Fluorescently Labeled Pulmonary Surfactant Protein C in Spread Phospholipid Monolayers

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ABSTRACT Pulmonary surfactant, a lipid–protein complex, secreted into the fluid lining of lungs prevents alveolar collapse at low lung volumes. Pulmonary surfactant protein C (SP-C), an acylated, hydrophobic, α-helical peptide, enhances the surface activity of pulmonary surfactant lipids. Fluorescein-labeled SP-C (F-SP-C) (3, 6, 12 wt%) in dipalmitoylphosphatidylcholine (DPPC), and DPPC:dipalmitoylphosphatidylglycerol (DPPG) [DPPC:DPPG 7:3 mol/mol] in spread monolayers was studied by epifluorescence microscopy. Mass spectrometry of F-SP-C indicated that the protein is partially deacylated and labeled with 1 mol fluorescein/1 mol protein. The protein partitioned into the fluid, or liquid expanded, phase. Increasing amounts of F-SP-C in DPPC or DPPC:DPPG monolayers decreased the size and total amounts of the condensed phase at all surface pressures. Calcium (1.6 mM) increased the amount of the condensed phase in monolayers of DPPC:DPPG but not of DPPC alone, and such monolayers were also perturbed by F-SP-C. The study indicates that SP-C perturbs the packing of neutral and anionic phospholipid monolayers even when the latter systems are condensed by calcium, indicating that interactions between SP-C and the lipids are predominantly hydrophobic in nature.

INTRODUCTION

Pulmonary surfactant (PS), a complex lipid–protein material lining the air–alveolar fluid interface, forms putative monolayers at that interface, preventing alveolar collapse at low volumes. PS is secreted by type-II pneumocytes, transformed into an unusual form called tubular myelin, which is perceived to be the precursor for the surface film. In vitro PS rapidly adsorbs to an air–water interface, forms stable monolayers that can be compressed to high surface pressures (π) or low surface tensions, and respreads readily from three-dimensional phases that are formed on overcompression (Goerke and Clements, 1986; Keough, 1992). PS contains a large amount of dipalmitoylphosphatidylcholine (DPPC), significant amounts of phosphatidylglycerol (PG), and some specific proteins. The alveolar fluid also contains monovalent and divalent ions, which have been conjectured to interact with PS lipids. Some of the biophysical properties of individual PS components that are related to surfactant extracellular activity are not well defined.

PS contains specific proteins, categorized as surfactant proteins A–D (SP-A, SP-B, SP-C, and SP-D) (Possmayer, 1988; see Johansson et al. (1994a) for a recent review). Hydrophobic proteins of PS, SP-B and SP-C together make up ~2 wt % of the lipids, although the exact amount of each has not yet been determined with certainty. The precise amount of the proteins that may be associated with the films at the air–water interface is even less well defined, although recent experiments suggest that at least SP-C can adsorb into the layer (Nag et al., unpublished results), and small amounts can remain in the film even under high compression (Taneva and Keough, 1994a). Other experiments suggest that if SP-B and SP-C do reach the surface, small amounts might remain associated with the surface layer, even under high compression (Taneva and Keough, 1994a,b; 1995). Porcine SP-C is a 4.2-kDa, dipalmitoylated, 35-residue peptide, of which 23 residues are hydrophobic (Beers and Fisher, 1992; Curstedt et al., 1990; Hawgood and Shiffer, 1991). In vitro studies have shown that SP-C facilitates PS lipid adsorption into an air–water interface (Notter et al., 1987; Perez-Gil et al., 1992b; Takahashi and Fujiwara, 1986; Yu and Possmayer, 1988), mediates lipid insertion into preformed lipid monolayers (Creuwels et al., 1993; Oosterlaken-Dijksterhuis et al., 1991), and facilitates respreading of lipids from collapsed phases of monolayers (Taneva and Keough, 1994b). SP-C also can alter the elasticity and viscosity of lipid monolayers (Pastrana et al., 1991). Some of these processes were enhanced in the presence of anionic lipids and calcium. Porcine SP-C has positively charged residues near its *N* terminal, and these may interact with anionic phospholipids (Shiffer et al., 1988; 1993). Although studies have indicated roles for SP-C in pulmonary surfactant dynamics, only recently have investigations indicated how SP-C may structurally interact with some PS lipids (Horowitz et al., 1993; Krill and Gupta, 1994; Morrow et al., 1993; Pastrana et al., 1991; Shiffer et al., 1993; Simatos et al., 1990; Vandenbussche et al., 1992; Williams et al., 1991).

A recent high-resolution nuclear magnetic resonance (NMR) study showed that SP-C in an apolar solvent has a 37-Å long α-helix between residues 9 and 34 (Johansson et al., 1994b). Other studies have indicated that SP-C has

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~60% α-helical conformation in lipid bilayers (Pastrana et al., 1991; Vandenbussche et al., 1992) or monolayers (Creuwels et al., 1993) and that it has a transbilayer orientation similar to that of integral membrane proteins (Morrow et al., 1993). It was suggested that the larger precursor protein from which mature SP-C is formed contains a transmembrane α-helical region, which may be embedded in the membrane of type-II cells, with the *N* terminal of this precursor located in the cytosol (Keller et al., 1991).

Monolayers of lipid–protein systems have long served as models of biological membranes (Cadenhead, 1985; Cserhati and Szogyi, 1994; Möhwald, 1990) and as models of serum lipoprotein particles (Ibadah et al., 1989; Krebs and Phillips, 1983; Krebs et al., 1988). This study of SP-C with lipids in monolayers is relevant to lipid–protein interactions of integral membrane proteins in model membranes as well as to the interactions of the components in surfactant monolayers and bilayers.

Epifluorescence microscopy of monolayers has been particularly useful in studying lipid and lipid–protein interactions of model biological membranes (Möhwald, 1990). Although a drawback of the system is that the monolayers have to be visualized with a small amount of fluorescent probe, an advantage is that proteins labeled with different fluorophores can be identified in such environments (Grainger et al., 1989; Möhwald, 1990). By epifluorescence microscopy the influence of proteins on lipid monolayer phase packing and the transition from liquid expanded (LE) to liquid condensed (LC) phase have been semiquantitatively assessed (Heckl et al., 1987; Möhwald, 1990; Perez-Gil et al., 1992a; Peschke and Möhwald, 1987). The influence of ions or anionic lipids (Evert et al., 1994; Nag et al., 1994) and lipid–protein (Heckl et al., 1987) monolayers has been monitored by this technique. Similarities of pressure-induced monolayer changes with bilayer lipid systems undergoing thermal phase transitions have been noted (Möhwald, 1990; Nag and Keough, 1993). In an earlier study we found that SP-C caused lipid packing rearrangements and affected isothermal phase properties of DPPC in spread monolayers (Perez-Gil et al., 1992a) and bilayers (Keough et al., 1992). In this study we show directly how fluorescein-labeled SP-C (F-SP-C) organized in spread monolayers of DPPC, DPPC:DPPG (dipalmitoylphosphatidylglycerol) (7:3 mol/mol), under the influence of calcium.

MATERIALS AND METHODS

Materials

1, 2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1, 2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol-sodium salt (DPPG-Na), and 1-palmitoyl-2-{12-[(7-nitro-2–1, 3-benzoxadiazole-4-yl)amino] dodecanoyl} phosphatidylcholine (NBD-PC) were purchased from Avanti Polar Lipids (Pelham, AL). The lipids were found to be pure by thin-layer chromatography and used as received. Fluorescein isothiocyanate was purchased from Molecular Probes (Eugene, OR).

