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ARTICLE

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Microstructure and dynamic surface properties of surfactant protein SP-B/dipalmitoylphosphatidylcholine interfacial films spread from lipid-protein bilayers

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Abstract Suspensions of dipalmitoylphosphatidylcholine (DPPC) bilayers containing 5, 10 or 20% (w/w) surfactant protein SP-B have been reconstituted and spread at air-liquid interfaces. Compression isotherms of DPPC/SP-B monolayers spread from these preparations were qualitatively comparable to the isotherms of the corresponding DPPC/SP-B monolayers spread from solvents. SP-B was squeezed-out at higher pressures from vesicle-spread films than from solvent-spread monolayers. SP-B caused a marked decrease on the rate of relaxation of DPPC collapse phases to equilibrium pressures in all the lipid/protein films assayed. This stabilizing effect was higher in vesicle-spread than in solvent-spread monolayers. Inclusion in the films of traces of the fluorescent probe NBD-PC (1 mol%) and use of a fluorescent derivative of SP-B labeled with a rhodamine derivative, Texas Red, allowed for direct observation of protein and lipid domains at the interface by epifluorescence microscopy. Upon compression, SP-B altered the packing of phospholipids in the bilayer-spread films, observed as a SP-B-induced reduction of the area of liquid-condensed domains, in a way similar to its effect in solvent-spread monolayers. SP-B was not associated with condensed regions of the films. Fluorescence images from vesicle-spread films showed discrete fluorescent aggregates that could be consistent with the existence of lipid-protein vesicles in close association with the monolayer. Both the retention of SP-B at higher surface pressures and the greater stability of collapse phases of DPPC/SP-B films prepared by spreading from liposomes in comparison to those spread from solvents can be interpreted as a consequence of formation of complex bilayer-monolayer interacting systems.

Key words Pulmonary surfactant · Lipid-protein interactions · Interfacial films · Lipid-protein monolayers

Abbreviations *DPPC* 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine · *NBD-PC* 1-palmitoyl-2-{12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl} phosphatidylcholine · *SP-B* surfactant Protein B · *SP-C* surfactant Protein C · *SDS-PAGE* sodium dodecyl sulfate polyacrylamide gel electrophoresis · *TR* sulforhodamine 101 sulfonyl chloride (Texas Red) · *TR-SP-B* Texas Red-labeled SP-B

Introduction

The ability of pulmonary surfactant to stabilize the lung surface against physical forces is critically dependent on the presence of some specialized proteins which promote rapid formation of a phospholipid-based monolayer, possibly in association with bilayers, at the air-liquid interface (for a review see Pérez-Gil and Keough 1998). Hydrophobic surfactant-associated proteins SP-B and SP-C are synthesized and co-assembled with surfactant phospholipids in type II cells in the form of bilayer-based structures, called lamellar bodies, which are secreted into the alveolar aqueous hypophase. Although the physiological functions of SP-B and SP-C have been related to the ability of surfactant to form and sustain surface active films, the molecular mechanisms by which the proteins transport, arrange, and modulate surfactant lipids are still relatively unknown (Hawgood et al. 1998; Johansson 1998; Pérez-Gil and Keough 1998). Both SP-B and SP-C improve surface activity of surfactant phospholipids as assayed in vitro but specific functional differences between the two proteins are not well understood. In humans, inherited deficiencies in SP-B are

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lethal at birth, as are engineered SP-B "knock-out" pathologies in mice (Clark et al. 1995; Nogee et al. 1993). However, SP-B deficiencies are accompanied by defective processing of SP-C, making it difficult to assign the observed pathological effects unequivocally to dysfunction of only one of the proteins.

Surfactant protein SP-B has been implicated in the transport and transfer of surfactant phospholipid molecules from bilayer-based structures in the hypophase to the air-liquid interface of lungs, where they form stable monolayers capable of reducing surface tension to very low values, a capacity conveyed by the main surface-active phospholipid, dipalmitoylphosphatidylcholine (DPPC). SP-B could participate in (1) bilayer-monolayer contacts, (2) bilayer destabilization, and (3) direct interfacial insertion of phospholipid. Several studies have reported that SP-B perturbs the packing of phospholipid bilayers that could be related to promotion of bilayer-monolayer transitions (Cochrane and Revak 1991; Oosterlaken-Dijkterhuis et al. 1991a, b; Pérez-Gil et al. 1995; Poulain et al. 1992; Wang et al. 1996). The presence of SP-B in phospholipid monolayers catalyzes interfacial insertion of phospholipid (Oosterlaken-Dijkterhuis et al. 1991a, b) and SP-B improves respreading of surfactant phospholipid monolayers compressed to collapse (Nag et al. 1997; Taneva and Keough 1994). Most of the studies on the effects of SP-B in surfactant films have been carried out in monolayers spread from organic solvents. Although previous studies have shown that solvent-spread SP-C/phospholipid monolayers are similar to monolayers adsorbed from bilayers (Nag et al. 1996), this has not been demonstrated for SP-B-containing systems. In determining potential equivalence for SP-B-containing films formed by spreading versus adsorption, one must consider that the nature of the interaction of SP-B with phospholipid bilayers and its consequent surface activity is dependent on the method used to reconstitute lipid-protein samples (Cruz et al. 1997, 1998).

Since films are formed from bilayer structures in vivo, the objective of the present work has been the anaylsis and comparison of the structure and dynamic behavior of SP-B-containing DPPC monolayers spread from vesicles or organic solvents.

Materials and methods

Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-{12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl}phosphatidylcholine (NBD-PC) were purchased from Avanti Polar Lipids (Pelham, Alao, USA). Lipids were checked for purity by silica-gel thin layer chromatography and used as received. The fluorescent-labeling chemical sulforhodamine 101 sulfonyl chloride, Texas Red (TR), was obtained from Molecular Probes (Eugene, Oreo, USA). Chloroform and methanol were HPLC grade solvents from Fisher Scientific (Ottawa, Ontario, Canada) or from Scharlau (Barcelona, Spain).

