein mixtures have potential for use as artificial surfactants in PS replacement therapies (Smith et al., 1988; Suzuki et al., 1986; Tanaka et al., 1986) for treating patients with surfactant disorders and deficiencies, such as occur in respiratory distress syndrome. Furthermore, synthetic peptides from various structural domains of SP-B and SP-C in conjunction with DPPC can reproduce some of the surface properties of native PS (see McLean and Lewis, 1995, for review). From in vitro dynamic cycling experiments of monolayers containing DPPC and SP-B or SP-C, it was suggested that such films can easily attain high π. Some of the proteins get "squeezed out" of the monolayer at higher π, but because some small amounts of proteins may remain in the monolayers at high π, they could rapidly respread the lipid from

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highly compressed collapsed-phase states (Takahasi and Fujiwara, 1986; Taneva and Keough, 1994a; Yu and Possmayer, 1990).

SP-B and SP-C are highly conserved, extremely hydrophobic proteins that are extracted with the lipid components of PS. Although structure-function studies of SP-B and SP-C have been performed in solvents and lipid vesicles or dispersions (Baatz et al., 1991; Johansson et al., 1994; Morrow et al., 1993a,b; Pastrana et al., 1991; Shiffer et al., 1993), studies have also been performed in monolayers (Cochrane and Revak, 1991; Creuwels et al., 1993, 1995; Lipp et al., 1996; Longo et al., 1993; Nag et al., 1996a; Oosterlaken-Dijksterhuis et al., 1991a; Pastrana-Rios et al., 1995; Perez-Gil et al., 1992; Post et al., 1995; Taneva and Keough, 1994b,c,d). Monolayer studies with synthetic segments of SP-B suggested that amphipathic α-helices interact with anionic lipid headgroups (Lipp et al., 1996; Longo et al., 1993), and such segments are located mostly at the polar interfacial area of lipid monolayers (Cochrane and Revak, 1991). Other investigations have suggested that SP-C is oriented with its α-helix parallel to the plane of the air-water interface (Oosterlaken-Dijksterhuis et al., 1991b; Pastrana-Rios et al., 1995). Recently infrared spectroscopy of the proteins in DPPC monolayers indicated differential orientation and lipid association between SP-B and SP-C at the interface (Pastrana-Rios et al., 1995), supporting some previous suggestions made about such lipid-protein systems (Taneva and Keough, 1994d). Most of these monolayer studies were performed using solvent-spread lipid-protein films, which may be somewhat different from the alveolar PS film that is apparently formed by adsorption of material from below the surface. Recent evidence, however, suggests that lipid and lipid-protein films that are spread from solvents or adsorbed from liposomal suspensions are structurally similar or equivalent at similar packing densities (Nag et al., 1996b).

Epifluorescence microscopy of lipid-protein monolayers has become a convenient tool for studying protein distribution and lipid-protein interactions at the air-water interface (Ahlers et al., 1991; Lipp et al., 1996; Möhwald, 1990; see Stine, 1994, for a recent review). By labeling proteins and lipids with different fluorophores, researchers can observe individual partitioning and distribution of lipids or protein in monolayers in condensed or expanded phases (Ahlers et al., 1991; Dietrich et al., 1993; Heckl et al., 1987; Lipp et al., 1996; Möhwald, 1990). Protein-induced effects on lipid packing can be measured by estimating the amounts of each surface phase as a function of protein concentration at different monolayer lipid packing densities (Heckl et al., 1987; Nag et al., 1996a,b; Perez-Gil et al., 1992; Peschke and Möhwald, 1987). Most of the lipid-protein monolayers have been studied as models for biological membranes under quasiequilibrium conditions of compression (Möhwald, 1990); we developed an epifluorescence balance system in which such monolayers could be studied, also under certain dynamic conditions (compression and expansion and multiple π-A cycling) relevant to studies of PS in vitro (Nag et al., 1990, 1991; Nag and Keough, 1993). Using this system we have studied SP-B and SP-C labeled with different fluorophores in spread DPPC monolayers during dynamic cyclic compression and expansion. By quantitatively analyzing the visual features observed in the DPPC/protein monolayers at different π, lipid molecular areas, degrees of crystallization, and protein concentrations, we could determine the packing changes induced by the proteins on the lipid. The changes are directly relevant to pulmonary surfactant, and because the two proteins present good models for transmembrane and ampipathic, surface-seeking peptides in bilayers, these studies are also relevant to understanding lipid-protein interactions in biological membranes.

MATERIALS AND METHODS

Materials

Dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma Chemical Co. (St. Louis, MO), and fluorescein-5-isothiocyanate (FITC) and Texas-Red sulfonyl chloride (TR) were from Molecular Probes (Eugene, OR). SP-B and SP-C were isolated from porcine lung, by a modification of the method of Curstedt et al. (1987), which is discussed elsewhere (Perez-Gil et al., 1993). The saline subphases on which the monolayers were spread were made with doubly glass-distilled water, the second distillation performed from dilute potassium permanganate.

Fluorescent labeling of SP-B and SP-C

Isolated SP-B and SP-C were labeled with TR and FITC, respectively, by the following procedures. About 300 μg of SP-B or SP-C in 2 ml of chloroform:methanol (2:1 v/v) was adjusted to a pH of 7.8 by adding appropriate amounts of 50 mM Tris in methanol. These protein solutions were then incubated with 10 mM solutions of TR for SP-B, or of FITC for SP-C, in chloroform:methanol 2:1 (v/v) overnight at 4°C. The pH of the solutions was readjusted to 2 by adding appropriate amounts of 0.2 M HCl, and the solutions were concentrated under a stream of N_2 to give a final solvent volume of 0.5 ml. The solutions were then applied to and eluted from a column of LH-20 (Pharamacia LKB, Uppsala, Sweden) to remove the unreacted probes. The chromatographic profiles were followed by measuring absorption at 250 nm (protein) and 450 nm (fluorescein) or 520 nm (Texas Red). Analysis of the phosphorus by a modified method of Bartlett (1959) (Keough and Kariel, 1987) in the fluorescently labeled SP-B and SP-C preparations showed less than 0.3 mol phospholipid/mol proteins, respectively. The amounts of labeled proteins were estimated by quantitative amino acid analysis as discussed elsewhere (Perez-Gil et al., 1993).

Mass spectrometry of proteins

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was performed on a VG TofSpec spectrometer (Manchester, England) on a cyano-4-hydroxy cinnamic acid matrix by the methods of Hillenkamp et al. (1991). External calibration of the spectrometer was performed with recombinant eglin-C or trypsinogen by methods discussed elsewhere (Hillenkamp et al., 1991). Typically, 100 pmol of R-SP-B or F-SP-C in chloroform:methanol (3:1 v/v) were mixed with the cinnamic acid solution in a 1:1 ratio (v/v), and 2 μl of the mixed solution was placed on a stainless steel support. About 20 laser shots were averaged over a molecular mass/charge range of 2000–10,000 for F-SP-C and 6000–20,000 for R-SP-B.