The subphase buffer for the monolayers was 0.15 M NaCl and 5 mM Tris dissolved in deionized doubly distilled water, the second distillation performed from dilute $KMnO_4$, containing 1.6 mM calcium ions when required. The pH was adjusted to 6.9 with 0.1N HCl.

Pulmonary surfactant protein SP-C was isolated from porcine lungs by the method of Curstedt et al. (1990), as discussed elsewhere (Perez-Gil et al., 1993).

Fluorescent labeling of SP-C

Purified, native SP-C was reacted with fluorescein isothiocyanate by the following method. The pH of a solution of 200 μg of purified SP-C in 2 ml of chloroform: methanol (2:1 vol/vol) was adjusted to 7.8 by addition of appropriate amounts of 50 mM Tris in methanol. Fluorescent labeling was achieved by incubation of the SP-C solution with 20 μl of 10 mM FITC at 4°C overnight. The pH of the solution was readjusted to 2 and the proteins eluted from an LH-20 Sephadex (Pharmacia LKB, Sweden) column to remove the unreacted fluorescein isothiocyanate. The chromatographic profile of the LH-20 effluent was followed by absorbance at 250 nm (unreacted SP-C) and 450 nm (fluorescein-labeled SP-C). F-SP-C showed a single band on SDS-gel electrophoresis near 5000 Da and was not further purified.

We scanned native and fluorescein-labeled SP-C (F-SP-C) by matrix-assisted laser desorption/ionization (MALDI) spectrometry to determine their respective molecular weights by procedures discussed by Hillenkamp et al. (1991). A similar type of spectrometry, matrix-assisted plasma desorption, was used previously by Curstedt et al. (1990) to determine the molecular weight of SP-C, and the analytical procedures are given in detail by those authors.

Protein and lipid quantitation

SP-C and F-SP-C were quantitated by amino acid analysis. DPPC, DPPG, and NBD-PC were assayed by estimation of the total amount of phosphorus in the lipids by a modified method of Bartlett (1959) as described elsewhere (Keough and Kariel, 1987).

Monolayer formation and observation

The lipids, NBD-PC (fluorescent lipid probe), and protein F-SP-C (fluorescein-labeled SP-C) were dissolved in chloroform:methanol (3:1 vol/vol) and mixed in the desired proportions. 1 mol % of NBD-PC was included in DPPC and DPPG:DPPG (7:3 mol/mol) mixtures. No lipid probe was used when F-SP-C (3, 6, and 12 wt % or 0.5, 1.07, and 2.14 mol %) was present in lipid monolayers because fluorescence emission from F-SP-C was found to be sufficient for the monolayers to be observed. Monolayers were spread on a buffered saline subphase from the chloroform:methanol (3:1 vol/vol) solution in an epifluorescence microscopic surface balance whose design and construction are described elsewhere (Nag et al., 1990). The balance was equipped with a tight-fitting Teflon barrier for compression of the surface film and a Wilhelmy plate connected to a pressure transducer for measurement of surface pressure in addition to the epifluorescence microscopic attachment to monitor surface fluorescence (Nag et al., 1990). All experiments were carried out at a temperature of 21 ± 1°C. The total monolayer area was 158 cm^2. Monolayers were compressed in steps at a rate of 20 mm^2/s or an initial rate of 0.13 $Å^2$ $molecule^{-1}$ s^{-1}, compression stopped for 1 min, and monolayer images obtained during that minute by a video camera and recorder (Nag et al., 1990). The images were analyzed with digital image processing by methods discussed in detail elsewhere (Nag et al., 1991). We estimated the percentage of condensed phase as (total area of black regions per image/total area of the image × 100) in a manner that was used previously (Heckl et al., 1987; Nag et al., 1991; Nag and Keough, 1993; Perez-Gil et al., 1992a; Peschke and Möhwald, 1987) to characterize quantitatively the monolayer phase properties.

RESULTS

The MALDI mass spectra of SP-C (top panel) and its fluorescein-labeled analog F-SP-C (bottom panel) are shown in Fig. 1. As shown in the top panel of Fig. 1, native SP-C had a main peak at 4213 Da and minor ones at 4023 and 8081 Da. The F-SP-C spectrum in the bottom panel showed a major peak at 4117 Da and smaller ones at 3747, 4007, and 4360 Da. The formula weights of SP-C from amino acid analysis are 4186 Da (+2 palmitates), 3948 Da (−1 palmitate), and 3710 Da (−2 palmitates). Curstedt et al. (1990), using similar mass spectrometry of SP-C, have shown that the native protein had spectral peaks at 4210 Da (+2 palmitates), 3971 Da (−1 palmitate), and 3733 Da (−2 palmitates), the main peak of SP-C (4210 Da) being close to our SP-C peak at 4213 Da. The difference in MALDI spectrometrically determined molecular mass and the formula weight of SP-C may be due to sodium adjuncts of various forms of SP-C being detected spectrometrically, as has been discussed by Curstedt et al. (1990). The spectrum in Fig. 1 (top panel) indicates that SP-C is mainly in its dipalmitoylated, native, monomeric form (4213 Da), with minor amounts of SP-C in other forms such as SP-C dimer (8081 Da) and monopalmitoyl SP-C (4023 Da). Similarly, the F-SP-C spectra can be explained as displaying some

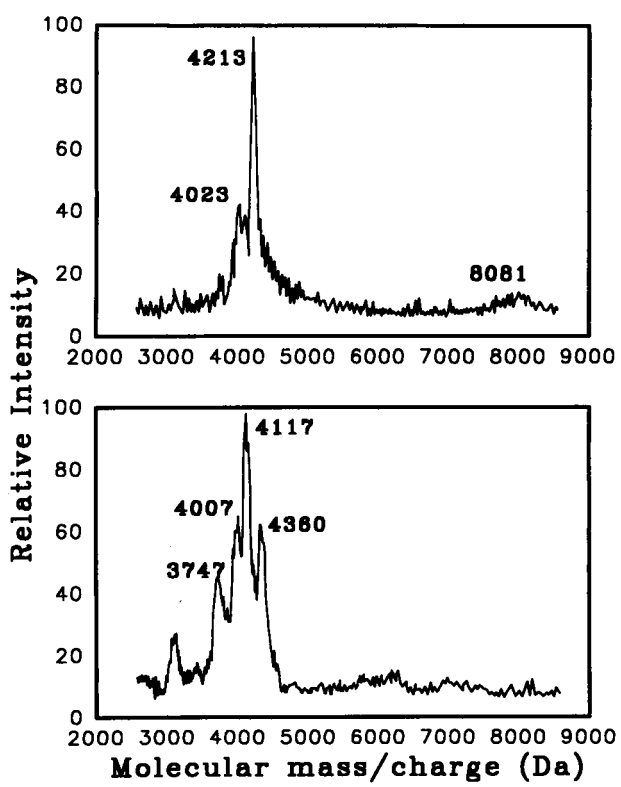

FIGURE 1 MALDI mass spectra of porcine SP-C (*top*) and fluorescein-labeled deacylated F-SP-C (*bottom*). The main peak at 4213 Da (*top*) is from the dipalmitoylated monomer of the protein, and the one at 4117 Da (*bottom*) is from the deacylated, fluorescein-labeled protein (F-SP-C). The other minor peaks are for various forms (see main text) of SP-C present in the extracted protein.

deacylated form of SP-C from the peak at 3747 Da [4213 Da − 476 Da (2 palmitates) = 3737 Da] and a main peak at 4117 Da, which corresponds to this deacylated form labeled with 1 mol fluorescein/1 mol protein [3737 Da + 376 Da (FW of fluorescein) = 4113 Da for F-SP-C]. The other peaks in the F-SP-C spectrum indicate minor amounts of SP-C at its various levels of acylation.