Isolation and labeling of SP-B

Surfactant protein SP-B was isolated from minced porcine lungs as described elsewhere (Pérez-Gil et al. 1993) and stored at −20 °C in chloroform-methanol (2:1, vol/vol). Purity of the protein was routinely checked by SDS-PAGE and quantitated by amino acid analysis. Labeling of SP-B with TR was carried out as previously described (Nag et al. 1997). Briefly, the apparent pH of a solution containing 500 μg of purified SP-B in 6 mL of chloroform-methanol (2:1, vol/vol) was adjusted to 7.8 by addition of aliquots of 50 mM Tris in methanol. Derivatization at pH 7.8 has been demonstrated to preferentially procede through the N-terminal amine group of the protein, which is less alkaline than lysine sidechain amines. Fluorescent labeling was initiated by addition of 20 μL of 10 mM TR and the mixture was incubated at 4 °C overnight. The pH of the solution was adjusted to 2 with 0.1 M HCl and the protein eluted through a column of Sephadex LH-20 (Pharmacia LKB, Sweden) to remove unreacted TR. The chromatographic profile of the LH-20 effluent was followed by absorbance at 240 nm (protein) and 580 nm (TR). Evaluation of the incorporated label was achieved by amino acid analysis and spectroscopic determination of attached TR ($\varepsilon_{580} = 144 \times 10^3\ M^{-1}\ cm^{-1}$ for TR in chloroform-methanol). Analysis of TR-SP-B by SDS-PAGE and matrix-assisted laser desorption ionization mass spectrometry confirmed that SP-B remained dimeric after derivatization and that it incorporated ~1 mol of TR per monomer.

Reconstitution of SP-B/DPPC bilayers

SP-B-containing DPPC bilayers were prepared either by sequential or simultaneous injection of methanolic lipid and protein solutions into aqueous buffer as previously described (Cruz et al. 1997, 1998). Stock solutions of SP-B (2 mg/mL) and DPPC (50 mg/mL) were prepared in methanol. DPPC bilayers were formed by injecting methanolic phospholipid solution with vigorous vortex-mixing into 0.7 mL of 50 mM Hepes buffer in 150 mM NaCl, pH 7, at 45 °C. This method has been demonstrated to produce homogeneous uni- or pauci-lamellar vesicles of about 100–200 nm in diameter (Cruz et al. 1997; Woodle and Papahadjopoulos 1989). Then 5–10 μL of SP-B in methanol was injected into the suspension. Alternatively, lipid and protein were first mixed in methanol and then injected into the buffer. Controls were prepared by injecting identical volumes of methanol in the absence of protein and/or lipid.

Interfacial adsorption assays

Interfacial adsorption was assayed using a specially designed surface balance similar to that described previously (Camacho et al. 1996; Cruz et al. 1997; Nag et al. 1996; Pérez-Gil et al. 1992a). This balance contained 1.5 mL of subphase at 21 °C, and the change of surface pressure (π) during adsorption (spreading) from vesicles was monitored with a dipping plate of Whatman no. 1 paper attached to the pressure transducer. Assays were done in this balance in two different modes. In one case, samples were analyzed for interfacial adsorption activity by injecting a defined volume of suspended lipid or lipid-protein material into the subphase while stirring continuously. An amount of 1.5 mL of 150 mM NaCl, 5 mM Tris-HCl (pH 7) was placed in the balance, and the baseline surface pressure was recorded for 5–10 min. At zero time, 80 μL of suspension of 80 nmol of DPPC, in the absence or in the presence of 5, 10 or 20% SP-B by weight and suspended by either of the two methods described above, were injected into the subphase. Adsorption was followed by measuring the change in surface pressure with time. This method will be referred to as "adsorption" from vesicles. In the other case, the interfacial spreading activity of the samples was assayed by spreading a small volume of lipid-protein suspension, typically 40 μL containing 40 nmol of DPPC plus the appropriate amount of SP-B, at the subphase surface by gently deposing the suspension drop by drop with a microsyringe. This method, which we shall refer to as "spreading" from vesicles to differentiate it from the former method, still requires transfer of material from the bilayer phase into the monolayer at the interface.

At least three independent preparations were assayed immediately after their formulation for each sample ($n \geq 3$). In some figures, just two of them (representing the extremes) have been presented for clarity. The curves presented are the result of subtracting a baseline measured after injection or deposition of the corresponding volumes of buffer containing proper amounts of methanol.

Formation of monolayers using organic solvents

Solvent-spread monolayers were prepared by carefully depositing 20–30 μL of a DPPC solution (1 mg/mL) in chloroform-methanol (3:1, vol/vol), containing the desired amount of SP-B on a subphase of 150 mM NaCl, 5 mM Tris-HCl (pH 7). All the subphases were made with doubly glass-distilled water, the second distillation performed from dilute potassium permanganate. After spreading of each monolayer, the organic solvent was allowed to evaporate and the monolayer to equilibrate for 30 min.

Monolayers formed by spreading from vesicles

Monolayers were also formed by spreading of suspensions of lipid-protein vesicles. A volume of 100–500 μL of suspensions of DPPC vesicles (1 mg/mL), prepared by the different methods described above and containing various amounts of SP-B, were carefully deposited with a microsyringe on top of the saline subphase until the surface pressure was raised to 0.5 mN/m. Then, the monolayer was equilibrated for 30 min before measuring the isotherms.

π-A and monolayer relaxation measurements

Solvent-spread or vesicle-spread monolayers were tested on an epifluorescence surface balance (Nag et al. 1990). The trough of the balance had a total surface of 160 cm^2 and all experiments were performed at a temperature of 21 ± 1 °C. To obtain compression isotherms, monolayers were compressed at 707 mm^2/s until collapse, which occurred at π over 65 mN/m in all the films assayed.

