Interactions of Pulmonary Surfactant Protein A with Phospholipid Monolayers Change with pH

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ABSTRACT   The interaction of pulmonary surfactant protein A (SP-A) labeled with Texas Red (TR-SP-A) with monolayers containing zwitterionic and acidic phospholipids has been studied at pH 7.4 and 4.5 using epifluorescence microscopy. At pH 7.4, TR-SP-A expanded the π-A isotherms of film of dipalmitoylphosphatidylcholine (DPPC). It interacted at high concentration at the edges of condensed-expanded phase domains, and distributed evenly at lower concentration into the fluid phase with increasing pressure. At pH 4.5, TR-SP-A expanded DPPC monolayers to a slightly lower extent than at pH 7.4. It interacted primarily at the phase boundaries but it did not distribute into the fluid phase with increasing pressure. Films of DPPC/dipalmitoylphosphatidylglycerol (DPPG) 7:3 mol/mol were somewhat expanded by TR-SP-A at pH 7.4. The protein was distributed in aggregates only at the condensed-expanded phase boundaries at all surface pressures. At pH 4.5 TR-SP-A caused no expansion of the π-A isotherm of DPPC/DPPG, but its fluorescence was relatively homogeneously distributed throughout the expanded phase at all pressures studied. These observations can be explained by a combination of factors including the preference for SP-A aggregates to enter monolayers at packing dislocations and their disaggregation in the presence of lipid under increasing pressure, together with the influence of pH on the aggregation state of SP-A and the interaction of SP-A with zwitterionic and acidic lipid.

INTRODUCTION

The collectin surfactant protein A (SP-A) is, by weight, the major protein associated with pulmonary surfactant, a lipid-protein material that lines the respiratory epithelium, the main biophysical function of which is to reduce the surface tension at the air-liquid interface of alveoli and thereby stabilize the respiratory surface. Most of the surface active properties of surfactant are associated with its phospholipid components. The major lipid, dipalmitoylphosphatidylcholine (DPPC) is responsible for the ability of this material to produce very low surface tensions at low lung volumes (for a recent review, see Johansson and Curstedt, 1997). Other phospholipid species of surfactant, including phosphatidylglycerol (PG), as well as some specific hydrophobic proteins have a role in modulating the physical properties of DPPC to allow for rapid and continuous formation of DPPC-enriched monolayers at the air-liquid interface of alveoli. SP-A improves the adsorption to the surface of preparations composed of the main surfactant phospholipids and the hydrophobic surfactant proteins (Hawgood et al., 1987). SP-A promotes interfacial adsorption of lipid suspensions in the presence but not in the absence of the surfactant proteins SP-B and SP-C (Schürch et al., 1992). In addition, SP-A is

necessary for the formation of tubular myelin (Suzuki et al., 1989; Williams et al., 1991; Korfhagen et al., 1996), a unique structure of surfactant in the alveolar spaces, whose presence can be correlated with high surface activity. SP-A-defective surfactant isolated from SP-A knock-out mice had impaired properties compared with normal SP-A-containing surfactant when assayed under limited concentration conditions (Korfhagen et al., 1996). This fact suggests that SP-A could improve surface activity of surfactant under certain circumstances such as pathologically limited availability of surfactant or the presence of inhibitory compounds in the airways. Yu and Possmayer (1996) have suggested that SP-A promotes formation of DPPC-enriched reservoirs attached to the surface that would provide a mechanism of monolayer formation during respiratory mechanics. SP-A and the other collectin present in surfactant, SP-D, possess several activities related to primary host-defense mechanisms in the alveolar spaces (van Golde, 1995; Wright, 1997).

By using epifluorescence microscopy we have recently observed the interaction of SP-A monolayers of DPPC, DPPC/dipalmitoylphosphatidylglycerol (DPPG), and surfactant lipid extracts (Ruano et al., 1998a; Nag et al., 1998). This technique allows direct observation of structural transitions in monolayers such as between liquid-expanded and liquid-condensed states and of fluorescently labeled proteins in regions of the monolayer (Möhwald, 1990; Pérez-Gil et al., 1992; Nag et al., 1996–1998). SP-A associates with fluid regions of DPPC monolayers, but accumulates at boundaries between fluid and condensed domains under conditions of phase coexistence (Ruano et al., 1998a). In DPPC/DPPG monolayers SP-A is effectively excluded even from fluid regions (Taneva et al., 1995; Ruano et al.,