Fig. 2 *a* shows the surface pressure–area per molecule (π-A) isotherms of DPPC monolayers containing 0, 3, 6, and 12 wt % or 0, 0.5, 1.07, and 2.05 mol% of F-SP-C (Fig. 2 *a*), and the typical images obtained from a monolayer containing 3 wt % F-SP-C are shown in Fig. 2 *b*. The isotherm in the inset of Fig. 2 *a* is for pure F-SP-C. The monolayer without F-SP-C contained 1 mol % of the fluorescent lipid probe NBD-PC. The letters in Fig. 2 *a* (arrows) indicate the π at which the images in Fig. 2 *b* from a DPPC+ 3 wt % F-SP-C monolayer were obtained. The LE to LC phase transition for all monolayers occurred at a π of 5–7 mN/m, as indicated by the plateau regions in the isotherms in Fig. 2 *a*. The protein isotherm (Fig. 2 *a*, inset) showed behavior similar to that of native SP-C monolayers studied previously (Perez-Gil et al., 1992a; Taneva and Keough, 1994a). The isotherms also showed a leveling at $\pi \sim 50$ mN/m that corresponded to the exclusion of F-SP-C-lipid units or monolayer collapse in the case of pure SP-C (Taneva and Keough, 1994a). Plots of area/molecule at π of 9, 18, and 27 mN/m for the monolayers containing F-SP-C showed that the protein produced a small positive deviation from ideal mixing plots for such mixtures (data not shown). This indicated that a small expansion of the lipid monolayers occurred at a π of 0–30 mN/m because of the influence of the protein and is also consistent with behavior seen previously with the native protein (Taneva and Keough, 1994b).

The typical images in Fig. 2 *b* of a monolayer of DPPC + 3 wt % F-SP-C showed that LE (bright regions) and condensed phase (dark regions) of DPPC could be visualized from the fluorescence of the fluorescein-labeled protein. Image *A* in Fig. 2 *b* shows mainly LE or fluid phase for the lipid monolayer and suggests that F-SP-C is in that phase. Image *E* indicates that F-SP-C (light regions) remained in the monolayer at high surface pressures (π = 64 mN/m). The bright regions in image *E* may be residual fluid phase containing protein or regions of excluded protein (plus or minus small amounts of adherent lipids). Image *B* indicates that the shapes of the LC domains of DPPC + 3 wt % F-SP-C monolayers had small protrusions at the LE/LC boundaries and were different from the more elliptical or kidney-bean shapes generally observed in pure DPPC monolayers (Möhwald et al., 1988; Nag et al., 1991).

The isotherm of F-SP-C (Fig. 2 *a*, inset) indicated that the protein occupied molecular areas of 640 Å^2 molecule^{-1} at "lift-off" π and 380 Å^2 molecule^{-1} at π of 25 mN/m. These values are in agreement with ones previously found for α-helical apolipoproteins (Krebs et al., 1988) and close to those seen with native acylated SP-C (Taneva and Keough, 1994a). Others have shown by circular dichroism that SP-C

has a high amount of α-helix in DPPC monolayers (Oosterlaken-Dijksterhuis et al., 1991; Pastrana-Rios et al., 1995). The isotherms in Fig. 2 *a* can be recalculated in terms of π versus area per molecule of DPPC only (as opposed to the average area per molecule of DPPC plus protein as in Fig. 2 *a*. Then the apparent change in area of a DPPC molecule that is induced by the protein can be calculated by subtraction of the recalculated isotherms of DPPC plus protein from the isotherm of DPPC alone (Heckl et al., 1987). Such a presentation is made in Fig. 3. The change of area of the lipid–protein monolayers from that of DPPC (open circles, Fig. 3) was nonlinear between 83 and 40 Å^2 molecule^{-1} of DPPC (Fig. 3). This difference in area was low at higher areas per molecule of lipid, reached a maximum at 55 Å^2 molecule^{-1} (π = 5 mN/m), and then declined to become negligible at 40 Å^2 molecule^{-1}, for all concentrations of the protein. One can use the change of the area between the lipid–protein isotherms and those of the pure lipid to approximate the partial molar areas of the protein at the different areas per molecule of DPPC in the lipid–protein isotherms by dividing the area change by the molar amount of protein in the monolayers. This assumes that all the area difference is due to the presence of the protein. The average nominal area of the protein at 5 mN/m (which corresponds to an area of DPPC of 83 Å^2 molecule^{-1}) so calculated from the change of area at different protein concentrations was 512 ± 103 Å^2 molecule^{-1}, an area close to that of the protein observed from the isotherm of the pure protein at that π (Fig. 2 *a*, inset). This would indicate that, at least at low π, the protein at all concentrations in the phospholipid monolayers occupied the same area as it did in the pure protein monolayer. At a higher π of ~ 25 mN/m (corresponding to 55 Å^2 molecule^{-1} for DPPC), where the