To measure relaxation kinetics, DPPC and DPPC/SP-B monolayers, prepared by spreading from solvent or adsorption from vesicles, were compressed at 707 mm^2/s until they reached maximal surface pressure, π of 65–70 mN/m. Then compression was stopped and surface pressure was monitored for 30 min.

Epifluorescence experiments

To visualize monolayers with the epifluorescence microscope, the films were labeled with 1 mol% NBD-PC or the fluorescently labelled form of SP-B (TR-SP-B) or both. Switching fluorescence filter combinations allowed for observation of fluorescence emissions from either NBD-PC (at 520 nm) or TR-SP-B (at 590 nm). Monolayers in these experiments were compressed at a slow speed (20 mm^2/s). At selected surface pressures, compression was stopped and, after 3 min of equilibration, a video recording was made for a 1-min period for both NBD and TR fluorescence. Images were digitized and analyzed using the program Scion Image (Scion Corporation, Md., USA). Quantitation of the properties of images from the compression isotherms was achieved by estimation of the total amount of nonfluorescent areas (presumably probe-excluding, condensed regions) relative to the total monolayer area scanned (Heckl et al. 1987; Nag et al. 1991; Pérez-Gil et al. 1992b; Peschke and Möhwald 1987).

Results

Figure 1 shows isotherms illustrating the kinetics of formation of surface films from suspensions of DPPC bilayers in the absence or presence of 5, 10 or 20% (w/w) SP-B. The protein dramatically improves the ability of DPPC to spread and adsorb at the air-liquid interface in all the preparations assayed. In the absence of protein,

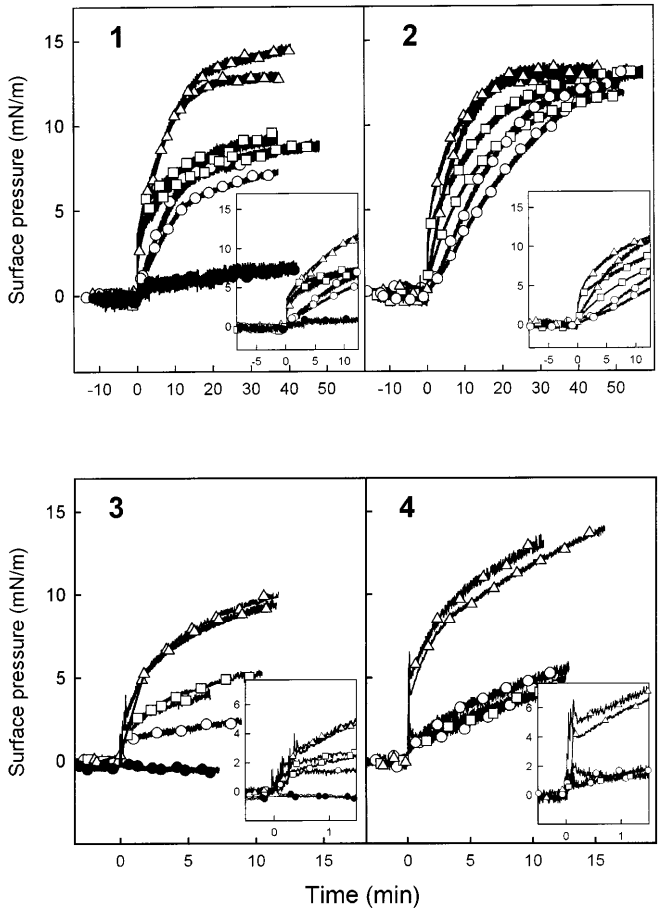

Fig. 1 Adsorption kinetics π-t isotherms of dispersions of DPPC (●) and DPPC plus 5% (○), 10% (□), or 20% (△) w/w SP-B, prepared by addition of the protein to preformed lipid vesicles (panels **1** and **3**) or by injection of lipid-protein mixtures in methanol into aqueous buffer (panels **2** and **4**). Adsorption assays were carried out at 21 °C as described in Materials and methods, either by injection of suspensions into 1.5 mL of a subphase of 150 mM NaCl in 5 mM Tris buffer (pH 7) (panels **1** and **2**) or by directly depositing and spreading the samples on top of the subphase (panels **3** and **4**). *Inserts* expand the time scale of the respective plots

suspensions of DPPC bilayers hardly adsorbed to the interface, no matter if the lipid suspension was introduced into the subphase or directly deposited at the surface. The presence of increasing amounts of SP-B in DPPC bilayers caused a progressive improvement of the adsorption kinetics of the lipid. Both the extent of SP-B-induced DPPC adsorption to the interface (evaluated by the increment in surface pressure) and the dependence of this activity on the protein content seem to be comparable in the two types of bilayer preparations, formed either by injection of lipid-protein organic mixtures into buffer or prepared by addition of SP-B to preformed bilayers. Similar surface activity of the two types of DPPC/SP-B assemblies, at short but not long times after sample reconstitution, was reported previously for a single lipid-protein ratio (Cruz et al. 1997).

Adsorption kinetics of samples directly deposited at the interface show two distinct stages. Initially, there is a steep increase in surface pressure, probably a consequence of immediate transfer of some of the lipid and protein molecules to the open surface. Then, in a second stage, there is a further increase in surface pressure which is qualitatively and quantitatively similar to the π-t kinetics observed in samples injected into the subphase. This fact suggests that the limiting step in monolayer formation occurs at the interface, and probably consists of the opening of bilayers and transfer of assemblages of lipid molecules to the interfacial monolayer. Considering these results, all other monolayers studied in the present work have been prepared by spreading lipid-protein suspensions at the interface, because the amount of material required to form the monolayers this way is much smaller than required to form them by dispersing the material into bulk subphase, especially in large surface balances such as those used to run compression isotherms and epifluorescence experiments.