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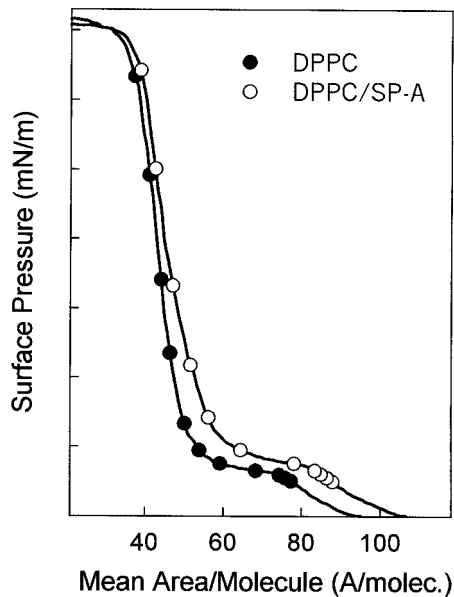
FIGURE 1 Typical Π-A isotherms of DPPC monolayers containing 1 mol % NBD-PC spread on subphases of 150 mM NaCl, pH 4.5 in the absence (*closed symbols*) or presence (*open symbols*) of TR-SP-A at 0.13 μg/ml.

1998a), accumulating in aggregates at boundaries between fluid and condensed regions. This immiscibility has been explained as resulting from electrostatic repulsion between lipids and proteins at physiological pH (Ruano et al., 1998b). Preferential interaction of SP-A with DPPC in comparison to acidic phospholipids has also been detected in phospholipid bilayers (Casals et al., 1993). The preference for DPPC could be the basis for SP-A-directed mechanisms of interfacial DPPC enrichment.

During inter and extracellular trafficking SP-A is likely to encounter milieus in which the pH might vary from ~5.5 to ~7. Changes in the environment from neutral to acidic pH would be expected to potentially modulate SP-A interactions with lipids because of effects on protein change. A recent study has shown that an acidic environment induced changes in the structure and lipid-binding properties of SP-A in bilayers (Ruano et al., 1998b) with the maximal effects being reached at pH 4.5. At that pH, the protein was able to bind to both neutral and acidic phospholipid vesicles, but the occurrence of SP-A-induced membrane aggregation was critically dependent on the presence of negatively charged lipids. To better understand the nature of and to
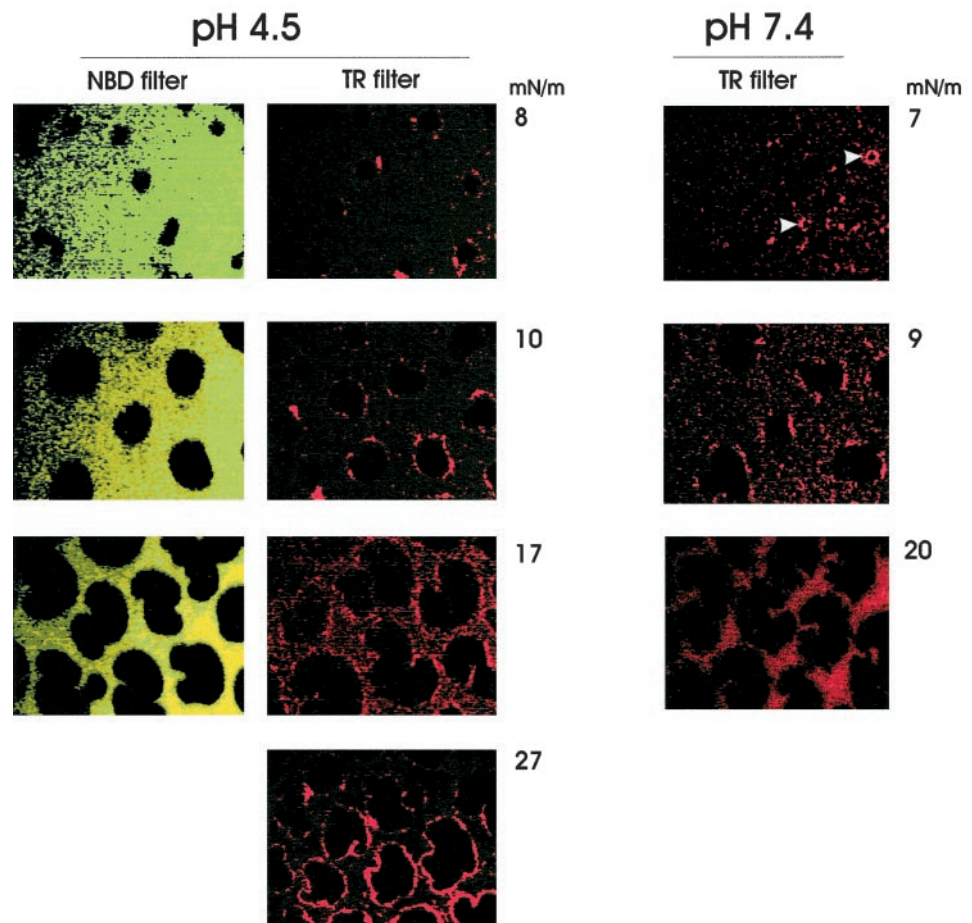