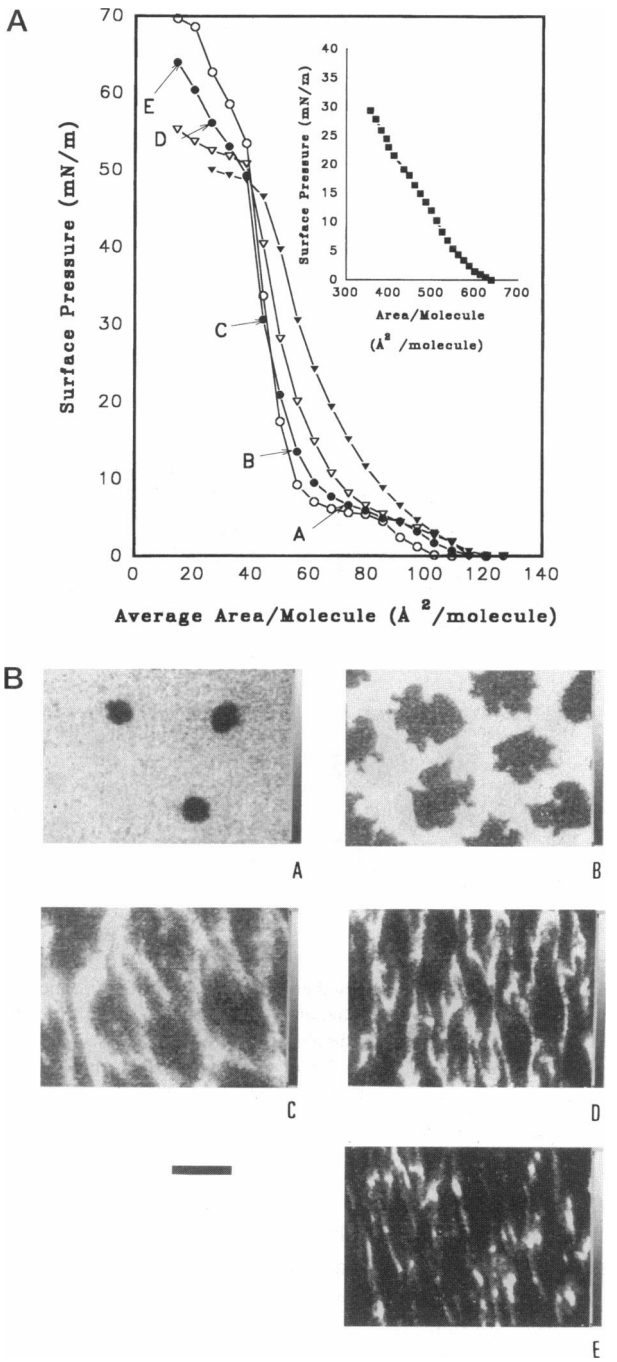

FIGURE 2 (*a*) Typical surface pressure–area/molecule (π-A) isotherms of DPPC containing 0 (○); 3 wt %., 0.5 mol% (●); 6 wt %, 1.07 mol% (▽); and 12 wt %, 2.05 mol% (▼) of F-SP-C and 100% F-SP-C (■, *inset*). (*b*)Typical images obtained from a DPPC monolayer containing 3 wt % F-SP-C at various π. The monolayers containing 0% F-SP-C contained 1 mol% NBD-PC. *A–E* in (*a*) represent the π at which the images in (*b*) were obtained. The light areas in the images represent the LE phase and were observed from the emission of the fluorescently labeled protein F-SP-C. The scale bar is 25 μm.

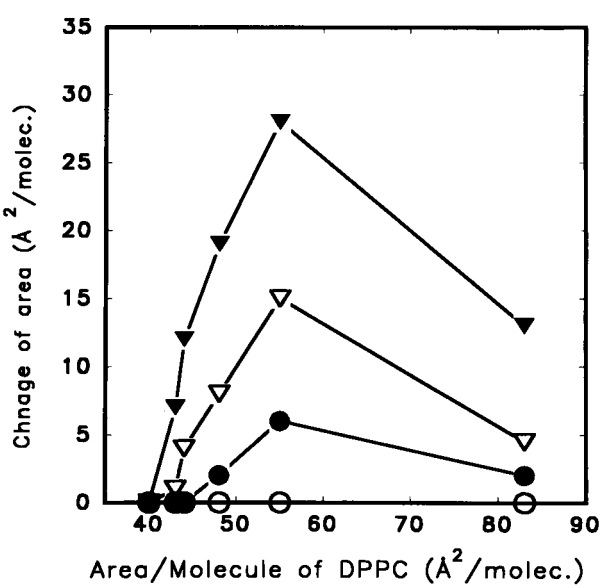

FIGURE 3 Nominal change in the area per molecule of DPPC induced by the protein plotted as a function of the area per molecule of DPPC. The change of area was obtained by subtracting the area per molecule of pure DPPC from the nominal area per molecule of DPPC in the presence of the proteins at six different surface pressures. The symbols correspond to systems containing DPPC plus 0 wt % (○), 3 wt % (●), 6 wt % (▽), and 12 wt % (▼) of F-SP-C.

change of area induced in the protein–DPPC isotherms was maximum (Fig. 3), the nominal area of the protein molecule in the mixed films was determined as 1306 ± 100 Å^2 molecule^{-1}, whereas the protein had an area of only 370 Å^2 molecule^{-1} in the pure protein monolayer (Fig. 2 *a*, inset). This implies that the protein had a greater partial area at the higher π in the mixed films than it would occupy in a monolayer of the protein alone. It was shown recently by ellipsometry and infrared spectroscopy (Post et al., 1995, Pastrana-Rios et al., 1995) that native acylated SP-C in lipid films possibly undergoes conformational changes at the air–water interface in the surface pressure range of 10–25 mN/m, accompanied by a possible change of orientation of the α-helix. Whereas such changes might be responsible for an increase in partial area of the protein, the size of the change estimated from the surface data is large, and it is difficult to envisage how changes of such a magnitude would accompany the conformational and orientational effects noted by Post et al. (1995) and Pastrana-Rios et al. (1995). It is possible to interpret the data in Fig. 3 to indicate that at low areas per molecule of DPPC, where the lipid is in the LE phase, the protein and the lipid effect limited perturbation on each other. At higher pressures, at which more lipid would be in the LC phase, more protein would be excluded into the LE phase, causing greater perturbation of packing of lipid in these domains and an apparent increase in the calculated protein area because of the initial assumption that the area change is all due to protein in the system.

Fig. 4*a* shows typical π-A isotherms of DPPC:DPPG (7:3 mol/mol) containing 0, 3, 6, and 12 wt % of F-SP-C (DPPC:DPPG/F-SP-C) without calcium, and Fig. 4 *b* shows the typical images observed in such monolayers at a π of 15 mN/m (increasing amounts of protein from *A* to *D*). In the absence of calcium DPPC:DPPG/F-SP-C monolayers displayed isotherms similar to those obtained for the DPPC/F-SP-C system (Fig. 2 *a*), except that the LE–LC plateau occurred at a higher π of 8–9 mN/m. The isotherms in the inset of Fig. 4 *a* are for similar DPPC:DPPG/F-SP-C monolayers in the presence of 1.6 mM $CaCl_2$ in the subphase (DPPC:DPPG-Ca). The tendency for the isotherms obtained with these mixtures in the presence of calcium to show plateaus near 50 mN/m could be partly due to the design of the balance and the high viscosity of these monolayers at high π. The balance was designed with a channel between the main surface and the observing regions (Nag et al., 1990; Peters and Beck, 1983). For highly viscous monolayers (DPPC:DPPG-Ca) the channel produced an apparent reduction of π compared with that observed in such monolayers on a balance of different design (Taneva and Keough, 1995). The effect was ascribed, likely improperly, by us to monolayer collapse when such systems were first studied in this balance (Nag et al., 1994).

The DPPC:DPPG/F-SP-C isotherms obtained in the presence of 1.6 mM calcium (Fig. 4 *a*, inset) showed LE–LC phase transition plateaus at a lower π compared with the monolayers without calcium. This behavior of DPPC:DPPG monolayers under the influence of calcium was consistent