Figure 2 shows surface pressure-area per molecule (π-A) isotherms of DPPC monolayers in the absence or in the presence of 5, 10 and 20 wt% SP-B. The figure compares behavior under compression of solvent-spread monolayers and monolayers formed by spreading lipid-protein suspensions. Compression isotherms of monolayers adsorbed from DPPC/SP-B bilayers are qualitatively similar to those of DPPC/SP-B monolayers formed from chloroformic lipid-protein mixtures. As previously seen in solvent-spread films (Nag et al. 1997), the presence of increasing amounts of SP-B caused expansion of the isotherms. All the films showed liquid expanded to liquid condensed plateaus in the region of 7–10 mN/m. Lipid-protein monolayers spread from

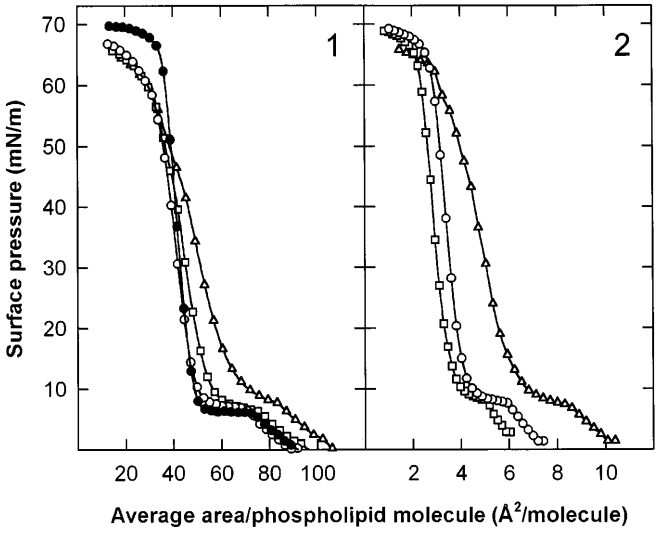

Fig. 2 Compression π-A isotherms of DPPC monolayers (●) and DPPC monolayers containing 5% (○), 10% (□), or 20% (△) w/w SP-B, prepared by spreading lipid-protein mixtures in chloroform-methanol 3:1 (vol/vol) (panel **1**) or by spreading DPPC/SP-B vesicles reconstituted by hydration of methanolic lipid-protein mixtures (panel **2**). Subphase was 150 mM NaCl in 5 mM Tris buffer (pH 7), temperature 21 °C, and compression rate of 707 mm^2/s (corresponding to 4.3 $Å^2$/mol phospholipid/s in solvent-spread films)

vesicles showed much lower apparent area per molecule, presumably because a substantial part of the deposited material did not remain at the air-liquid interface. Some of the lipid-protein vesicles probably diffused to the bulk hypophase. Some of the bilayers might form associations with the interfacial monolayer. In either of these cases the apparent area per molecule in the monolayer, based on the amount deposited, would be low. Isotherms of monolayers adsorped form DPPC/SP-B bilayers prepared by injection of SP-B in methanol into preformed phospholipid vesicles were qualitatively and quantitatively similar to those shown in Fig. 2, corresponding to monolayers adsorbed from lipid-protein bilayers prepared from methanolic lipid-protein mixtures (data not shown).

Isotherms of solvent-spread SP-B-containing DPPC monolayers show inflections at π around 46–48 mN/m (Fig. 2, panel 1) that probably correspond to the exclusion of SP-B molecules from the interface. Exclusion of native SP-B in solvent-spread DPPC monolayers under quasistatic compression has been found to occur at around 40 mN/m (Taneva and Keough 1994), but at higher pressures (around 46 mN/m) under conditions of compression similar to those used in the present work. (Nag et al. 1997). Isotherms of DPPC/SP-B films formed from bilayers show some evidence of protein squeeze-out near 48 mN/m, as noted by the existence of an inflection at that pressure. However, the π-A isotherms of films formed from the DPPC/SP-B bilayers containing different protein proportions converge at somewhat higher pressures than those formed by spreading different DPPC/SP-B organic mixtures (seen in panel 1). If convergence of isotherms of the films containing different amount of protein is interpreted as a consequence of the total exclusion of the protein from the interface occurring at those pressures, DPPC/SP-B films formed from bilayers retain at least part of the protein at higher pressures, above 60 mN/m, than do the films spread from solvents. Since films of DPPC may be in long-lived metastable states (Horn and Gershfed 1977), it may be also possible that the inflections in the isotherms at high pressures are the result of partial film collapse promoted by the presence of SP-B, rather than just exclusion of SP-B alone.

The effect of SP-B on the relaxation kinetics of the different DPPC-based films, when compressed to the collapse point of the film, is shown in Fig. 3. Although it has been seen that pure DPPC films compressed to collapse pressure can sustain very high surface pressures, in the range of 70–72 mN/m, over moderately long periods of time (Notter et al. 1980; Schürch et al. 1978), solvent-spread DPPC monolayers relaxed to equilibrium pressures in the surface balance used in this work in about 15 min. This difference may have occurred because of differences in compression rates and extent of compression before reaching the collapse plateaus, and for reasons of balance design. The presence of 5% by weight of SP-B in DPPC significantly slowed film relaxation. Higher amounts of protein, 10 or 20%, caused