Dynamic π-A measurements of monolayers

The R-SP-B and F-SP-C in chloroform:methanol (3:1, v/v) were mixed with DPPC in the same solvent to give weight proportions of DPPC + 10 or 20 wt% of either R-SP-B (0.43 and 1.0 mol% respectively, based on MW of SP-B dimer) or F-SP-C (1.9 and 4.2 mol%, MW of SP-C monomer). Additional samples were made that contained DPPC + 10 wt% R-SP-B + 10 wt% F-SP-C and DPPC + 1 mol% NBD-PC.

The mixtures were spread on an unbuffered 150 mM NaCl solution containing 2 mM $CaCl_2$ with the pH adjusted to 6.9. Monolayers of the same DPPC and DPPC-protein mixtures were tested simultaneously on a Teflon ribbon-barrier surface balance (Taneva and Keough, 1994a), which prevents leakage of monolayer components at high π, and on a balance that had a tightly fitting compression barrier equipped with an epifluorescence microscopic attachment (Nag et al., 1990). All experiments were performed at a temperature of 22 ± 1°C. The monolayers were compressed to a surface pressure (π) of ~65 mN/m and expanded to 0 mN/m for four cycles at an initial rate of 333 mm^2/s (0.64 $Å^2 \cdot molecule^{-1} \cdot s^{-1}$) in both surface balances, and the surface pressure-area (π-A) data were collected using a Wilhelmy dipping plate attached to a force transducer (Nag et al., 1990). The surface pressure was plotted as a function of area per amino acid residue of the proteins, using 1 residue for DPPC, 35 residues for F-SP-C, and 79 for R-SP-B monomers. The details of such measurements have been discussed previously (Taneva and Keough, 1994b,c,d). In the surface balance that was equipped with the fluorescence microscope, the monolayers were compressed or expanded in 20 steps, and 5 s was introduced at each step to monitor and record the visual features of the monolayer as described previously (Nag et al., 1990).

Visual observations and analysis of monolayers

Switching fluorescence filter combinations allowed for observation of fluorescence emissions from either R-SP-B at 590 nm or F-SP-C at 520 nm. This technique has previously been used by us and others to observe various fluorophores simultaneously in lipid monolayers (Grainger et al., 1990; Lipp et al., 1996; Maloney and Grainger, 1993; Montero et al., 1994). The images of the monolayers were video recorded for 5 s at each π, and the stored images were later processed using JAVA 1.3 image analysis software (Jandel Scientific, Corte Madera, CA). The relative amounts of dark (presumably condensed) phase were estimated from measuring the total amount of nonfluorescent areas of each image divided by the total area of the image. An average of five images was analyzed at each π, and the data were represented as a percentage of condensed phase as a function of π, or area per molecule of the lipid as discussed elsewhere (Nag and Keough, 1993).

The percentage of dark or condensed phase was also converted to the degree of crystallization or gelation, ϕ, by the method of Heckl et al. (1987). The degree of crystallization was calculated as $\phi = 1 - (1 - X/100) \cdot A/A_{fl}$, where X is the percentage of condensed phase, A is the area per molecule of DPPC, and A_{fl} is the area per molecule of DPPC at π just below the onset of appearance of the condensed (black) phase. This technique has previously been used in the quantitative analysis of lipid-protein distributions as observed by fluorescence microscopy in monolayers (Heckl et al., 1987; Möhwald, 1990; Nag et al., 1996a).

RESULTS

Fig. 1 shows the MALDI spectra of fluorescently labeled SP-B (*top*) and SP-C (*bottom*). The SP-B spectrum showed a major peak at 8777 Da corresponding to the FW of SP-B monomer (8.7 kDa) (Curstedt et al., 1990). This peak might arise because of fragmentation of the SP-B monomer in the MALDI process, or it may come from the dimer with two excess charges (one per monomer) instead of one per dimer. Sodium dodecyl sulfate gels of this material indicated that it

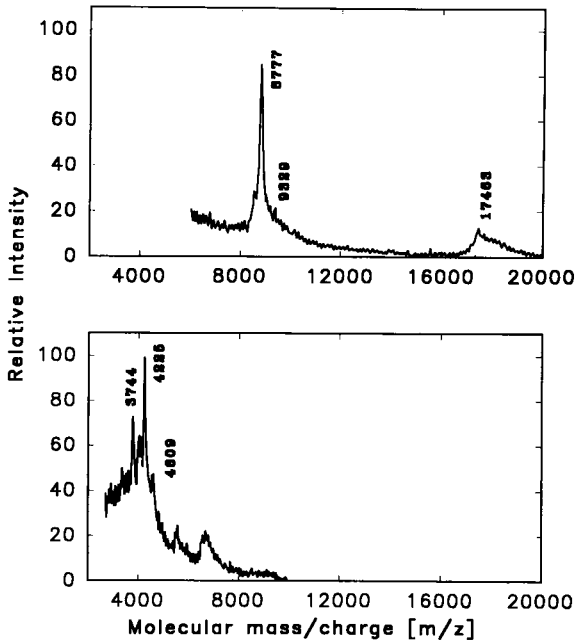

FIGURE 1 Typical MALDI mass spectra of Texas Red-SP-B (R-SP-B, *top*) and fluoresceinated SP-C (F-SP-C, *bottom*) plotted as relative intensity as a function of mass/charge (*m/z*) ratio. In the top panel the peaks at 8777 and 17,463 come from the unlabeled form of SP-B, and the one at 9329 Da comes from R-SP-B. In the bottom panel the peak at 4226 Da arises from the unlabeled, acylated form of SP-C; the one at 3744 Da from the deacylated protein; and that at 4609 Da from F-SP-C. The other minor peaks indicate the presence of different forms of the labeled or unlabeled proteins (see text for details).

was essentially all in the dimeric form. The peak at 9329 Da corresponds to the FW of labeled SP-B [8700 Da + 625 (Texas-Red-sulfonyl)] and the one at 17,463 to the dimer of the protein. The SP-C spectrum (Fig. 1, *bottom*) showed sharp peaks at 3744 Da and 4225 Da, corresponding to the formula weight of the amino acid sequence of SP-C (3.7 kDa) and the dipalmitoylated native form (4.2 kDa) (Curstedt et al., 1990). The peak at 4609 Da indicates the presence of the fluorescein-labeled form of F-SP-C [4200 Da (native) + 376 Da (fluorescein)]. The other diffuse smaller peaks at higher molecular weights are possibly SP-C labeled with more than 1 mole of fluorescein. The molecular weights of SP-B and SP-C are in relatively close agreement with the ones previously measured by Curstedt et al. (1990) using plasma desorption mass spectrometry. The MALDI spectra indicated that the proteins were intact and minimally labeled, and sodium dodecyl sulfate gels indicated that SP-C was predominantly in its monomeric form and SP-B in its dimeric form.