FIGURE 2 Typical images obtained from DPPC monolayers containing 1 mol % NBD-PC spread on a subphase containing 0.13 μg/ml TR-SP-A at pH 4.5 (*left*) or 7.4 (*right*). Images were recorded through filters selecting fluorescence from either NBD-PC (*green*, emission maximum at 520 nm) or TR-SP-A (*red*, emission maximum at 590 nm) at the indicated surface pressures. For pH 7.4, only the TR images are given, but the condensed domains are clearly visible.

visualize the interactions involved in the association of SP-A with surfactant films, the forces driving formation of SP-A domains at the interface and the nature of pH and lipid composition on SP-A self-aggregation, we have studied the interaction of SP-A with surfactant phospholipid monolayers at acidic pH by analyzing the association with and the location of the protein in zwitterionic (DPPC) or acidic (DPPC/DPPG) monolayers using epifluorescence microscopy. This would allow direct visual examination of the hypothesis (Ruano et al., 1998b) that association of SP-A with anionic lipids at low pH could reduce its aggregation state. Because the maximal effects of such changes were seen at pH 4.5 in the previous work with bilayers (Ruano et al., 1998b), we have selected that pH along with pH 7.4 (Ruano et al., 1998a) to make relevant comparisons. It is noted also that interactions in the extracellular milieu are important. While the pH of the aqueous lining layer or hypophase in the alveoli is neutral (Nielson et al., 1981), it can become very acidic in some pathological conditions, such as in hydrochloric acid aspiration trauma (Eijking et al., 1993).

EXPERIMENTAL

Materials

The lipids used in the experiments of this study, 1,2- dipalmitoylphosphatidylcholine (DPPC) and 1,2-dipalmitoylphosphatidylglycerol (DPPG), were from Sigma Chemical Co. (St. Louis, MO). The fluorescent lipid probe 1-palmitoyl-1-{12-[(7-nitro-2–1,3-benzoxadizole-1-yl)amino]dodecanoyl} phosphatidylcholine (NBD-PC) was from Avanti Polar Lipids (Birmingham, AL). The fluorescent labeling chemical sulforhodamine 101 sulfonyl chloride, Texas Red (TR), was obtained from Molecular Probes Inc. (Eugene, OR). Chloroform and methanol were HPLC grade solvents from Fisher Scientific Co. (Ottawa, ON) and all other reagents were analytical grade chemicals from Merck (Darmstadt, Germany).

Isolation and labeling of SP-A

SP-A was purified from pulmonary surfactant prepared from porcine broncheoalveolar lavage as previously described (Casals et al., 1989), by sequential butanol and octylglucoside extractions (Casals et al., 1993), and stored in solution of 5 mM Tris-HCl buffer, pH 7.4, at $-20°C$. Purity of the protein preparations was routinely checked by SDS-PAGE under reducing conditions followed by Coomassie Blue staining. SP-A was quantitated by amino acid analysis after hydrolysis of protein samples in 0.2 ml of 6 M HCl containing 0.1% (w/v) phenol in evacuated and sealed tubes at 108°C for 24 h followed by analysis in a Beckman System 6300 High Performance amino acid analysis. Fluorescently labeled SP-A was prepared as previously described (Ruano et al., 1998a).

Epifluorescence experiments

Surface pressure-area measurements and microscopic observations of monolayers were performed on an epifluorescence microscopic surface balance, the construction and operation of which have been described elsewhere (Nag et al., 1990, 1991). Phospholipid monolayers including 1 mol % NBD-PC were formed on subphases that did or did not contain SP-A as previously described (Ruano et al., 1998a). Monolayers were spread by depositing aliquots of chloroform/methanol solutions of the lipids on subphases containing 150 mM NaCl, adjusted to pH of either 7.4 or 4.5, in the absence or presence of TR-SP-A 0.13 µg/ml. All subphases

were prepared with double-distilled water, the second distillation being from dilute potassium permanganate. After spreading a monolayer the organic solvent was allowed to evaporate for 5 min, and to facilitate SP-A adsorption to the air-liquid interface, the monolayer was compressed rapidly (707 mm²/s) to a surface pressure of 10 mN/m and then expanded again to 0 mN/m. After a 1-h period allowing for observation of penetration of the protein into the gas or gas-liquid expanded coexistence phases (e.g., Maloney et al., 1995), compression of the monolayer at slow speed (20 mm²/s or an initial rate of 0.13 Å²/molecule/s) at 23 ± 1°C was begun. At selected surface pressures, a visual recording was made on videotape for a 1-min period for both NBD and TR fluorescence by switching fluorescence filter combinations to select fluorescence emission in the proper wavelength range. The images obtained were analyzed with digital image processing using JAVA 1.3 software (Jandel Scientific, San Rafael, CA) as discussed elsewhere (Nag et al., 1991; Pérez-Gil et al., 1992).