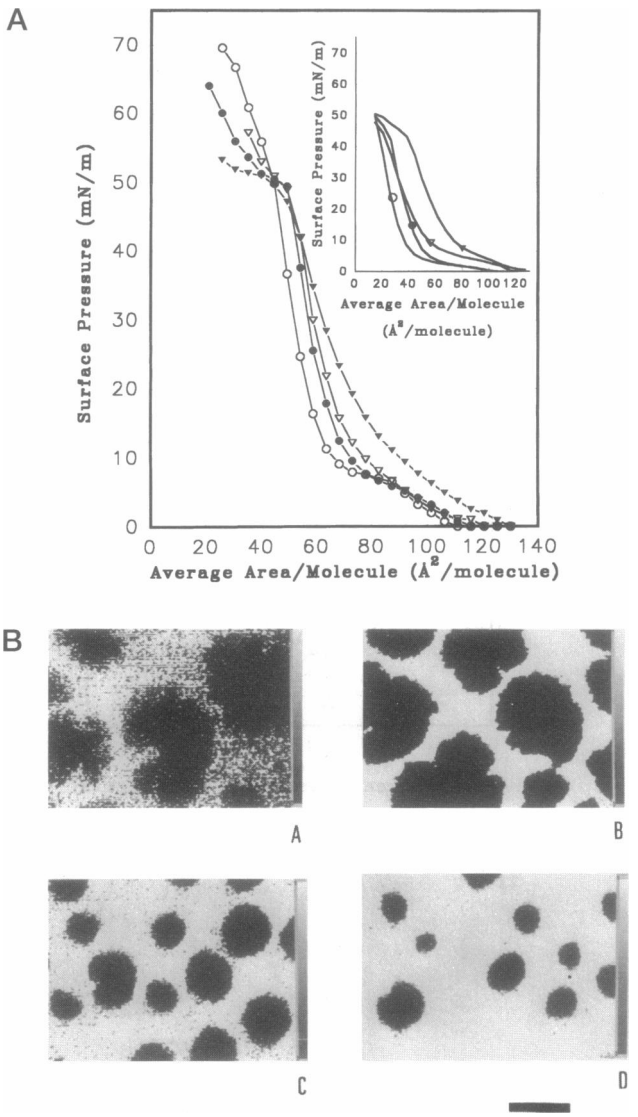

FIGURE 4 (*a*) Typical π-A isotherms of DPPC:DPPG (7:3, mol/mol) containing amounts of F-SP-C similar to those in Fig. 2, with (inset) and without 1.6 mM calcium in the subphase. (*b*) Typical images seen at $\pi \sim$ 15 mN/m in such monolayers without calcium. *A*, *B*, *C*, and *D* in the images in (*b*) are in the direction of increasing concentration for 0, 3, 6, and 12 wt % protein concentration, respectively. The scale bar is 25 μm.

with that of similar anionic lipid monolayer systems studied previously (Flach et al., 1993; Losche and Möhwald, 1989; Nag et al., 1994; Taneva and Keough, 1995). The images in Fig. 4 *b* indicate that with increasing amounts of F-SP-C (*A–D*) the average size of the LC domains of the lipids decreased. This pattern of decreasing size of LC domains with increasing amounts of protein in the monolayer was also observed in the DPPC/F-SP-C system (data not shown) and previously in DPPC monolayers containing a native, unlabeled, and acylated SP-C (Perez-Gil et al., 1992a).

The total amount of condensed phase as a percentage of the total monolayer (% condensed) for DPPC/F-SP-C (top panel) and DPPC:DPPG/F-SP-C (bottom panel) monolayers without calcium plotted as a function of π are shown in Fig.

5. At comparable π, increasing amounts of F-SP-C decreased the total amounts of condensed phase for both DPPC and DPPC:DPPG monolayers. It can be observed from Fig. 5 (top panel) that, although as much as 6 wt % of F-SP-C caused smaller condensed domains, it did not substantially reduce the amount of condensed phase of DPPC monolayers, whereas 12 wt% of F-SP-C did reduce that total amount. In a previous study (Perez-Gil et al., 1992a) a similar perturbation of DPPC monolayers by native SP-C was observed. The total amounts of condensed phase in DPPC:DPPG monolayers were reduced by F-SP-C.

Fig. 6 shows the relative amounts of condensed phase (left panels) and the degree of crystallization ϕ (right panels) plotted as a function of molecular area of the lipid. The degree of crystallization ϕ was calculated by the method of Heckl et al., (1987), where $\phi = 1 - (1 - X)A/A_{fl}$, where X is the ratio of the dark (condensed) area to the total area, A is the observed area per lipid molecule, and A_{fl} is the area of the lipid molecule in the fluid region just below the liquid expanded to the LC phase transition region. The former estimates give the proportion of the total area that is in the condensed phase, whereas the latter give the proportion of the total number of lipid molecules that are in that phase. Both plots show a difference in the influence of 12 wt % protein compared with that of 3 and 6 wt %.

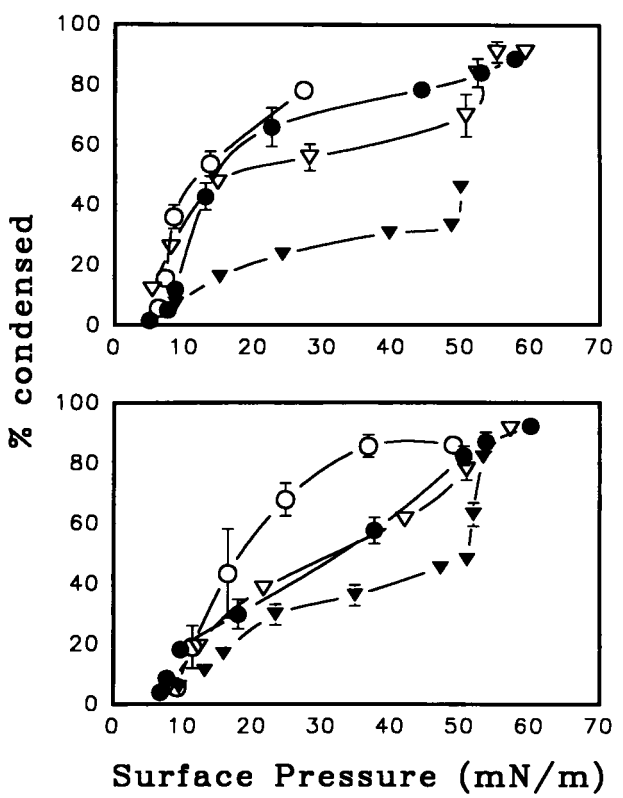

FIGURE 5 Percentage of the monolayers in condensed phase (% condensed) as a function of π for DPPC (*top*) and DPPC:DPPG (*bottom*) containing F-SP-C in the absence of calcium. The symbols represent the same lipid–protein mixtures as in Figs. 2 *a* and 4 *a*. Error bars represent standard deviations of 10 frames analyzed at each π from an individual monolayer.

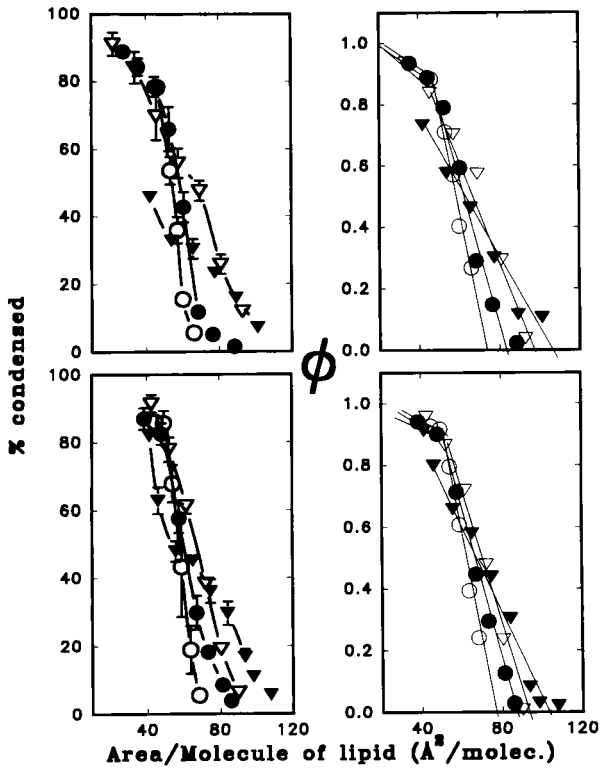

FIGURE 6 Percentage of condensed phase as in Fig. 5 (left panels) and degree of crystallization or ϕ (right panels) plotted as a function of molecular area of the lipids. The degree of crystallization was obtained by conversion of the percentage condensed (Fig. 5) to the amount of gel phase by methods used previously by Heckl et al. (1987) (see text for details) to estimate partial molar areas occupied by the protein in the monolayers. The top panels are for DPPC and the bottom ones are for DPPC:DPPG. The symbols represent the same concentrations of protein as in the previous figures.