Typical isotherms of surface pressure-average area per residue (π-A) (a residue is taken to be one amino acid residue of the protein or one DPPC molecule) of monolayers of DPPC (*dotted lines, top*), DPPC plus 10 wt% of either R-SP-B (*top*) and F-SP-C (*bottom*) are displayed in Fig. 2 *a*. For π-A isotherms based on lipid only, the reader is referred to Taneva and Keough (1994b,c,d), and for the effect of the

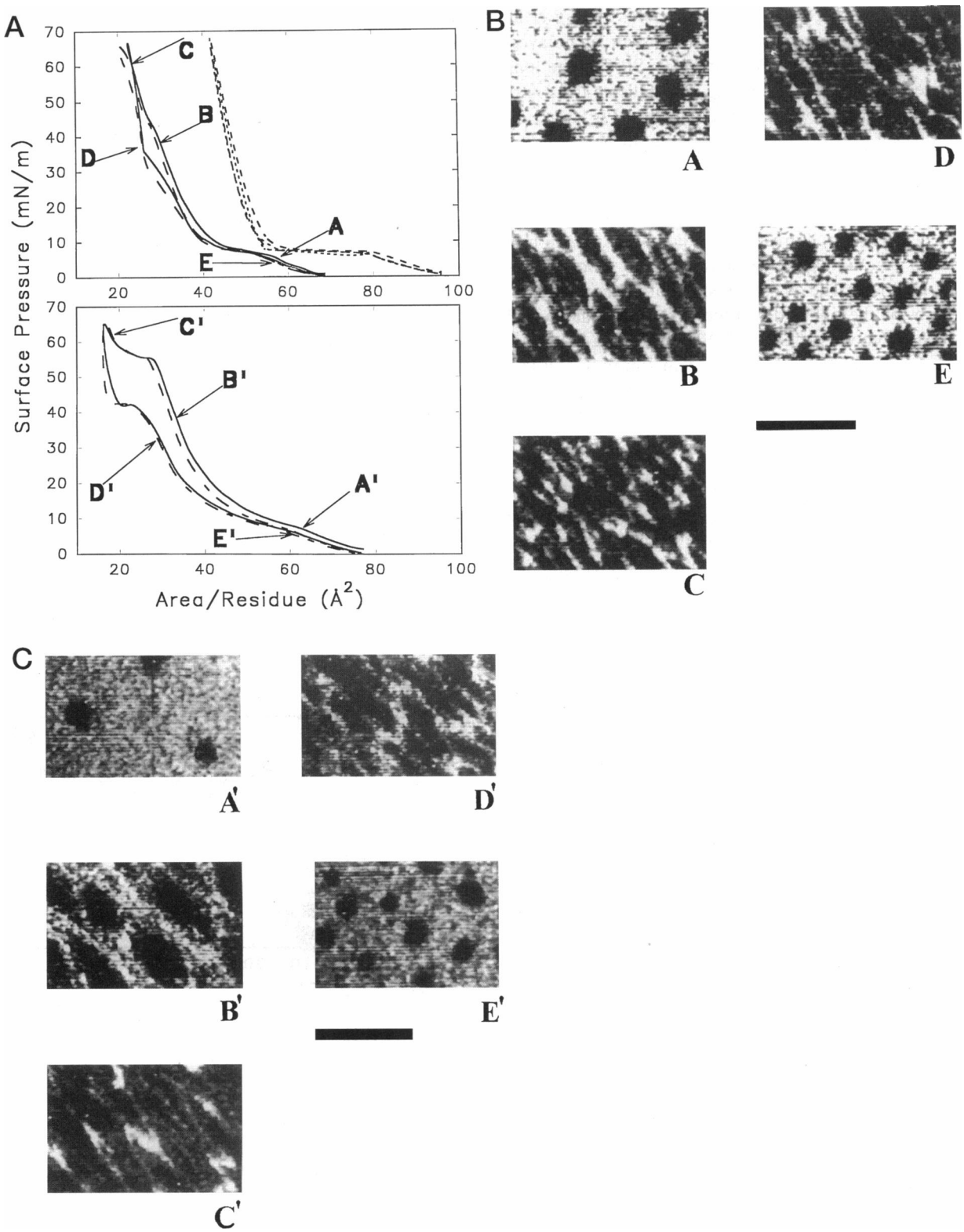

FIGURE 2 (*a*) Surface pressure (π) as a function of area per residue (π-A, a residue is one amino acid residue or one DPPC molecule) for DPPC + 1 mol% NBD-PC (*top*, –·–), DPPC plus 10 weight% R-SP-B (*top*, ——), and a 10 wt% F-SP-C (*bottom*) monolayer; typical fluorescence images seen at π indicated by letters A–E in the isotherms in *a* are shown in *b* for R-SP-B and *c* for F-SP-C. The right-hand limbs of the cycles represent the compression part of the isotherm and the left-hand limbs, the expansion. The long dashed lines in *a* indicate the fourth compression-expansion isotherms. The bright regions in *b* and *c* indicate the fluorescence from the labeled proteins. The scale bar is 25 μm.

probe on the isotherms, which is negligible, to Nag et al. (1996a). Typical images observed from such monolayers are shown in Fig. 2, *b* (R-SP-B) and *c* (F-SP-C). The long dashed lines in the isotherms in Fig. 2 *a* indicate the fourth compression-expansion cycles. The letters in Fig. 2 *a* indicate the surface pressure (π) at which the images in Fig. 2 *b* (from R-SP-B fluorescence) and Fig. 2 *c* (from F-SP-C fluorescence) were obtained, from the first compression-expansion cycles. The plateau regions in the isotherms at lower π (7–10 mN/m) correspond to the phase transition of DPPC from a liquid expanded phase (LE) to a liquid condensed (LC) phase. The change in slope of the compression limbs (limbs on the right side) of the isotherms near π of 43 mN/m in the isotherms of DPPC/R-SP-B (Fig. 2 *a, top, solid line*) and the plateau occurring at ~55 mN/m in the DPPC/F-SP-C monolayers (Fig 2 a, *bottom*) are thought to arise from the "squeeze out" or selective elimination of the proteins from the monolayers. The typical collapse π of native SP-B is around 40 mN/m and SP-C around 48 mN/m under quasistatic compression (Taneva and Keough, 1994b,c,d). These squeeze-out plateaus seen at the high π end of the compression limbs of the compression isotherms were seen previously in DPPC monolayers containing similar amounts of native SP-B or SP-C (Taneva and Keough, 1994a). The similarity of the isotherms suggested that the labeled proteins behaved like the native unlabeled ones (Taneva and Keough, 1994a).