The video images were obtained with a CCD camera, which records in black and white. The images presented in the figures have been false-colored to display them as they appear approximately to the eye in the microscope.

RESULTS

Fig. 1 shows typical compression π-A isotherms of DPPC monolayers spread on subphases of 150 mM NaCl, adjusted to pH of 4.5, in the presence or absence of TR-SP-A at 0.13 µg/ml. As observed at neutral pH (Ruano et al., 1998a), SP-A expanded the isotherms of DPPC at pH 4.5, indicating that either protein insertion at the air-liquid interface or perturbation of lipid packing by the protein, or both, had occurred. Protein-containing monolayers also displayed clear plateaus in the range of 7–9 mN/m, indicative of liquid-expanded to liquid-condensed phase transitions.
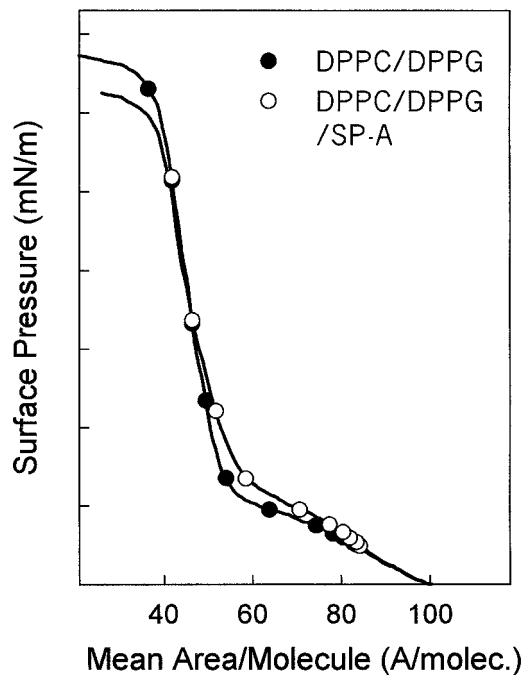
FIGURE 3   Typical Π-A isotherms of DPPC/DPPG (7:3, w/w) monolayers containing 1 mol % NBD-PC spread on subphases of 150 mM NaCl, 5 mM Tris-HCl, pH 4.5 in the absence (*closed symbols*) or presence (*open symbols*) of TR-SP-A at 0.13 µg/ml.
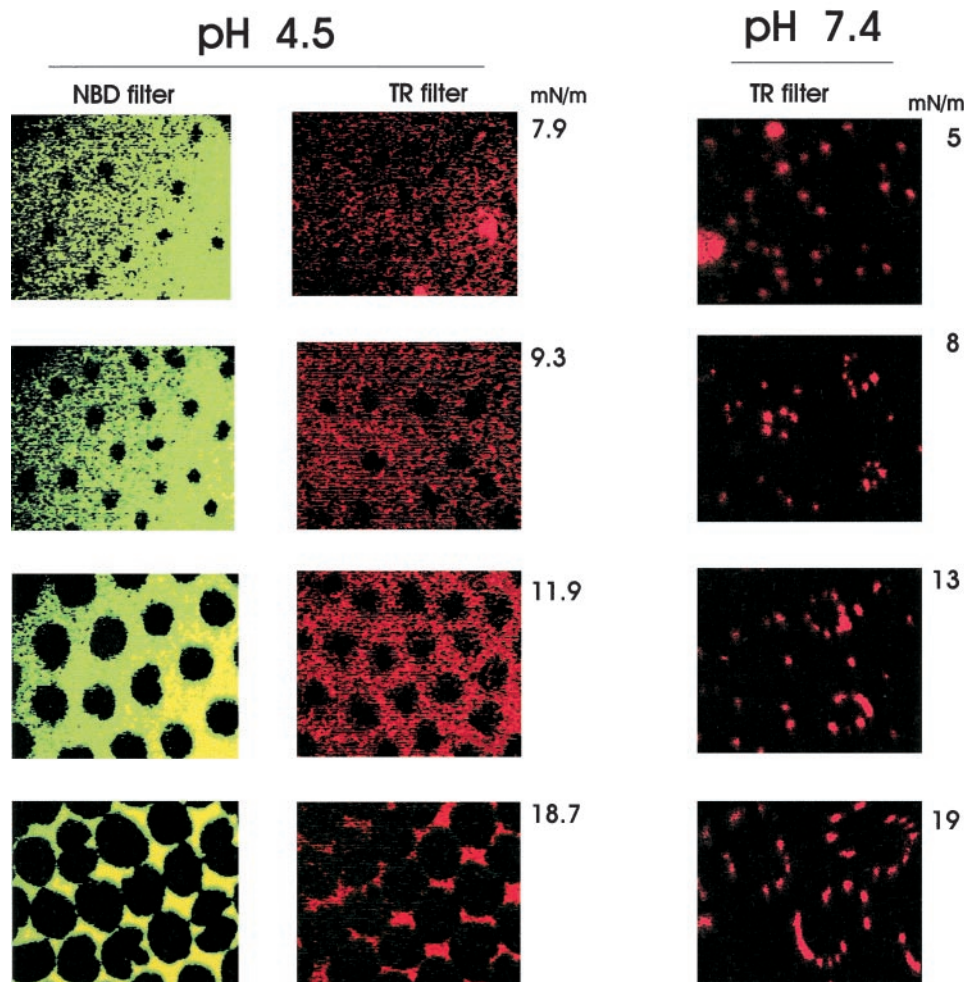
DPPC/DPPG/SP-A