The plots of degree of crystallization (right panels, Fig. 6) can be used to estimate the area occupied by the lipid in the gel or crystalline (condensed) phase or in the fluid phase by extrapolation of the linear portions of the plots to ϕ of 1 or 0, respectively, as previously discussed by Heckl et. al (1987). For the pure DPPC monolayers (open circles in Fig. 6, right panels) an area per molecule of 75 ± 2 Å^2 molecule^{-1} [95% confidence limits] (ϕ = 0) was obtained for the lipids in the fluid phase and 46 ± 2 Å^2 molecule^{-1} (ϕ = 1) for the lipids in the gel or condensed phase, and the values for the mixed DPPC/DPPG monolayers were very close to those of the DPPC monolayers. These values are in close agreement with those found previously for DPPC by others (Heckl et al., 1987; Möhwald, 1990). The plots of the degree of crystallization are linear up to ϕ = 0.9, and for monolayers containing 0, 3, and 6 wt % of F-SP-C they converge to a similar area per molecule of lipid (of ~55 Å^2 molecule^{-1}). Beyond this point there is a change of slope in most of the systems, except in the lipid monolayers containing 12 wt % F-SP-C. The slope change is observed for lipids with and without protein, possibly indicating that there was little or no effect of the protein on the gelation

patterns above ϕ of 0.9. This would be consistent with the protein's being mostly squeezed out of the condensed lipids above $\phi \sim 0.9$. In contrast, when the plots were extrapolated to $\phi = 0$ the indication was that the area of the lipids in the fluid or expanded phase increased by ~ 10 Å^2 molecule^{-1} with each increment of protein concentration. This suggests that the protein changes the packing of the lipid in the expanded or fluid phase. Extrapolation of the lines to the upper axis of $\phi = 1$ for 0–6 wt % protein revealed a value of $\sim 45 \pm 2$ Å^2 molecule^{-1} (95% confidence limits), which suggests that the partial molar areas of the lipids in the gel phase for the pure lipids and the lipid–protein systems are similar. This would suggest that the protein up to 6 wt % did not affect the gel phase. The systems with 12 wt % protein showed lower slopes for ϕ-versus-area plots, which did not extrapolate to real areas per molecule at $\phi = 1$, behavior that suggests that at the high protein concentration the gel phase packing could be influenced by the protein.

The percentages of condensed phase as a function of surface pressure of DPPC:DPPG monolayers in the presence of 1.6 mM calcium are shown in Fig. 7*a*, and typical images observed in such monolayers at $\pi = 8$ mN/m are seen in Fig. 7 *b* (increasing amounts of F-SP-C from *A* to *D*). The calcium produced higher amounts of condensed phase in DPPC:DPPG monolayers at any given π compared with the ones without calcium, but the F-SP-C continued to induce a decrease in the size of condensed phase domains. The images in Fig. 7 *b* also indicate that the LC domains in DPPC:DPPG monolayers had a different shape (flowerlike) in the presence of calcium (± F-SP-C) from the shapes seen in DPPC or DPPC:DPPG systems without calcium (Figs. 2 *b* and 4 *b*). Flowerlike LC domains were observed previously in anionic lipid monolayers containing divalent cations (Losche and Möhwald, 1989). In the presence of calcium, increasing amounts of F-SP-C decreased the amounts LC phase of DPPC:DPPG monolayers (Fig. 7 *a*), as had been seen in the systems without calcium (Fig. 4 *b*).

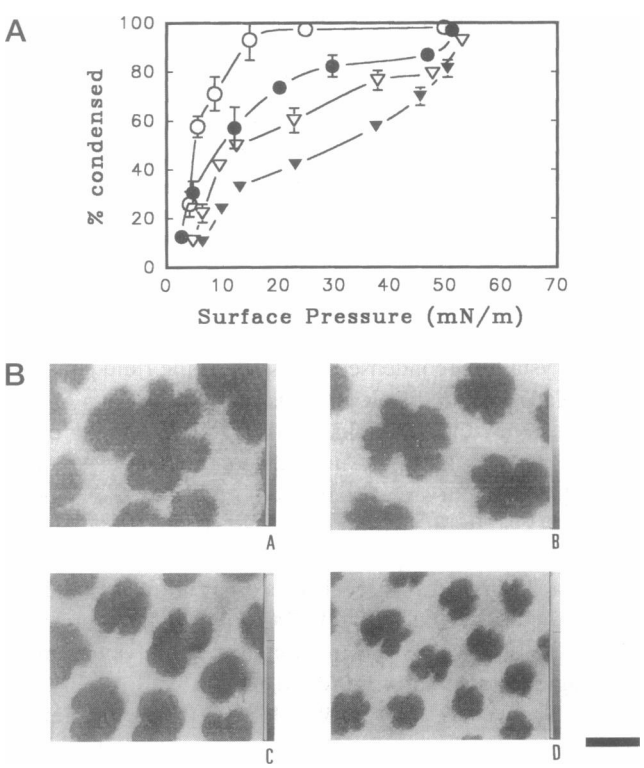

FIGURE 7 (*a*) Percentage of condensed phase plotted as a function of π for DPPC:DPPG/F-SP-C monolayers in the presence of 1.6 mM $CaCl_2$. (*b*) Typical images from such monolayers obtained at a π of 8 mN/m. *A*, *B*, *C*, and *D* in (*b*) indicate the direction of increasing protein concentration (0, 3, 6, and 12 wt % F-SP-C, respectively). The scale bar is 25 μm. Error bars indicate standard deviations of 10 images analyzed at each π from an individual monolayer.

DISCUSSION

Pulmonary surfactant protein SP-C is difficult to characterize because of its extremely hydrophobic nature and insolubility in the polar solvents usually employed in structural characterization of proteins (Beers and Fisher, 1992; Keough, 1992). Extracted native SP-C was found to contain forms that differed in their levels of acylation, truncation, and dimerization; these differences were easily detected by MALDI-type mass spectrometry (Curstedt et al., 1990). In this study we used MALDI analysis of SP-C and F-SP-C to determine the degree of acylation, dimerization, and fluorescent labeling of the protein. During the fluorescent labeling process the protein underwent partial deacylation along with complete labeling with 1 mol fluorescein (F-SP-C)/1 mol SP-C. This partially deacylated, fluorescein-labeled F-SP-C was found not to exhibit any significant differences in its surface pressure-area monolayer characteristics (Fig. 2 *a*, inset) or in its interaction with DPPC in monolayers from properties observed with native SP-C studied previously (Perez-Gil et al., 1992a). Recently it was also shown that in the absence of calcium the level of SP-C acylation does not significantly alter its function in lipid adsorption and insertion in preformed monolayers (Creuwels et al., 1993). In a recent study in this laboratory F-SP-C was found to increase the rate of adsorption of DPPC to an air–water interface similarly to native nonlabeled SP-C (Nag et al., unpublished results). In that same study, evidence was obtained that spread films and those adsorbed from liposomes displayed similar surface textures and domain distributions. Nevertheless, it is noted that the solvent-spread films of simple systems used here may not totally reflect the adsorbed film of pulmonary surfactant, which may potentially have more-complex composition in situ. But understanding of the complex systems will be furthered by investigation of the simple systems such as those used here.