The typical images observed in the lipid-protein monolayers showed distinct LC domains (dark regions) of DPPC (A, A′) in a homogeneous fluorescent background, indicating that the R-SP-B or F-SP-C partitioned into the liquid expanded (LE) (fluid) phase of the DPPC monolayers. The images that were selected for analysis were chosen randomly. The reproduced images in the figures are of somewhat lower quality than some earlier publications (Nag et al., 1996a; Perez-Gil et al., 1992), because the total number of images that were obtained at any observation point was lower than in previous studies (Nag et al., 1996a). This meant that fewer choices of images with high definition were available to show as being illustrative of the randomly chosen images, as was the case in previous studies. Increasing π from 14 mN/m to 63 mN/m (going from points A or A′ to C or C′ in Fig. 2 *a*) resulted in the LC domains growing in size and amount and their shapes changing from more circular to elongated forms. This latter effect might have been due to directional shear forces, such as those from the compression barrier at high π, but we are unable to say so with certainty. The effect may also be due to changing line tension between the condensed phase and fluid phases at the higher π. The images also indicated that at high π (~63 mN/m; C, C′), some of the proteins remained in or kept in very close association with the monolayers. This conclusion was also reached by Taneva and Keough (1994a), based on the π-A behavior of the isotherms of DPPC containing small amounts of native, unlabeled SP-B and SP-C. Upon expansion of the film with a consequent decrease in π (going from C to E and C′ to E′), the images at E and E′ were similar to those at A and A′. This was consistent with the proteins redistributing or redispersing in the expanded phase. Visual features of the monolayers on the fourth cycle (*dashed isotherms* in Fig. 2 *a*) were similar to those shown for the first cycle, suggesting that only minor changes occurred in the lipid-protein distribution in those monolayers over those cycles. The visual features did not indicate any significant difference in the distribution of R-SP-B and F-SP-C in DPPC monolayers at the different π, with both proteins being associated with the fluid or LE phase.

Fig. 3 shows the relative amounts (percentages) of condensed phase or black regions seen in monolayers of DPPC, and DPPC containing 10 wt% and 20 wt% of R-SP-B (*top*) or F-SP-C (*bottom*) as a function of π, during the first cycle of compression (*closed symbols*) and expansion (*open symbols*). The data were obtained by analyzing five randomly selected images at each π. The properties of DPPC monolayers without fluorescent protein were observed by using low amounts (1 mol%) of the fluorescent lipid probe 1-palmitoyl-2-nitro-benoxadiazole-phosphatidylcholine (NBD-PC). The condensed phase packing of DPPC was perturbed by either protein because at equivalent π, smaller amounts of condensed phase were formed in the lipid-protein films than those seen in films of the lipid alone. A similar pattern of perturbation of condensed phase by na-

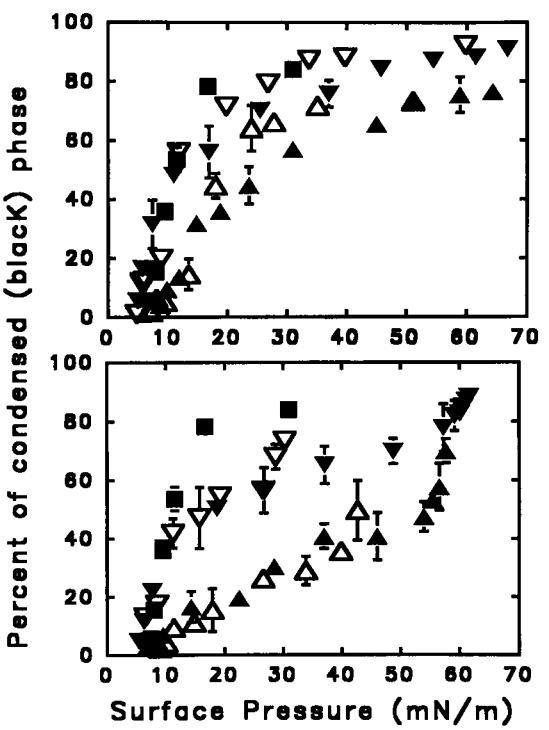

FIGURE 3 The percentage of condensed phase plotted as a function of surface pressure for monolayers of DPPC + 1 mol% NBD-PC (■), DPPC + 10 wt% (▼), and 20 wt% (▲) of R-SP-B (*top*) or F-SP-C (*bottom*). Open symbols indicate data obtained from the expansion of the monolayers. The error bars indicate ±1 standard deviation for five images analyzed at each π.

tive, acylated SP-C has previously been observed (Perez-Gil et al., 1992). Increasing the amount of the protein from 10 to 20 wt% decreased the total amounts of condensed phase. The perturbation of total condensed phase seen in DPPC/R-SP-B monolayers (Fig. 3, *top*) was less than that seen for F-SP-C (Fig. 3, *bottom*). In the DPPC/F-SP-C monolayers (Fig. 3, *bottom*), similar amounts of condensed phase were observed at comparable π during either compression (*closed symbols*) and expansion (*open symbols*). This suggested that similar lipid-protein distributions occurred at comparable π, during the compression or expansion process. In the monolayers containing R-SP-B (Fig. 3, *top*), however, the amount of dark or condensed phase (*open symbols*) seemed to be slightly but consistently higher on compression (*solid symbols*) than on expansion. These results indicate that both proteins perturbed the packing of DPPC, SP-C more so than SP-B.

Fig. 4 shows the amounts of condensed phase seen in DPPC monolayers containing 10 wt% of either protein from the first (*solid lines*) and the fourth (*dashed lines*) compressions. For monolayers containing either protein, the percentages that existed in condensed phase in the first and fourth cycles were very similar, indicating that little change occurred in lipid and protein distributions between successive cycles. This observation also seems to suggest that irreversible loss of materials from the monolayers to the subphase was small between successive cycles.

Fig. 5 displays the amount of condensed (dark) phase (*left*) and the degree of crystallization or gelation, ϕ (*right*), of DPPC, and DPPC plus R-SP-B (*top*) and F-SP-C (*bottom*) plotted as a function of nominal area per molecule of the lipid. The plots of percentage of condensed phase (*left*) indicate that the amounts of the condensed phase formed at equivalent area per molecule of the lipid are increased from that of the lipid alone, between 90 $Å^2 \cdot molecule^{-1}$ and 60 $Å^2 \cdot molecule^{-1}$. The plots of degree of crystallization or ϕ versus nominal area per molecule of these systems can be used to give estimates of the amount of lipids in the different phases, and of whether the proteins alter the packing of the lipids in such phases (Heckl et al., 1987; Möhwald, 1990). The patterns of the degree of crystallization for DPPC or DPPC plus F-SP-C and R-SP-B are linear between 90 and 70 $Å^2 \cdot molecule^{-1}$ (Fig. 5, *right*). Below 70 $Å^2 \cdot molecule^{-1}$, which occurred at $\phi \approx 0.9$, except for the monolayer with 20 wt% F-SP-C, where it occurred at $\phi \approx 0.6$, the slopes of the plots decreased abruptly. The plots of the degree of crystallization for DPPC are similar to the ones seen previously by Heckl et al. (1987). In our systems this gelation process for DPPC did not seem to be substantially affected by 10 wt% of either protein, suggesting that possibly most of the protein had left the monolayer system by that point in compression. Extrapolating the lines to $\phi = 0$ provided an estimate of the area of the lipids in the fluid phase. The values obtained (80 $Å^2 \cdot molecule^{-1}$ for R-SP-B