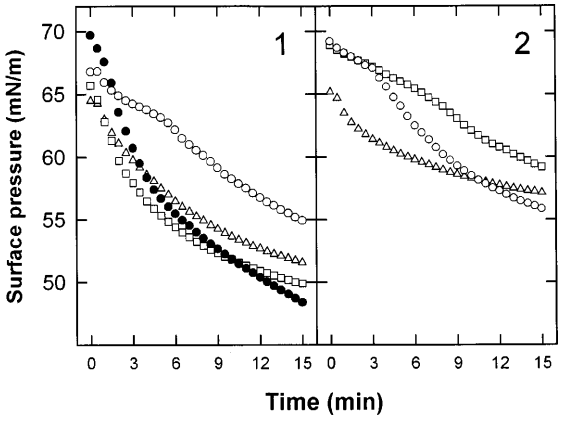

Fig. 3 Relaxation kinetics π-t isotherms of DPPC monolayers (●) and DPPC monolayers containing 5% (○), 10% (□), or 20% (△) w/w SP-B, after compressed to the collapse pressure. Monolayers were formed by spreading from organic solvents (panel **1**) or by spreading lipid-protein vesicles reconstituted by injection of organic lipid-protein mixtures into aqueous buffer (panel **2**)

negligible effects on the stability of film collapse, suggesting that a balance between the presence of SP-B and high DPPC concentration at the interface influences stability of the highest pressures. The stabilizing effect of SP-B on DPPC compressed to collapse was remarkably greater in the films formed by adsorption from lipid-protein bilayers. DPPC films formed from liposomes containing SP-B relaxed very slowly in comparison to protein-free monolayers.

Figure 4 shows typical images observed by epifluorescence microscopy during compression of DPPC and DPPC/SP-B monolayers prepared by solvent-spreading or spreading from lipid-protein vesicles. The presence of SP-B caused a typical reduction in the size and increased the number of liquid condensed domains (dark regions). A similar effect has been described for other proteins upon interaction with phospholipid monolayers (Heckl et al. 1987; Pérez-Gil et al. 1992b; Ruano et al. 1998). Inclusion of TR-SP-B confirmed that the protein partitioned into the liquid-expanded regions of DPPC monolayers (bright regions seen with NBD-PC). The effect of SP-B in increasing the number of condensed domains and the preferential location of the protein in fluid lipid areas were similar in solvent-spread monolayers and monolayers adsorbed from bilayers.

The effect of the protein on the condensation of DPPC monolayers was quantitated by (1) the total amount of condensed phase as a percentage of the total monolayer area (% condensed); (2) the average area of the domains; and (3) the average number of condensed domains per frame, as a function of the surface pressure (Fig. 5). The quantitative data confirmed that SP-B caused a significant reduction in size of the condensed domains in solvent-spread monolayers, as previously reported (Nag et al. 1997). This effect was very similar with native and rhodamine-labeled proteins, indicating that derivatization of the protein did not significantly alter its properties. Condensed domains of DPPC in the