The expansion of the DPPC and DPPC:DPPG monolayers by F-SP-C was similar to that in previously published results on such monolayer systems containing a native acylated SP-C (Taneva and Keough, 1994a). Low amounts of SP-C were found to mix almost ideally in DPPC and DPPC:DPPG lipid environments and caused only a small increase in ideal average area/molecule in such monolayers with

increasing protein concentration (Perez-Gil et al., 1992a; Taneva and Keough, 1994a). Other proteins such as glycophorin (Jones and Davis, 1987), bombilitin III (Signor et al., 1994), and cytochrome C (Peschke and Möhwald, 1987) were found also to cause changes in the isotherms of lipid monolayers. Up to π of 30 mN/m F-SP-C produced a slight expansion of the monolayers compared with that expected for ideal mixing of lipids and proteins, irrespective of the lipid and ionic conditions used. Assuming that all the nominal change in the area is due to the area occupied by the protein, the data in Fig. 3 could be interpreted to indicate that the protein occupied a partial molar area of ~512 Å^2 molecule^{-1} in the lipid–protein monolayers at low π at all protein concentrations, whereas at higher π its calculated partial molar areas appeared to increase substantially. This change in the apparent partial molar area of the protein at higher π may be suggestive of a change of conformation or alignment of the protein in the monolayers, or a change of lipid area, or both. The area changes of the DPPC monolayers induced by SP-C at lower π are, however, smaller than the ones seen when water-soluble cytochrome C protein adsorbed to DPPC monolayers. This protein was also found to insert in the fluid phase of DPPC (Heckl et al., 1987). Hydrophobic SP-C may have a different organization from water-soluble proteins in phospholipid monolayers. Recently Post et al. (1995) showed by ellipsometry of DPPC/SP-C monolayers that a change of thickness of the lipid–protein monolayers occurred between 10 and 25 mN/m in the presence of SP-C, possibly indicating that some change of molecular alignment of this protein in monolayers occurred between those π. SP-C is insoluble in aqueous systems, so it can be spread in monolayers in pure form (Fig. 2 *a*, inset). Monolayers of pure protein formed through spreading from solvent do not show an increase in partial molar areas with compression. So, although it might be possible that the lipid causes a large change in the area of the protein, it seems more likely that the assumption that the lipid is unaffected by the protein at the higher pressure (and lower areas per lipid molecule) is not warranted and that SP-C perturbs the lipid packing, especially of the liquid expanded phase.

F-SP-C occupied the LE or fluid phase of all the phospholipid monolayers studied, as shown in the images in Figs. 2 *b*, 4 *b*, and 7 *b*. Previous studies showed that fluorescently labeled cytochrome C inserted into the LE phase of lipid monolayers (Peschke and Möhwald, 1987; Möhwald, 1990). In lipid bilayers, bacteriorhodopsin and calcium ATPase have been shown by electron microscopy to be excluded from the gel phase of lipids and to be distributed in the fluid phase (Kleeman and McConnell, 1976; Cherry et al., 1980). Recent studies of the amphipathic helical peptide bombilitin-III in DPPC monolayers also indicate that the protein is excluded from the LC phase of lipids (Signor et al., 1994). Horowitz et al. (1993) recently showed by fluorescence energy transfer methods that SP-C is excluded from the gel phase of DPPC:DPPG bilayers, and the exclusion prompts the protein to self-associate in the fluid phase. The preference of F-SP-C for the LE phase thus seems to be a general property of proteins to occupy loosely packed regions of bilayers or monolayers. The pattern of plots of degree of crystallization as a function of molecular area (Fig. 6) also possibly supports the idea that F-SP-C occupied the fluid phase, because the areas per molecule of the lipids were changed by F-SP-C when they were in the expanded or fluid phase ($\phi = 0$), whereas the induced change at 100% lipid gelation ($\phi = 1$) was estimated to be minimal. This also possibly indicates that the protein (as much as 6 wt %) does not penetrate the gel or condensed phase and does not perturb the molecular areas of the lipids in that phase significantly. At 12 wt %, the lipid–protein packing appears to follow a different pattern in which the F-SP-C does somehow influence the gel phase packing in addition to its effect on the fluid phase lipid. This result is consistent with previous ones for high amounts of nonfluorescent, native SP-C in DPPC monolayers (Perez-Gil et al., 1992a).

F-SP-C altered the packing of monolayer phases, as indicated by the decrease in domain size and amount of the condensed phase shown in Figs. 4 *b*, 5, and 7, a behavior consistent with that seen for native SP-C in DPPC monolayers reported previously (Perez-Gil et al., 1992a). Pastrana et al. (1990) have shown by Fourier-transform infrared spectroscopy that SP-C alters the packing of DPPC:DPPG bilayers so the lipids in lipid/SP-C mixtures exhibit more fluid characteristics than the lipids alone. In bilayers of saturated lipids, increasing amounts of SP-C broadened the range of the gel-to-fluid transition, decreased the cooperativity, and caused a reduction in the calorimetrically detectable enthalpy of the transition in a concentration-dependent manner (Shiffer et al., 1993; Simatos et al., 1990; Vandenbussche et al., 1992). These studies indicate that SP-C can alter lipid packing in bilayers. A decrease of cooperativity of the gel-to-fluid transition is associated with the protein's "removing" a certain number of lipids that take part in that transition or modifying the way in which the overall chain melting occurs. As has been shown in this study, F-SP-C affected the condensed phase formation and growth in DPPC and DPPC:DPPG monolayers in a concentration-dependent manner (Figs. 4 *b*, 5, and 7). Reaching similar amounts of condensed phase with increasing amounts of protein in the lipid monolayers required higher surface pressures. Fig. 5 shows that 12 wt % F-SP-C in DPPC and DPPC:DPPG monolayers decreased the % condensed from 80% to 30% at a π of 30 mN/m. As discussed previously, the appearance of more, smaller domains in the presence of protein is likely due to a competition between the disrupting effect of SP-C on the packing and the need to pack into condensed phase caused by increasing applied pressure, resulting in more nucleation sites, which grow in size with increasing π (Perez-Gil et al., 1992a). These experiments provide direct evidence for the location of SP-C in the LE phase, something that could only be implied in the previous research (Perez-Gil et al., 1992a).

The LC domain shapes seen in the DPPC/F-SP-C monolayers (Fig. 2 *b*, image *B*) showed protrusions on their boundaries and were different from the more circular or

elliptical ones found in pure DPPC monolayers. This is consistent with the presence of lipid–protein association in the monolayers studied. Similar LC domain shapes (with protrusions) have also been observed in lipid–protein monolayers by others; it has been suggested that these shapes are due to protein-mediated instabilities occurring at the LC/LE domain boundaries (Möhwald et al., 1988). The qualitative effects of F-SP-C on DPPC and DPPC:DPPG systems were similar in both the presence and the absence of calcium; that is, the protein produced more, smaller LC domains in all cases. This suggests that the interaction of SP-C with the lipids in these systems is governed primarily by hydrophobic forces, as implied from studies with native SP-C (Taneva and Keough, 1994a; Taneva and Keough, 1995).