FIGURE 4 Typical images obtained from DPPC/DPPG (7:3, w/w) monolayers containing 1 mol % NBD-PC spread on a subphase containing 0.13 μg/ml TR-SP-A at pH 4.5 (*left*) or 7.4 (*right*). Images were recorded through filters selecting fluorescence coming either from NBD-PC (*green*) or TR-SP-A (*red*) at the indicated surface pressures.

Microscopic images of DPPC monolayers containing 1 mol % NBD-PC in the presence of 0.13 μg/ml TR-SP-A in the subphase at acidic pH, compared with those at neutral pH, are shown in Fig. 2. Fluorescence coming from either the lipid probe or the fluorescently labeled protein was selectively recorded from the same monolayers by switching the filters. Our apparatus does not allow, however, for instantaneous recording of images at two wavelengths. At low pressures there is considerable movement of the monolayer so that fields change more rapidly than filters can be changed. Even at higher pressures the time needed to change filters, possibly refocus, and collect visual data for processing means that fields will have changed somewhat for sequential images. The times involved for the changes are in the range of 5–15 s. The association and correlation of regions of fields can be made more rapidly by eye almost immediately upon changing the filters, but images at the two wavelengths are not superimposable because of monolayer movement between the acquisition of the images.

The association of TR-SP-A with DPPC monolayers at acidic pH is similar to that observed at neutral pH at low surface pressures (Fig. 2). At both pH values the protein starts to interact with the monolayer when condensed solid domains of lipid begin to nucleate (in the range of 6–8 mN/m). This observation has been interpreted as a requirement for gel-like condensed regions or, likely, condensed/fluid coexistence for the interaction of TR-SP-A with monolayers to occur (Ruano et al., 1998a). At surface pressures up to 15 mN/m, TR-SP-A fluorescence appeared at acidic pH in fluid-expanded regions with increased intensity at condensed/fluid boundaries. However, in contrast with the behavior observed at pH 7.4, TR-SP-A accumulation at the boundaries of the DPPC condensed domains never decreased under compression at acidic pH. Compression up to 27 mN/m produced monolayers in which nearly all protein fluorescence accumulated at the perimeters of the solid regions, forming fluorescent rings with the shape of the condensed areas.

Fig. 3 presents Π-A isotherms of DPPC/DPPG 7:3 (w/w) monolayers spread on subphases of NaCl 150 mM, at pH 4.5, in the absence or presence of TR-SP-A 0.13 µg/ml. In contrast with the behavior observed at neutral pH (Ruano et al., 1998a), TR-SP-A hardly expanded the isotherms of DPPC/DPPG monolayers at acidic pH, suggesting that at pH 4.5 the protein does not insert very much into the negatively charged interface. Microscopic images of those monolayers show dramatic differences in the distribution of TR-SP-A in acidic phospholipid-containing monolayers (Fig. 4) compared with the zwitterionic ones (Fig. 2). The distribution of TR-SP-A into DPPC/DPPG monolayers at acidic pH was dramatically different to that observed at neutral pH. At pH 4.5, TR-SP-A appeared to homogeneously distribute into the fluid or expanded phospholipid at any surface pressure up to 25 mN/m, while it was excluded from the interior of the condensed domains. No selective accumulation of TR-SP-A was detected at the solid/fluid boundaries or at any other location in the interface. By contrast, at neutral pH, TR-SP-A was present in DPPC/ DPPG monolayers as discrete aggregates, more or less regular in size and distributed preferentially around the