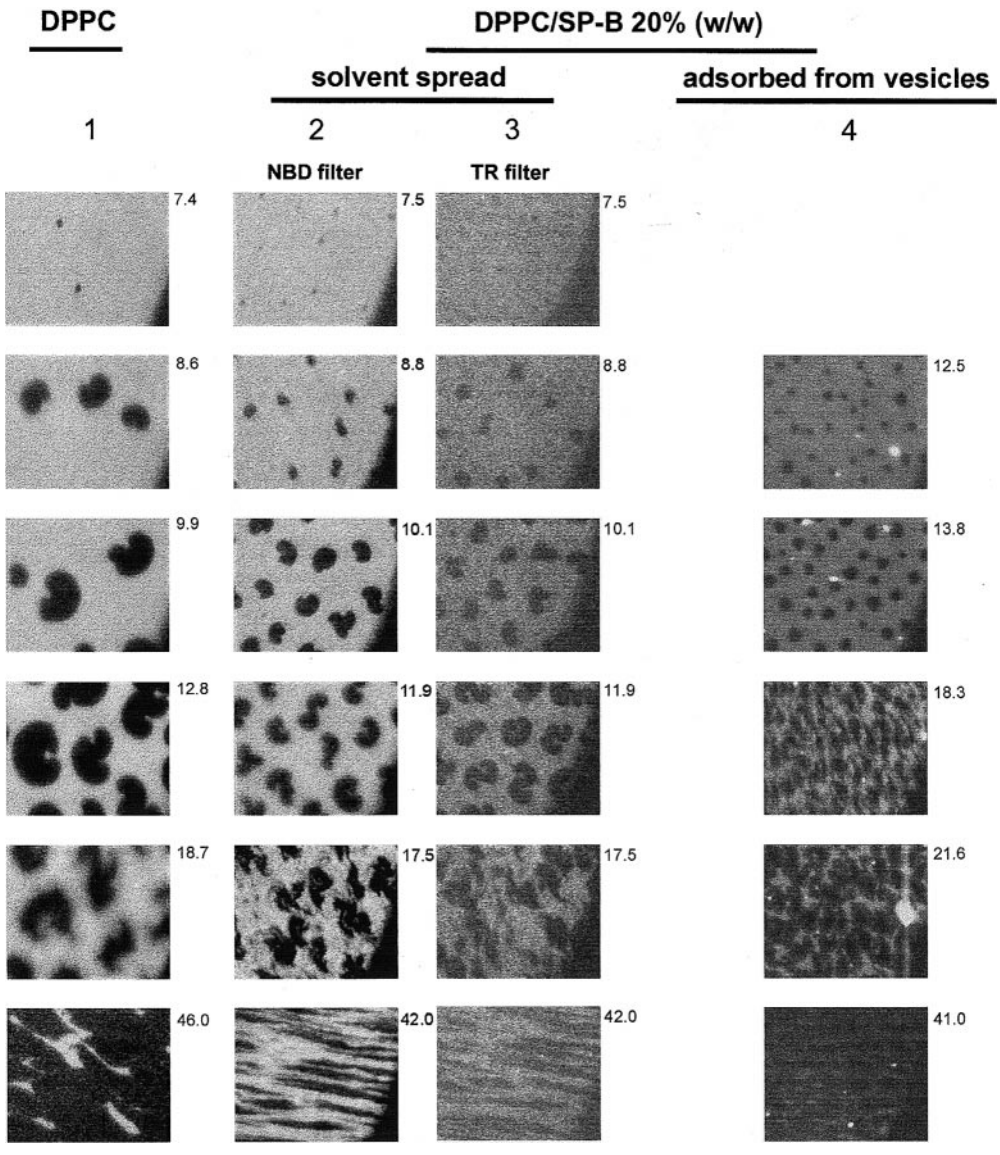

Fig. 4 Typical epifluorescence microscopy images from DPPC monolayers containing 1 mol% NBD-PC in the absence (column *1*) or presence of 20% (w/w) SP-B (columns *2–4*). Images were recorded through filters selecting fluorescence coming either from NBD-PC (emission centered at 520 nm) (columns *1*, *2*, and *4*) or TR-SP-B (centered at 590 nm) (column *3*). Monolayers were prepared by spreading of organic solutions (columns 1–3) or lipid-protein vesicles formed by co-injection of lipid and protein into buffer (column *4*)

presence of SP-B were smaller in monolayers adsorbed from vesicles than in solvent-spread monolayers. However, there were greater numbers of those smaller domains in the vesicle-spread monolayers, resulting in similar extents of total condensation. Monolayers prepared by spreading SP-B/DPPC bilayers were similar in both distribution of fluid and condensed regions and preferential distribution of SP-B into lipid-expanded areas at pressure where there was expanded and condensed phase coexistence, no matter the method used to prepare lipid-protein suspensions (data not shown). All films, those of pure DPPC and those containing SP-B, showed similar pressure-dependent condensation curves. This indicates that SP-B does not perturb the packing of DPPC molecules as much as surfactant protein SP-C, a finding consistent with previous observations (Morrow et al. 1993; Nag et al. 1997; Pérez-Gil et al. 1992b).

Observation of DPPC/SP-B monolayers using NBD-PC adsorbed from fluorescently labeled vesicles revealed the presence of highly fluorescent aggregates, the sizes and distribution of which (Fig. 6) did not seem to depend on surface pressure (see Fig. 4). These fluorescent spots were absent in solvent-spread lipid-protein films. The sizes and relative frequencies of such fluorescent aggregates from the two kinds of vesicle-adsorbed monolayers is presented in Fig. 6. The population of fluorescent aggregates in monolayers formed from bilayers prepared by addition of protein to preformed lipid vesicles had higher mean and standard deviation than that of the population of fluorescent aggregates observed in monolayers adsorbed from DPPC/SP-B vesicles formed from methanolic lipid-protein solutions. The two observed distributions resemble the differences in size of the lipid-protein vesicles in the two assayed preparations (Cruz et al. 1997). The fluorescent spots observed in the images could therefore come from the presence of lipid-protein vesicles or aggregates directly associated with the interfacial films.

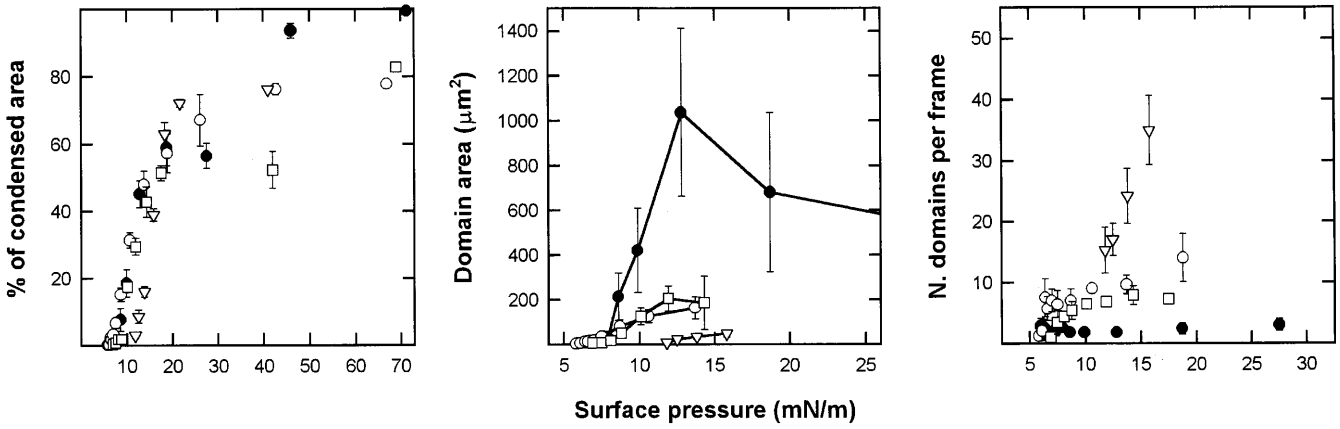

Fig. 5 Quantitative analysis of condensed phase in monolayers of DPPC (●) and monolayers of DPPC containing 20% (w/w) SP-B (○, ▽) or TR-SP-B (□), as observed under epifluorescence microscopy. Data presented are from solvent-spread monolayers (●, ○, □) or monolayers adsorbed from lipid-protein vesicles prepared by addition of SP-B to preformed DPPC bilayers (▽). Percent of total condensed area (*left*) and average area (*center*) and number (*right*) of the condensed domains are plotted against surface pressure for the various monolayers assayed. Values are $\bar{x} \pm$ SD for $n = 10$ images. Error bars not shown are within the symbol sizes

Discussion

The samples studied in this work confirm that surfactant protein SP-B catalyzes rapid transition of DPPC from bilayers to interfacial monolayers. In this work, we have compared monolayers adsorbed from lipid-protein bilayers, either reconstituted by addition of SP-B to preformed DPPC bilayers or prepared by injection of methanolic lipid-protein mixtures into aqueous buffer,