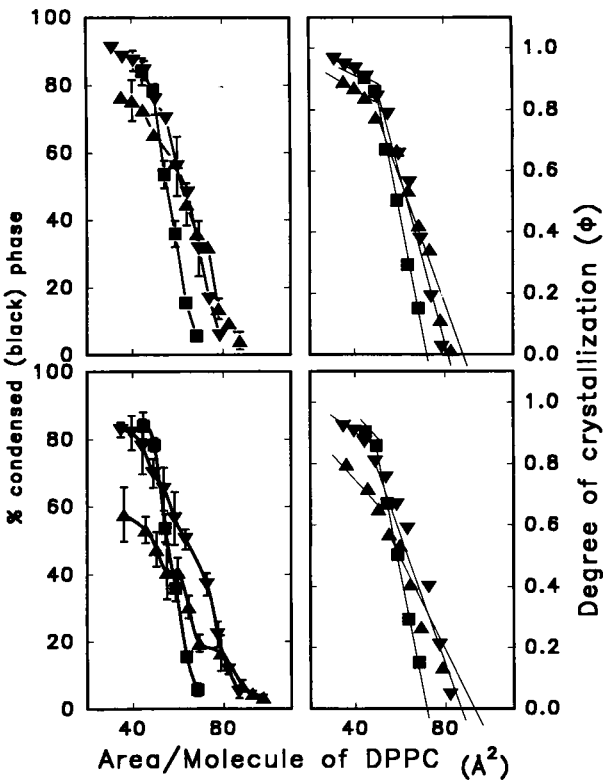

FIGURE 5 The total percentage of condensed phase (*left*) and the degree of crystallization ϕ (*right*) plotted as a function of nominal area per molecule of the lipid only, for monolayers of DPPC and DPPC plus R-SP-B (*top*) and F-SP-C (*bottom*). The nominal area of the lipid molecule was calculated from the amount of lipids in the lipid-protein mixtures spread at the air-water interface. The symbols are for system containing 0 wt% (■), 10 wt% (▼), and 20 wt% (▲) protein.

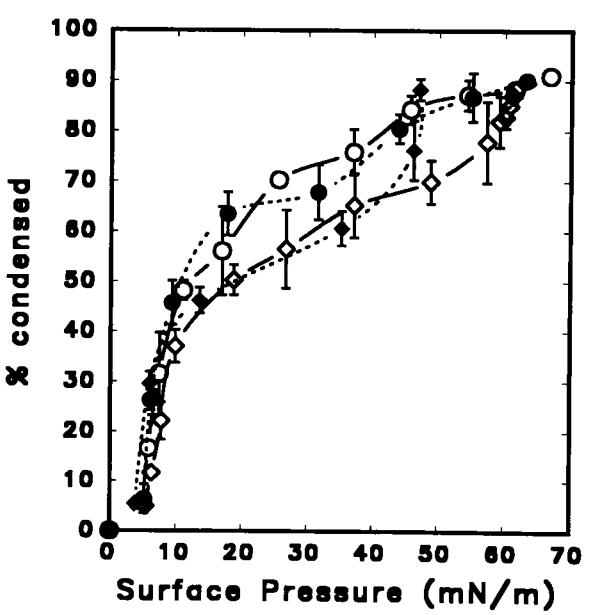

FIGURE 4 Total percentage of condensed phase plotted as a function of surface pressure for DPPC monolayers containing 10 wt% of R-SP-B (○, ●) or F-SP-C (◇, ◆) from the first (——, ○, ◇) and the fourth (– – –, ●, ◆) compression cycles. The error bars indicate standard deviations of five images analyzed at each π.

and 90 $Å^2 \cdot molecule^{-1}$ for F-SP-C) suggested that proteins disturbed the fluid phase and decreased the packing density of the lipid in that phase. By extrapolating the lines to $\phi = 1$, the area of the DPPC in the monolayers and the ones containing 10 wt% of either protein or 20 wt% of R-SP-B gave an area of 44 ± 2 $Å^2 \cdot molecule^{-1}$. This suggests that the proteins occupying the fluid phase changed the area of the lipid in that phase, but could not penetrate the gel or ordered phase. In the plots of ϕ versus area per molecule for monolayers containing 20 wt % F-SP-C (Fig. 5, *up triangle, bottom right*), the change of slope occurred at a lower ϕ of 0.6, implying that higher concentrations of F-SP-C substantially perturbed the ordering of DPPC. The difference between the monolayers containing higher amounts (20 wt%) of SP-B and SP-C reaffirms the interpretation of previous data (Morrow et al., 1993a,b; Simatos et al., 1990; Taneva and Keough, 1994a,d) that SP-C has a greater effect on packing than SP-B. The π-A isotherms of DPPC plus 10 wt% of R-SP-B plus 10 wt% F-SP-C are shown in Fig. 6 *a*. Typical images seen in such monolayers from the fluorescence of R-SP-B (*right*) or F-SP-C (*left*) are shown in Fig. 6 *b*, and the percentage of condensed phase as a function of π are shown in Fig. 6 *c* (the data from monolayers containing 20 wt% of either protein are shown for comparison). The fourth-cycle isotherms are shown as dashed lines in Fig. 6 *a*, and the letters indicate the surface pressures at which the images in Fig. 6 *b* were obtained. The first cycle isotherm in Fig. 6 *a* indicated that some "squeeze out" may occur at $\pi \approx 43$ mN/m for R-SP-B and at $\pi \approx 55$ mN/m for F-SP-C (Fig. 2 *a*), although the plateaus were smaller than the ones seen in DPPC monolayers plus equal amounts of the individual proteins (Fig. 2 *a*). The images in Fig. 6 *b* indicated that both proteins partitioned into the expanded phase of DPPC monolayers and mixed homogeneously in that phase at all π (A, A′). However, they remained present to some degree at high π (B, B′), and they reinserted into the monolayer upon expansion (C, C′). The plot of condensed phase as a function of π in Fig. 6 *c* compared to the ones obtained from monolayers containing 20 wt% of either protein suggested that the proteins perturbed the monolayer in a additive manner.