condensed lipid domains at any surface pressure. Virtually no protein fluorescence was detected in both condensed domains and fluid regions, indicating complete immiscibility of TR-SP-A and acidic phospholipid at neutral pH. The effect of TR-SP-A on the condensation of the monolayer under compression at acidic and neutral pH was quantitated by measuring the effect of the presence of the protein on the percent of total condensed area of the films. Fig. 5 shows the amount of condensation upon compression of DPPC and DPPC/DPPG monolayers at pH 7.4 or 4.5 in the absence or presence of TR-SP-A. The protein only produced a significant decrease in the total condensation when interacting with DPPC monolayers at neutral pH and at surface pressures in the range 10–30 mN/m. This effect has been attributed to protein-induced perturbation of lipid packing, which reduces condensation of lipid (Pérez-Gil et al., 1992; Nag et al., 1997). Similarly, Fig. 6 shows that TR-SP-A only produced a clear increase in the number of condensed domains in DPPC monolayers at pH 7.4. Similar effects of decreasing the total amount of condensed area while increasing the number of condensed domains have been found for hydrophobic proteins SP-B and SP-C in DPPC and
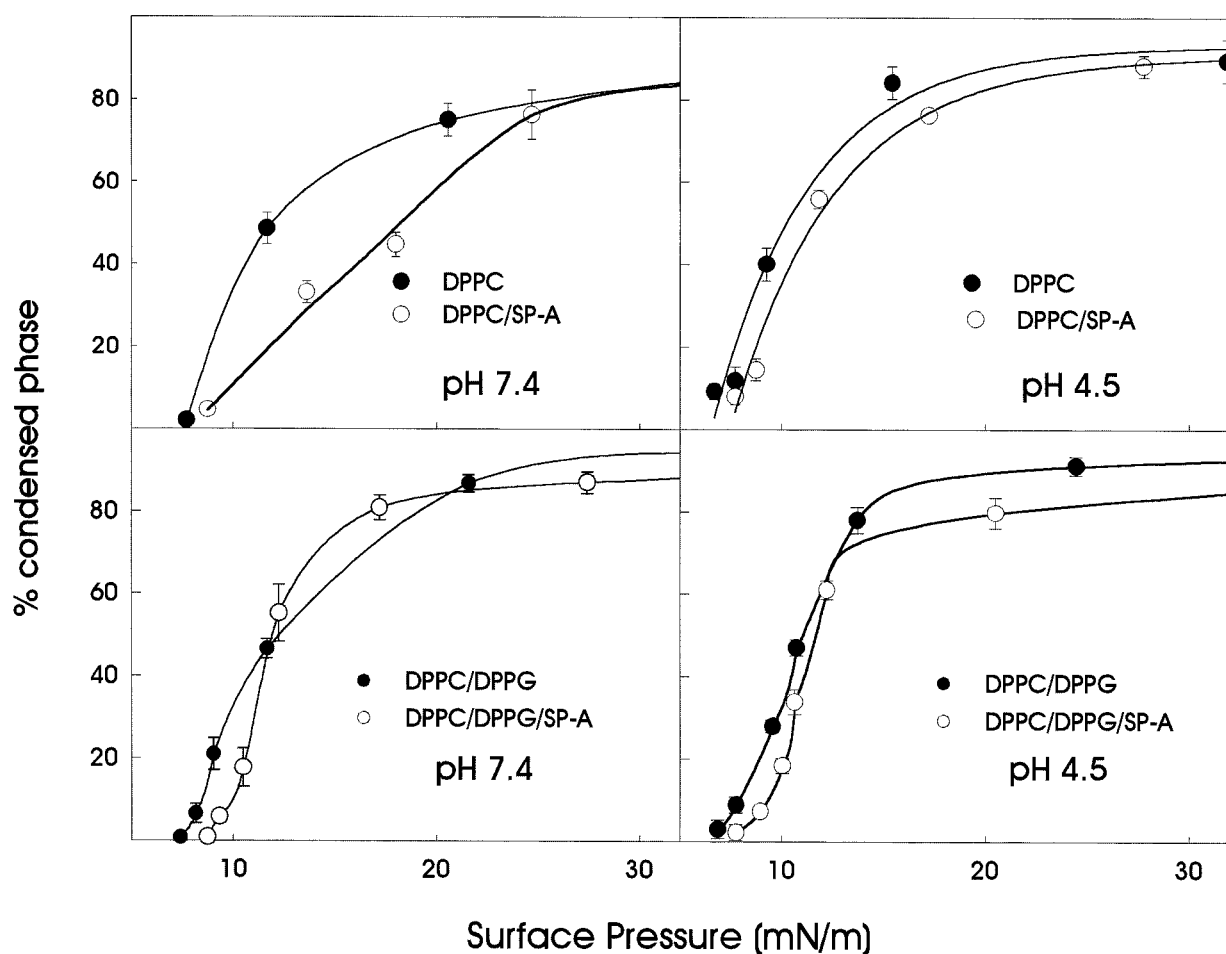