Pulmonary surfactant is presumed to provide a DPPC-rich monolayer at the air–alveolar fluid interface, which reduces surface tension of that interface. In the lung alveoli the surface tension has been directly measured to be very low (corresponding presumably to very high surface pressures) at low lung volumes (Schürch et al., 1976), and it is currently believed that the alveolar fluid interfacial surface pressure that is due to the PS monolayer varies from 40 to 70 mN/m during normal breathing (Goerke and Clements, 1986). Other PS components such as SP-C may be squeezed out of such monolayers during compression to maintain a low surface tension (or high π) at the air–alveolar-fluid interface. It is hard to estimate from this study the exact amounts of F-SP-C present in the monolayers at very high π because of the difficulty in obtaining quantitative estimates of the highly irregular fluorescently labeled areas at these pressures. The results, however, indicate that some SP-C may be present in the monolayers at high π (see, for example, Fig. 2 *b*, images *D* and *E*) and support other studies that also indicated that small amounts of protein could be associated with the films at high surface pressures (Taneva and Keough, 1994a,b). This fact is also reflected in the percent-condensed plots in Fig. 5, where ~90% of the monolayer area was occupied by the condensed phase above the collapse π of SP-C. These results are consistent with those of Taneva and Keough (1994a) that suggest that small amounts of SP-C remain in the monolayer up to very high π but that at high SP-C concentrations exclusion of some SP-C-lipid units occurs. The α-helix of SP-C can have preferred orientations in monolayers, depending on the π (Creuwels et al., 1993). With increase in π (from 10 to 30 mN/m), the α-helical axis could change from being parallel to the plane of the monolayer to being perpendicular to it (Creuwels et al., 1993). F-SP-C may be following a similar course, with the more perpendicular orientation allowing for some protein to remain in such monolayers at high π (above the collapse π of the protein). The presence of the protein at high π may also support a functional role for SP-C in rapidly respreading lipids from compressed monolayers, as suggested elsewhere (Taneva and Keough, 1994b).

A recent ^{2}H-NMR study indicated that the charged *N* terminal of SP-C is located at the surface of the DPPC bilayer, near the polar headgroup region of the lipid (Morrow et al., 1993). SP-C was found to order the bilayer membrane surface of DPPC:DPPG (7:1 mol/mol), partially immobilizing the lipid headgroups (Horowitz et al., 1992). Inasmuch as the *N*-terminal region of SP-C also contains the cationic residues arginine and lysine, the protein has the potential for electrostatic interactions with anionic lipids (Shiffer et al., 1993). Our monolayer data do not allow us to confirm that such an association occurs, but the percent-condensed-π plots (Fig. 5, bottom panel) may indicate some interaction of F-SP-C with the anionic headgroup of DPPG. At 12 wt % F-SP-C the percent condensed profile of DPPC:DPPG in Fig. 5 (bottom panel) indicates a slightly higher amount of gel phase formation in such monolayers than in DPPC at similar π. This observation could also be interpreted in terms of SP-C inducing a slight ordering effect on DPPC:DPPG monolayers compared with DPPC. In Fig. 7 *a* a similar ordering effect of pure DPPC:DPPG monolayers under the influence of calcium can be seen, as higher amounts of condensed phase were reached at a lower π than in the calcium-free system. Bilayers of DPPG plus SP-C have been shown to exhibit chain melting transitions at temperatures (T_c) 20–25°C higher than in DPPG alone (Perez-Gil et al., 1994; Shiffer et al., 1993). The higher T_c of the bilayers indicates that they are more densely packed and ordered than those in the absence of protein. Thus F-SP-C may have some electrostatic interactions with anionic lipid monolayers, but they cannot be isolated in this study, possibly because the hydrophobic lipid–protein interactions overshadow the electrostatic interactions.

Fluorescence microscopy of anionic lipid monolayers has shown that calcium can condense or increase the amount of LC phase of phosphatidylglycerol (Evert et al., 1994; Leckband et al., 1993; Nag et al., 1994) and other anionic lipids (Losche and Möhwald, 1989) compared with its effect on DPPC. This condensation was observed as an increase in formation and growth of LC regions of such monolayers at lower π compared with what was observed in DPPC (Evert et al., 1994; Losche and Möhwald, 1989; Nag et al., 1994). Our observations of DPPC:DPPG-Ca monolayers are similar to those found in the previous studies. As shown in Fig. 7 *a*, higher amounts of condensed phase were found in all DPPC:DPPG and DPPC:DPPG/F-SP-C monolayers in the presence of calcium than in the monolayers without calcium (Fig. 5, bottom panel). Also, the shapes of the LC domains in all DPPC:DPPG/F-SP-C monolayers in the presence of calcium (Fig. 7 *b*, images *A-D*) showed flowerlike structures and were different from those seen in monolayers without calcium. These structural changes in DPPC:DPPG/F-SP-C condensed domains may indicate that calcium-mediated effects on the lipid packing persist in monolayers containing the protein. The condensation of the acidic lipids in monolayers is possibly due to dehydration of the anionic lipid headgroups by calcium, which leads to an ordering of the lipid acyl chains at low π (Flach et al., 1993). The occurrence of a calcium-induced lateral phase segregation of PG-rich LC phase may also be possible (Leckband et al., 1993), where the total LC phase is a mixture of DPPG-Ca-

rich segregated phase plus the normal surface-pressure-induced LC phase.

The plots of % condensed π for DPPC:DPPG monolayers in the presence of calcium (Fig. 7 *a*) show that 95% condensed phase is reached at π of 15 mN/m, compared with a requirement for twice the π (30 mN/m) to reach similar amounts of condensed phase in the monolayers in the absence of the cation (Fig. 5). For the most part, inclusion of F-SP-C in DPPC:DPPG-Ca monolayers caused relatively larger reductions in the % condensed than in the DPPC:DPPG monolayers [Compare Figs. 5 (bottom panel) and 7 *a*.]. This observation suggests that the protein partially overrides the condensing effect of calcium and continues to perturb the monolayers' lipid packing. It has been suggested that α-helical peptides interact with an air–water or a lipid–water interface according to their hydrophobicity (Krebs and Phillips, 1983), and the highly hydrophobic F-SP-C even disrupted the monolayer packing of an ionically condensed monolayer. The perturbing effects of the protein over the condensing effect of cations demonstrated a predominance of hydrophobic forces over electrostatic ones in the lipid–protein interactions.

Recent studies of mellitin in lipid films have shown that a dehydration of the lipid headgroups is associated with the protein's assuming a different secondary structure in the lipid environment (De Jongh et al., 1994). Creuwels et al. (1993) have shown that with increase in π the α-helix of SP-C undergoes an orientational change from parallel to perpendicular relative to the monolayer plane. Because calcium can dehydrate the DPPG headgroup, F-SP-C might possibly have had a different orientation in the DPPC:DPPG monolayers when calcium was present and perturb packing in such monolayers differently from DPPC or PG systems without the cation.

SP-C can induce packing rearrangements (increase fluidity or decreased condensed phase formation) in neutral and anionic lipid arrays. Fluid lipid bilayers have higher rates of adsorption to the air–water interface than do gel state bilayers. SP-C may enhance the capacity of lipids, neutral or anionic, to adsorb at an air–water interface by causing packing perturbations and increasing fluidity in monolayers, or it might provide sites of dislocation in bilayers so cooperative lipid transfers to the interface might be initiated. When calcium was present the relative perturbation of the anionic lipid system by SP-C seemed to increase, consistent with enhanced adsorption and lipid insertive processes seen previously by others (Creuwels et al., 1993). This study indicates that the α-helical protein SP-C is dispersed in the fluid (LE) phase and confirms the prediction of a previous study (Perez-Gil et al., 1992a). It also confirms that such proteins may remain in the monolayer at high surface pressure, in regions laterally excluded from the condensed phase.

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