DISCUSSION

Pulmonary surfactant (PS) undergoes a number of transformations in the alveolar fluid and possibly in the alveolar air-fluid interface, such that during normal respiratory cycling a surface film is formed that is enriched in one component, DPPC. In vitro dynamic cycling of PS has indicated that the material undergoes transformation from heavy to light subtypes that can be separated by density gradient centrifugation (Gross, 1995; Gross and Schultz, 1992). The lighter subtypes are thought to be material eliminated from the interfacial monolayer, and they do not contain any detectable surfactant proteins (Gross, 1995). To maintain low surface tension at the interface, the monolayers are enriched in DPPC by surface refining where the unsaturated lipids and other components are possibly squeezed out of the monolayer (Goerke and Clements, 1986; Keough, 1992). Clements (1977) found evidence for a DPPC-rich material at the air-alveolar interface: the temperature dependence of transpulmonary pressure-volume relationships during lung deflation closely correlated with the thermotropic phase transition of DPPC but not that of PS separated from lavage fluid. It has also been shown that the surface tension at the air-alveolar fluid interface is near 0 mN/m (or $\pi \approx 70$ mN/m) at low lung volumes (Schürch et al., 1978). Other studies suggest that the film at the air-water interface that needed to reach such values upon compression must have been rich in DPPC (Clements, 1977; Hawco et al., 1981; Hildebran et al., 1979). The models proposed from in vitro studies for such DPPC enrichment of PS films usually involve the selective elimination of unsaturated and other non-DPPC lipids at intermediate π during compression of monolayers (Notter et al., 1980; Schürch et al., 1994; Snik et al., 1978) or after a number of cycles (Nag and Keough, 1993). Other models suggest that DPPC can be adsorbed in selective packets during expansion of PS monolayers (Schürch et al., 1994). Other studies have indicated that DPPC can be adsorbed into preformed monolayers (Oosterlaken-Dijksterhuis et al., 1991a,b) or be replenished or readsorbed into the monolayer from the highly compressed states formed on monolayer collapse (Notter et al., 1980; Taneva and Keough, 1994a). This study suggests that SP-B and SP-C may be present in small concentrations in DPPC monolayers at high π (Fig. 2 *b*), and the proteins are accommodated in or in association with DPPC monolayers, such that at high π (~65 mN/m; $\gamma = 5$ mN/m) high amounts (at least 85%) of condensed phase can be formed (Figs. 3 and 5). An advantage of having the proteins embedded in or associated with the DPPC matrix at high π may be aiding in the rapid respreading and replenishment of the monolayer with the lipid upon expansion (Taneva and Keough, 1994a). Other fluid components of pulmonary surfactant, such as unsaturated phosphatidylcholine and phosphatidylglycerol, may also enhance the surface activity of DPPC, and a combination of such lipids and hydrophobic proteins may also be involved in surfactant dynamics in situ. To produce observable differences, the amount of hydrophobic proteins used here (10–20 wt%) was higher than that usually associated with surfactant (1–2 wt%). It is assumed that the effects observed are qualitatively reflective of those in natural surfactant, although they may be of greater intensity in these models. The adsorption model may be further supported by the fact that SP-B and SP-C in preformed monolayers can induce the rapid transfer of phospholipid from vesicles in the subphase (Oosterlaken-Dijksterhuis et al., 1991a). The combined studies suggest that small amounts of the hydrophobic proteins, SP-B, SP-C, or both, can remain associated with DPPC monolayers, even under compression to high π, observations that support the suggestions made in a previous study (Taneva and Keough,

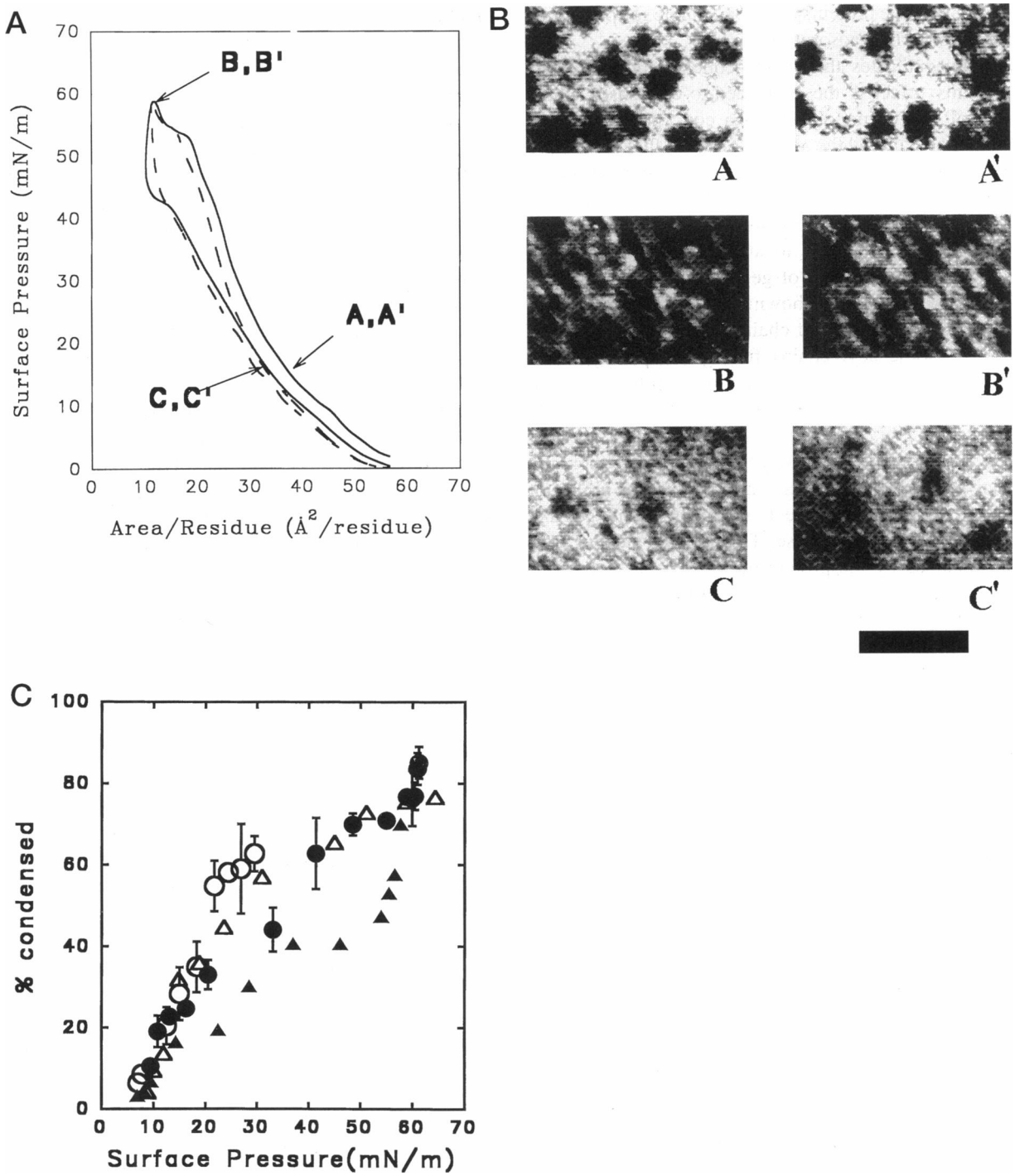

FIGURE 6 (*a*) Surface pressure as a function of area per residue (a residue is one amino acid residue or one DPPC molecule) for monolayers of DPPC + 10 wt% R-SP-B + 10 wt % F-SP-C, from the first (——) and the fourth compression-expansion cycle (– – –). (*b*) The typical images seen from R-SP-B (*right*) and F-SP-C fluorescence (*left*). The right-hand limbs of the isotherms represent compression, and the left-hand limbs, expansion. (*c*) The percentage of condensed phase is plotted as a function of π for the monolayers containing DPPC + 10 wt% R-SP-B + 10 wt% F-SP-C (●, ○) and monolayers containing 20 weight % of R-SP-B (△) or F-SP-C (▲). The letters in *a* represent the π at which images in *b* were obtained. The closed and open symbols (●, ○) in *c* represent the data from the compression and expansion cycles, respectively, and the error bars represent the standard deviations of five images analyzed at each π.