FIGURE 5   Percent of total condensed area plotted against surface pressure for different monolayers in the absence (●) and presence (○) of TR-SP-A at 0.13 µg/ml. Values are $x \pm$ SD for $n = 10$ images. Error bars not shown are within the symbol sizes.
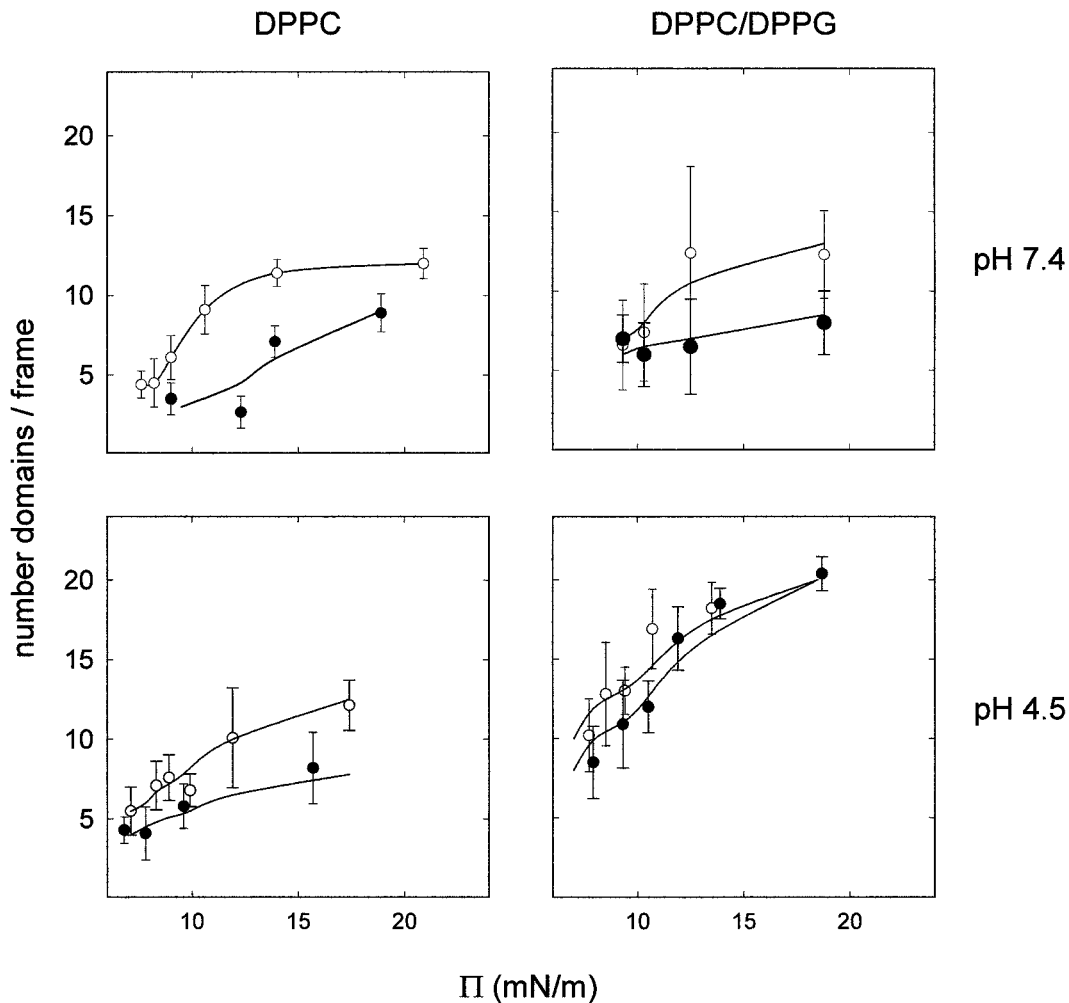
FIGURE 6   Dependence of the number of condensed domains per frame on the surface pressure for different monolayers in the absence (●) and presence (○) of TR-SP-A at 0.13 μg/ml. Values are $x \pm$ SD for $n = 10$ images. Error bars not shown are within the symbol sizes.

DPPG monolayers (Pérez-Gil et al., 1992; Nag et al., 1996, 1997).

DISCUSSION

We have previously found that fluorescently labeled SP-A associates with spread phospholipid monolayers at neutral pH (Ruano et al., 1998a). Features of the interactions of SP-A with DPPC or DPPC/DPPG monolayers at neutral pH correlated with previous observations on the interaction of SP-A with phospholipid bilayers and monolayers (King et al., 1986; Casals et al., 1993; Taneva et al., 1995; Ruano et al., 1996).