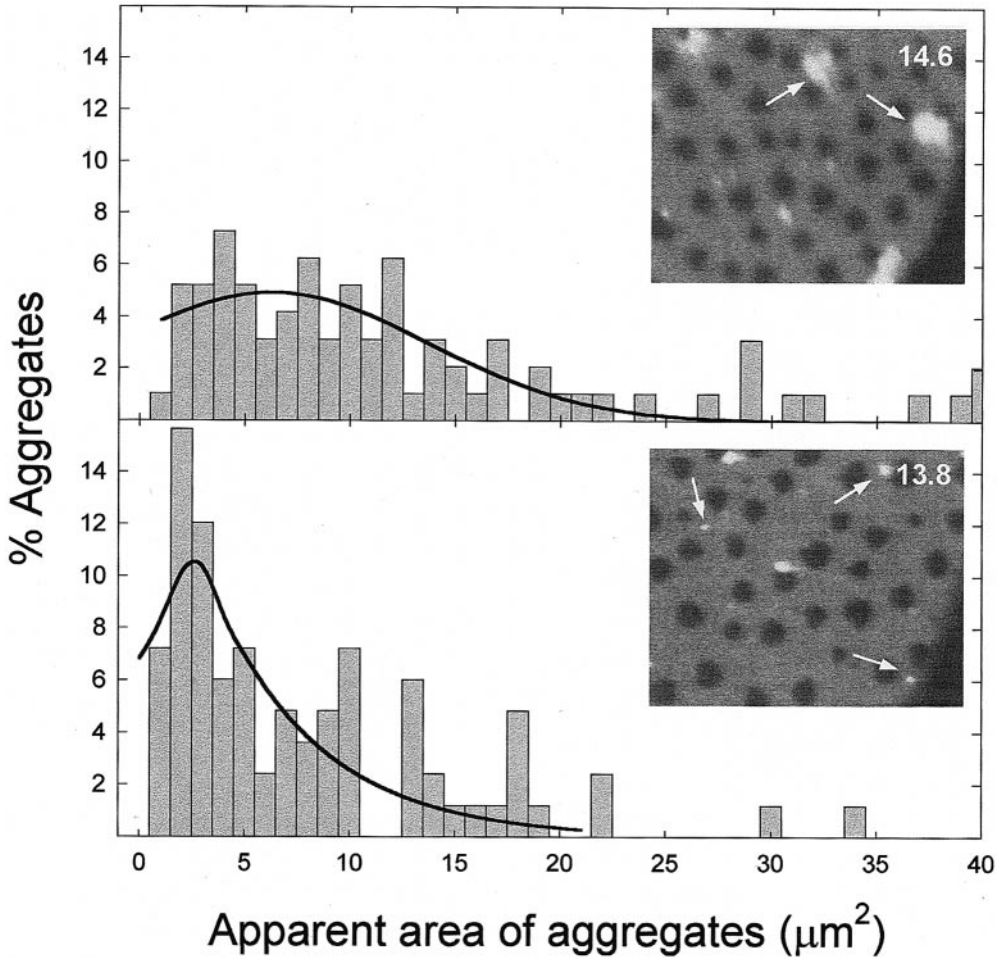

Fig. 6 Frequency distributions of areas of the fluorescent aggregates observed in DPPC monolayers containing 20% (w/w) SP-B formed by adsorption from lipid-protein vesicles prepared either by addition of SP-B to preformed DPPC vesicles (*upper panel*) or by co-injection of lipid and protein into aqueous buffer (*lower panel*). *Inserted micrographs* show representative fluorescent aggregates (*white arrows*) at the indicated surface pressures. As the size of the aggregates were independent of surface pressure, frequencies were calculated by measuring the aggregates observed at all the surface pressures analyzed in the isotherms

with DPPC/SP-B monolayers spread from solvents. Although the phospholipid profile of surfactant is much more complex than pure DPPC, we have centered the present study on characterizing films originated from DPPC/SP-B bilayers because surfactant films are supposed to be highly enriched in this phospholipid, especially after dynamic cycling. We have extensively characterized the mode and extent of interaction of SP-B with DPPC bilayers in previous studies (Cruz et al. 1997, 1998; Morrow et al. 1993; Pérez-Gil et al. 1995), and this system was therefore a good starting model to study both the structure and properties of films formed after bilayer-monolayer transitions. DPPC/SP-B bilayers showed good adsorption kinetics when assayed either by dispersion into saline subphases or by direct spreading on top of these subphases.

This work demonstrated that DPPC/SP-B monolayers originated from interfacial adsorption of vesicles are very similar in both microstructure and dynamic behavior to those spread from solvents. Similarities include: (1) expansion of the DPPC isotherm due to insertion or interaction of SP-B; (2) SP-B-induced stabilization of DPPC collapse phases against relaxation; (3) repose of SP-B in liquid-expanded regions of the monolayer; and (4) reduction of the size and increase in the number of condensed domains in monolayers being induced by SP-B without it altering significantly the overall total condensation of the films at any stage of compression. This suggests that solvent-spread and adsorbed films are very similar, if not equivalent. A comparative study of solvent-spread films and monolayers adsorbed after injection of vesicles from samples composed of DPPC and surfactant protein SP-C also concluded that both solvent-spread and adsorbed monolayers were analogous (Nag et al. 1996). In the case of SP-B, equivalence between solvent-spread and all the adsorbed monolayers assayed is of special interest as the extent of interaction of the protein with phospholipid bilayers has been shown to depend critically on the method used to reconstitute the samples (Cruz et al. 1997, 1998). The present results again confirm that solvent-spread interfacial films are good models for naturally formed surfactant monolayers, the latter films being formed in situ by processes of adsorption.