1994a), and thus the proteins can be available for promoting respreading or aiding adsorption.

This study indicates that at the concentrations studied, both proteins affected the expanded-to-condensed phase transition of DPPC monolayers in a concentration-dependent manner and altered the amount of lipids that could pack into the condensed phase at any given π or area per molecule (Figs. 3 and 5). This meant that with increasing amounts of protein, higher pressures or lower average areas per molecule were required to attain similar amounts of condensed phase or degrees of gelation. In DPPC bilayers SP-B and SP-C have been shown to reduce the calorimetrically detectable gel-to-fluid chain melting transition, and to "remove" some acyl chains from participating in the gel-to-liquid crystalline phase transition, results that complement this study (Morrow et al., 1993a,b; Shiffer et al., 1993; Simatos et al., 1990). In monolayers both proteins occupied the expanded (fluid) phase, perturbed the lipid molecular packing in that phase, and reduced the ability of the lipids to undergo a phase transition to more gel-like regions or the condensed phase. This type of packing perturbation induced by the protein in bilayers has been suggested to enhance the adsorptivity of DPPC to an air-water interface (Haagsman, 1994; Keough, 1992; Perez-Gil et al., 1992). Antibodies directed against SP-B inhibited rapid adsorption of pulmonary surfactant, confirming the role of the protein in the adsorption process (Suzuki et al., 1986). Protein-induced packing changes of surfactant protein-lipid monolayers may also enhance the adsorption of further lipids from the bulk in the subphase (Haagsman, 1994; Oosterlaken-Dijksterhuis et al., 1991a,b).

The influence of F-SP-C on packing appeared to be greater than that of R-SP-B, as seen from their effects on the percentage of total condensed phase or the degree of crystallization as functions of π or the nominal area per molecule of DPPC. The amount of condensed phase increased rapidly with π for the system with 10 wt% F-SP-C (Fig. 3) during compression between 10 mN/m and 50 mN/m, the point at which the squeeze-out of F-SP-C plus lipids probably began (Taneva and Keough, 1994a), whereas 20 wt% F-SP-C produced a more gradual increase in the amounts of the gel phase until the squeeze-out π was achieved. Above the squeeze-out π of ~55 mN/m monolayers containing 20 wt% F-SP-C rapidly approached the higher ratios of condensed phase seen in monolayers with 10 wt% F-SP-C or with DPPC alone. Furthermore, the degree of gelation patterns in Fig. 5 indicated that at nominal areas of lipid below 60 $Å^2 \cdot \text{molecule}^{-1}$, the percentage of condensed phase for the monolayers containing 20 wt% of the proteins was slightly lower than the pure lipid, with this effect being more pronounced for F-SP-C (Fig. 5, *bottom*) than R-SP-B (Fig. 5, *top*). This is consistent with a perturbation of high density packing by the proteins. The change of slope at $\phi \approx 0.9$ for the DPPC monolayer implied that the crystallization or gelation changed at or near the limiting molecular area of DPPC, and it could be an indication of the molecules in the gel or condensed phase undergoing a phase transition to a "solid-like" phase, which has been suggested previously by others (Möhwald, 1990). Moreover, the areas per molecule calculated by extrapolating the lines for degree of crystallization to $\phi = 0$ were higher in the case of F-SP-C than those for equivalent amounts of R-SP-B. This provides some indirect evidence that indicates that equal amounts (wt%) of SP-C perturbed the DPPC monolayers more than SP-B, correlating well with previous bilayer studies on such lipid-protein systems (Morrow et al., 1993a,b; Shiffer et al., 1993; Simatos et al., 1990). A recent study using fluorescence energy transfer of labeled SP-B and SP-C in DPPC bilayers showed that SP-C is excluded from the gel phase phospholipid and aggregates in the fluid phase, whereas SP-B has little or no preference for either phase (Horowitz, 1995).

Previous studies on SP-B/phospholipid bilayers have indicated that the protein that has a number of positively charged residues throughout its polypeptide chain can make several electrostatic interactions with anionic phospholipid headgroups (Johannson et al., 1994). About 11 wt% of SP-B only minimally affected the acyl chain mobility of DPPC in bilayers, as estimated from the first moments of ^2H NMR spectra (Morrow et al., 1993a) and by fluorescence anisotropy measurements in DPPC:PG systems (Baatz et al., 1991). Fluorescent antibody directed against SP-B bound to surfaces of giant bilayer vesicles, indicating that the protein (or at least the epitopes recognized by the antibody) was localized in the polar or headgroup region of the phospholipid bilayers rather than being deeply embedded in the hydrocarbon interior (Longo et al., 1992; Vincent et al., 1993). A current structural model of SP-B–lipid interactions suggests that the protein, which contains potential ampipathic helices, associates with phospholipids in bilayers by a combination of electrostatic interaction with headgroups and hydrophobic interactions with parts of the acyl chains near the headgroup (Baatz et al., 1991; Pastrana-Rios et al., 1995; Vandenbussche et al., 1992). SP-B is functionally more effective in inducing bilayer fusion and lipid insertion into preformed monolayers containing the protein than is SP-C (Oosterlaken-Dijksterhuis et al., 1991a,b). SP-C has a few positive charges localized at the N-terminal, a highly hydrophobic α-helical C-terminal region, and palmitoyl chains attached to the two cystines in the N-terminal region. It orients in bilayers with the α-helical axis parallel to the acyl chains, as do most transmembrane α-helical proteins (Clercyx et al., 1995; Horowitz et al., 1992; Johansson et al., 1995; Morrow et al., 1993a; Pastrana-Rios et al., 1995; Vandenbussche et al., 1992). Thus SP-C could be expected to affect the lipid packing in bilayers and monolayers in different ways than SP-B. This study is consistent with the previous ones, because it shows a difference in the patterns of perturbation of the DPPC packing in monolayers by the two proteins.