In the present work we have explored the effect of pH on both SP-A distribution in, and SP-A modifications of, the condensation during compression of monolayers of DPPC or DPPC/DPPG. In a recent paper we showed that the secondary structure and aggregation state of SP-A and its interaction with neutral and negatively charged phospholipid bilayers were dependent on pH (Ruano et al., 1998b). It was observed that SP-A bound to both neutral and neg-

atively charged vesicles at acidic pH. The binding of negatively charged vesicles to SP-A led, however, to a change in the secondary structure of the protein, which indicated that the level of SP-A self-aggregation decreased after the interaction of the lipid and protein.

In DPPC monolayers at neutral and acidic pH, aggregates of fluorescent SP-A appear at the liquid condensed/liquid expanded boundary regions, indicating that SP-A aggregates are segregated to the lipid packing defects in the monolayer (Ruano et al., 1998a). SP-A accumulation at condensed/fluid boundaries of DPPC monolayers persists even at pressures up to 30 mN/m. These results suggest that protein/protein interactions under such conditions are stronger than lipid/protein interactions, preventing mixing of SP-A with phospholipids. Results with DPPC monolayers correlate with those previously reported with DPPC bilayers, where we showed that the binding of DPPC vesicles to SP-A at pH 4.5 did not have any effect on the state of self-aggregation of the protein (Ruano et al., 1998b). However, the insertion of aggregates of protein in condensed/fluid boundaries of DPPC monolayers at pH 4.5 caused

almost no effect in both amount of total condensation and number and size of condensed domains. This would also be a consequence of nearly total segregation of lipid and protein at acidic pH.

As previously observed the distribution of SP-A in DPPC/DPPG monolayers at neutral pH indicated that protein was aggregated and there was, effectively, natural exclusion of protein and lipid (Ruano et al., 1998a). For DPPC/DPPG monolayers at pH 4.5, the distribution of SP-A was remarkably different from that at pH 7.4, the SP-A being fairly homogeneously disbursed in the liquid-expanded phase of the monolayers, suggesting that the interactions at lower pH came about through a more dispersed form of SP-A and the lipid. This would have occurred because lowering the pH would have reduced negative charge on the protein (and lipid), reducing mutual exclusion. Since the maximal self-aggregation of SP-A occurred at pH 4.5 (Ruano et al., 1998b), other forces, however, must also be dominating the distribution of SP-A in DPPC/DPPG at pH 4.5. These findings are consistent with the view that the adsorption of TR-fluorescent SP-A to DPPC/DPPG, but not to DPPC, monolayers reverses protein self-aggregation. Taken together, these results show different modes of interaction of SP-A with monolayers of DPPC and DPPC/DPPG at pH 4.5 and reinforce the results of previous studies on the interaction of SP-A with DPPC and DPPC/DPPG bilayers at the same pH. SP-A is able to decrease the amount of condensation upon compression and to increase the number of condensed domains in monolayers of DPPC, but not in those of DPPC/DPPG, at neutral pH. At acidic pH SP-A did not affect the amounts or numbers of domains of either type of monolayer. These observations are consistent with a selective interaction of SP-A with DPPC, which is pH-dependent. Perturbation of lipid packing was also reported for the hydrophobic surfactant protein SP-C, which decreased the total amount of condensed area and produced more, smaller condensed domains in monolayers of either DPPC or DPPC/DPPG (Pérez-Gil et al., 1992; Nag et al., 1996). The perturbing influence of SP-A in monolayers is more selective, since it is only observed in DPPC monolayers at neutral pH. Under such conditions, globular "headgroups" of SP-A (comprising lipid binding domains) would interact with acyl chains of phospholipid monolayers sufficiently to perturb the usual lipid packing. The perturbation of lipid packing induced by SP-A could be important for the selective insertion of DPPC into the monolayer at low compression rates (Schürch et al., 1992; Yu and Possmayer, 1996). Yu and Possmayer (1996) suggested that SP-A could form bridges between the interfacial monolayer and subphase bilayers, contributing to the establishment of a surfactant surface reservoir. In addition, it was recently showed that SP-A mediates transfer and exchange of phospholipids between the outer monolayers of membranes (Cajal et al., 1998). Taking into account that SP-A selectivity interacts with DPPC-rich bilayers (Casals et al., 1993) and monolayers (Ruano et al., 1998a) at neutral pH, it would be possible that contacts mediated by SP-A between the interfacial monolayer and subphase bilayers are important for replenishment of DPPC in the monolayer at physiological pH. The pH dependence of the interactions of SP-A with lipids may also have impact on the processing of surfactant in the endocytic pathway (Beers, 1996).

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