The images in Fig. 4 and the isotherms in Fig. 2 suggest that there is only a small change in π_c in the presence of SP-B. Some differences in the sizes and shapes of DPPC-rich domains are seen with DPPC/SP-B monolayers at pressures just above π_c. These suggest that the effect of SP-B in DPPC is small, a finding consistent with studies in bilayers of DPPC and SP-B (Baatz et al. 1990; Morrow et al. 1993). The effect of SP-B in reducing the size and increasing the number of condensed domains in DPPC monolayers appeared slightly more pronounced in monolayers adsorbed from lipid-protein vesicles than in solvent-spread films. This effect was also observed in DPPC/SP-C monolayers adsorbed from vesicles (Nag et al. 1996). The number and size of the solid domains in monolayers subjected to compression is critically dependent on the speed of compression (Nag et al. 1991). The faster the monolayer is compressed, the smaller are the condensed domains. As a monolayer is adsorbed, so its compression is increased. Rapid adsorption will therefore correspond to rapid compression.

In contrast to SP-C (Nag et al. 1996, 1997; Pérez-Gil et al. 1992b), and even to SP-A (Ruano et al. 1998), SP-B did not have substantial effects on the compression-driven condensation of DPPC films, a feature consistent with previous findings on the influence of SP-B on DPPC and DPPC/DPPG in solvent-spread films (Nag et al. 1997). The present results suggest that SP-B hardly penetrates into the acyl chain region of the monolayer. Other studies have suggested also that SP-B is located rather superficially in bilayers (Baatz et al. 1990; Cruz et al. 1997, 1998; Morrow et al. 1993; Vincent et al. 1991), or that it might interact with the lipid hydrophobic domains to form dimers and aggregates in a way in which only small proportions of the lipid domains are modified by the SP-B (Morrow et al. 1993; Williams et al. 1991). We have suggested that reconstitution of lipid-protein vesicles by co-injection of SP-B and DPPC in buffer leads to some protein penetration into the bilayers (Cruz et al. 1998). The present results suggest, however, that either penetration of SP-B into bilayers is still not enough to perturb acyl chain packing once both lipid and protein are transferred to the interface, or at the interface SP-B molecules preferentially adopt a shallow, nonperturbing, disposition with respect to lipids.

Nevertheless, SP-B stabilized DPPC collapse phases against relaxation. To maintain patency of smaller alveoli, surfactant films must sustain relatively high pressures for some periods of time (Schürch et al. 1978). DPPC-enriched films compressed to very high surface pressures are stable over periods of minutes. However, other lipid and protein components of the surfactant decrease the stability of DPPC films. Surfactant protein SP-C, for instance, has been reported to mobilize and relax DPPC monolayers (Pastrana et al. 1991), probably because its presence perturbs packing of DPPC acyl chains. The presence of SP-C has been proposed also to modulate the rheological properties of surfactant films which could then be easily respread during repetitive respiratory cycles (Nag et al. 1998; Von Nahmen et al. 1997). SP-B might have a role opposite to that of SP-C in modulating the properties of surfactant films at high pressures. Interaction of SP-B with the surface of the films, probably without significant penetration, could help to stabilize DPPC-enriched compressed phases. Effects of SP-B leading to increased lateral stability of the surface layer have been proposed (Cochrane and Revak 1991). A balance between the action of SP-B and SP-C could then lead to surfactant films stably compressible to high pressures but still flexible enough to work under the dynamic conditions imposed by the lung.

Some considerations can be made on the physiological significance of the effects of SP-B in lipid-protein

films shown in this work. The amount of SP-B present in surfactant in vivo is thought to be in the range of 1–2% protein to lipid by weight (Goerke 1998). Although the studies presented here have been conducted using higher protein concentrations with the purpose of increasing the patency of effects, these effects were consistently detected at all the protein concentrations assayed. As no special behavior was detected at protein contents lower than 5% (data not shown), the conclusions with respect to the shallow, nonperturbing disposition of SP-B molecules in the surface films should be essentially valid at more physiological lipid-protein ratios. Other authors have described special effects induced by the presence of SP-B/SP-C mixtures in the condensation of DPPC/DPPG films at protein to lipid ratios as low as 1% (w/w) (Krüger et al. 1999), opening the question of a possible concerted action of SP-B/SP-C complexes. Future studies should therefore explore how the particular behaviour of SP-B reported here or that of SP-C (Nag et al. 1996) is modulated by the simultaneous presence of both polypeptides.

DPPC/SP-B monolayers formed by adsorption of lipid-protein bilayers into the air-liquid interface have differences with respect to the corresponding solvent-spread films that could be of importance. SP-B is sustained in vesicle-adsorbed DPPC monolayers under compression at higher pressures than those needed to exclude it completely from solvent-spread monolayers (seen in isotherms of Fig. 2). Also, the stabilizing effect of SP-B on the condensed phases of DPPC is substantially higher in vesicle-adsorbed than in solvent-spread monolayers. We think that this feature could be explained as a consequence of the existence of vesicles attached to the interfacial monolayers in films formed by adsorption. The fluorescent aggregates observed in the epifluorescence images could be consistent with the existence of monolayer-liposome contacts.

The presence of lipid-protein vesicles attached to or otherwise associated with the interfacial monolayer seems to enhance stability of compressed DPPC phases against relaxation in the presence of SP-B. This feature suggests important implications for the properties of surfactant films in vivo. It has been suggested that the surfactant film in vivo should be considered as a continuous or a punctate multilayer rather than a pure single monolayer system (Schürch et al. 1995), in which a substantial amount of surfactant could be associated with the air-liquid interface in bilayer forms. SP-B has been proposed to catalyze bilayer-monolayer contacts (Oosterlaken-Dijkterhuis et al. 1991a, b). A recent study has shown that compression of phospholipid films containing both SP-B and SP-C leads to a special phase in the surface monolayer that includes formation of three-dimensional, surface-associated structures of micron dimensions (Krüger et al. 1999). The presence of SP-B in monolayer-bilayer complex systems could provide improved stability to surfactant films, especially at the highest surface pressures which are necessary during expiration. Such a property would be important for alveolar stability against collapse and the maintenance of patency and residual volume at end expiration.

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