The structural orientation of SP-C monomers or dimers (deacylated) in monolayers studied by circular dichroism showed that the proteins have a high amount of α-helical structure, and that the helix axis is oriented parallel to the

air-water interface (Creuwels et al., 1995; Oosterlaken-Dijksterhuis et al., 1991a; Pastrana-Rios et al., 1995). Our MALDI data indicate that SP-C was mainly monomeric and acylated, and was similar to the proteins studied by these authors. Ellipsometric studies with DPPC/SP-C monolayers indicate that with compression there is a slow increase in monolayer film thickness between 10 and 50 mN/m, followed by an abrupt increase at higher π (Post et al., 1995). The π-A isotherms shown in Fig. 2 *a* (*bottom*) show a distinct plateau at ~50 mN/m, assumed to be the squeeze-out of the protein from the monolayers, possibly accompanied by some lipid (Taneva and Keough, 1994d). Pastrana et al. (1991) had suggested that SP-C packing in the lipid matrix is very stable; even at high compression, the protein may not be squeezed out, but the lipid-protein complex might undergo some type of structural change. Others have suggested from collapse-plateau ratio measurements and infrared reflectance-absorbance spectroscopy studies of such lipid-protein monolayers that some protein is squeezed out in association with some lipid, and these complexes remain associated with the monolayer above the collapse π of the protein (Pastrana-Rios et al., 1995; Taneva and Keough, 1994a). Orientational changes of the SP-C molecule with respect to the monolayer plane may be associated with the phenomenon that accompanies squeeze-out. Recent neutron diffraction studies indicate that DPPC headgroups in monolayers undergo conformational changes and dehydration at $\pi > 30$ mN/m (Brumm et al., 1994). Others have indicated that there may be a change in orientation of DPPC in monolayers around that π, as inferred from anthraloxystearic acid probe orientation studies (Denicourt et al., 1994). Isotherms of pure SP-C in monolayers show a kink or plateau region around 18 mN/m (Creuwels et al., 1995; Nag et al., 1996a; Perez-Gil et al., 1992; Taneva and Keough, 1994c), which has been suggested to be an orientation change of the pure protein (Creuwels et al., 1995). These orientational changes may also occur in the presence of the lipids. Thus a combination of lipid-protein orientational changes may be involved in accommodating the protein in the lipid matrix at high π.

The structure of native SP-B in monolayers is mainly α-helical (~40%), the helix axis being oriented parallel to the interface (Oosterlaken-Dijksterhuis et al., 1991a,b). Because of the distribution of the charged amino acid residues throughout its sequence, the protein has been suggested to have regions of ampipathic-helical conformation (Bruni et al., 1991; Cochrane and Revak, 1991; Johansson et al., 1991; Longo et al., 1993; Takahasi et al., 1990; Vandenbussche et al., 1992; Waring et al., 1989). The N-terminal domain of SP-B has been suggested to contain ampipathic helical conformation in bilayer or monolayer systems (Fan et al., 1991). The positively charged synthetic 25-amino acid N-terminal peptide of SP-B interacts strongly with negatively charged palmitic acid in monolayers, increasing the collapse π of such monolayers (Lipp et al., 1996; Longo et al., 1993). When SP-B was used in DPPC monolayers, no significant secondary structural change from the predominant α-helical (47% in bilayers) conformation was observed by Fourier transform infrared spectroscopy (Pastrana-Rios et al., 1995; Vandenbussche et al., 1992), indicating that the protein probably has a stable native conformation in monolayers or bilayers. The R-SP-B/DPPC isotherms (Fig. 2 *a*) and the plots of percentage of condensed phase versus π (Fig. 3, *top*) showed that the amount of condensed phase increased with π in a pattern different from that seen in the F-SP-C/DPPC monolayers (Fig. 3, *bottom*). There were no abrupt increases in the condensed phase at any π, as was observed in the F-SP-C/DPPC monolayers near 50 mN/m, suggesting that the minimal perturbation of DPPC condensed phase by R-SP-B was relatively constant as a function of π. During the expansion of R-SP-B/DPPC monolayers, higher percentages of condensed phase were observed (Fig. 3, *top*, *open symbols*) than those seen in monolayers during compression at comparable π (*closed symbols*). This might indicate that upon expansion, SP-B spreads less readily back into the monolayer than does SP-C. Recent infrared spectroscopy of DPPC/SP-B films has indicated that a substantial amount of material squeezed out of such films is not readsorbed back upon expansion compared to films containing SP-C (Pastrana-Rios et al., 1995). Our study and the one by Pastrana-Rios et al. (1995) suggest that some SP-B may be irreversibly squeezed out of the lipid films at high π, and thus such films upon expansion have higher amounts of lipids than that found during compression at equivalent π. As spreading of phospholipids to an air-water interface is dependent on hydration, it is tempting to speculate that R-SP-B may replace the hydration of the DPPC headgroups, after they have been dehydrated because of compression (Brumm et al., 1994; Denicourt et al., 1994). Thus the orientation of SP-B in the headgroup or polar region of the phospholipid may be of significance to previous findings that SP-B was more effective in inserting phospholipid to preformed monolayers than SP-C, and SP-B-containing vesicles adhere to each other more than SP-C-containing ones (Oosterlaken-Dijksterhuis et al., 1991a,b). The results obtained here are consistent with SP-B being oriented differently in the monolayers than SP-C, and with its being excluded in a regime that did not require adherent lipids, which is different from the regime in which SP-C is excluded, because SP-C which has lipids associated with it on exclusion (Taneva and Keough, 1994a).

The study of the monolayers containing R-SP-B and F-SP-C together (Fig. 6) showed that the two proteins mixed homogeneously in the fluid or expanded phase of DPPC. The combinations of both proteins also reduced the ability of DPPC to form the condensed phase. A previous study on similar lipid-protein mixtures showed that the proteins and some accompanying lipids were squeezed out of the monolayer separately, SP-B at ~43 mN/m and SP-C at ~55 mN/m, indicating independent behavior of the proteins in the presence of each other (Taneva and Keough, 1994d). The percentages of condensed phase in the monolayers of DPPC + 10 wt% R-SP-B + 10 wt% F-SP-C (Fig. 6 *c*) are roughly intermediate between those obtained for DPPC

monolayers containing 20 wt% of either protein alone, indicating that the proteins may perturb the DPPC monolayers in an independent and additive fashion. However, some of both proteins remained associated with the DPPC monolayer at high π, as seen in the images B, B′ in Fig. 6 *b*, and both were located in the same regions of the monolayers under all conditions of compression and expansion (Fig. 6 *b*).

These results have significance for pulmonary function and dynamics at the air-alveolar fluid interface. Instillation of lipid-protein mixtures containing hydrophobic proteins into the alveoli has been reported to improve lung function in PS-deficient experimental animals (Smith et al., 1988; Suzuki et al., 1986; Tanaka et al., 1986; see McLean and Lewis, 1995, for a review). The hydrophobic proteins individually perturbed the monolayer packing of DPPC by dispersing in the expanded phase of the monolayers, but some of each of them remained in or near the monolayers at high π, where the monolayers essentially were in the condensed phase. These factors may allow for the replenishment of DPPC into the surface monolayer after monolayer collapse upon its overcompression at high π, because some hydrophobic protein that remained associated with the lipids could enhance respreading (Taneva and Keough, 1994a). When the films are not overcompressed beyond the point of collapse, as was the case here, the proteins that had been selectively excluded at $\pi \approx 42$ and 50 mN/m, for SP-B and SP-C, respectively, could also promote reinsertion of themselves plus any associated lipid during expansion, provided they remained associated with the monolayer. Transfer of DPPC from surfactant secreted complexes to a preformed monolayer (Haagsman, 1994; Oosterlaken-Dijksterhuis et al., 1991a), or movement of collective units of lipids from the subphase to the interface (Goerke and Clements, 1986; Schürch et al., 1994) to reach high π may be accomplished by the presence of the proteins. The results are consistent with the role ascribed to these proteins in vivo, and with the potential positive benefit of their inclusion in synthetic surfactant.

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