Interactions of Hydrophobic Lung Surfactant Proteins SP-B and SP-C with Dipalmitoylphosphatidylcholine and Dipalmitoylphosphatidylglycerol Bilayers Studied by Electron Spin Resonance Spectroscopy†

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ABSTRACT: Hydrophobic surfactant-associated proteins SP-B and SP-C have been isolated from porcine lungs and reconstituted in multilamellar vesicles of dipalmitoylphosphatidylcholine (DPPC) or dipalmitoylphosphatidylglycerol (DPPG) containing different phospholipid spin probes, in order to characterize the lipid−protein interactions by electron spin resonance (ESR) spectroscopy. Both proteins caused a significant increase in the outer hyperfine splittings of all the ESR spectra, indicating that SP-B and SP-C reduce the mobility of the phospholipid acyl chains. The more hydrophobic SP-C had greater effects on phospholipid bilayers than did SP-B. The effect was saturated at protein/lipid ratios of 20% and 30% (w/w) for SP-B and SP-C, respectively, in bilayers of DPPC. SP-B and SP-C increased the ordering and decreased the mobility of the lipid acyl chains in both DPPC and DPPG bilayers in the fluid phase, without affecting the gel phase on the conventional ESR time scale. On the other hand, both proteins induced a more homogeneous distribution of the phospholipid spin probes in the gel phase of DPPC. The selectivity of the interaction of SP-B and SP-C with different phospholipid species was determined from the ESR spectra of spin-labeled phospholipids with different headgroups in host bilayers of either DPPC or DPPG. SP-B showed a general preference to interact with negatively charged phospholipids, which was modulated in an ionic strength-dependent manner. At near-physiological ionic strength, SP-B showed selectivity for phosphatidylglycerol. SP-C did not show well-defined selectivity for a particular class of phospholipid, but it did show some preference for phosphatidic acid, which was partially abolished at physiological ionic strength. Finally, studies of the effects of the hydrophobic surfactant proteins on the ionization equilibrium of a stearic acid spin probe in bilayers of DPPC showed that both proteins altered the structure at the bilayer surface, causing an increase in the surface polarity. Such changes could be related to the changes in tensoactive properties induced by these proteins in surfactant phospholipids.

The alveolar epithelium of the lungs is the largest surface through which mammals communicate with the environment. Its integrity and stability are essential to achieve respiratory function. Such stability is maintained by the presence of a complex material, the pulmonary surfactant, that is synthesized and secreted by alveolar type II cells. This material predominantly consists of 90% lipids—the main components being dipalmitoylphosphatidylcholine (DPPC)[1] and smaller amounts of phosphatidylglycerol (PG)—and 8−10% of some surfactant-specific proteins (VanGolde et al., 1988). To achieve its function, the surfactant material must reach the air−water interface of the alveoli and spread, most likely as a monolayer that is able to reduce surface tension during dynamic compression and expansion of the lung (Goerke & Clements, 1986).

Although DPPC seems to be the main component responsible for the tensoactive properties of the surfactant in the monolayer, the other lipid and protein components are thought to be needed to facilitate the transport of lipid, through the aqueous hypophase lining the alveoli, to the air−water interface. Particularly important is the participation in this process of the two surfactant proteins SP-B and SP-C. These small proteins have unusual structural properties, including an strongly hydrophobic character [see Weaver and Whitsett (1991) or Hawgood and Shiffer (1991) for a review]. Both are water-insoluble, and they coisolate with lipids during the extraction of surfactant with organic solvents. SP-B consists of a dimer with an apparent molecular weight of 18 000. The monomer contains 79 amino acids, including seven cysteines, which form three intrachain and one interchain disulfide bond (Johansson et al., 1991), and several positively charged residues. SP-C is a small peptide of 35 amino acids with an apparent molecular weight of 4 000. Its hydrophobicity is extreme; it has a C-terminal region of 23 hydrophobic, aliphatic, branched residues, and the N-terminal end, which has a cationic character, is increased in

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[1] Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; ESR, electron spin resonance; *n*-PCSL, *n*-PESL, *n*-PGSL, *n*-PSSL, and *n*-PASL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, -phosphoglycerol, -phosphoserine, and -phosphoric acid; *n*-SASL, *n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid.

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hydrophobicity by palmitoylation of the two cysteines in this region of the molecule (Curstedt et al., 1990). It has recently been reported that, in hydrophobic environments, SP-C adopts an unusually regular α-helical structure (Johansson et al., 1994).

Both proteins, SP-B and SP-C, have been demonstrated to dramatically increase the rate of phospholipid surface film formation in vitro (Oosterlaken-Dijksterhuist et al., 1991; Yu & Possmayer, 1990; Pastrana et al., 1991; Pérez-Gil et al., 1992a), although the mechanism by which these proteins impart such biophysical properties to lipids is not yet understood. Study of the events involved in the interaction of these proteins with the surfactant-specific lipids will give information on the functionality of the surfactant system in molecular terms. In this sense, several studies focusing on the characterization of surfactant protein−lipid interactions have been published recently, including the application of techniques such as differential scanning calorimetry (Simatos et al., 1990; Shiffer et al., 1993; Morrow et al., 1993), Fourier transform infrared spectroscopy (Pastrana et al., 1991; Vandenbussche et al., 1992a,b), epifluorescence monolayer microscopy (Pérez-Gil et al., 1992b), deuterium NMR spectroscopy (Simatos et al., 1990; Morrow et al., 1993), Raman spectroscopy (Vincent et al., 1991), and several fluorescence methods (Baatz et al., 1990; Oosterlaken-Dijkterhuist et al., 1992).

The characteristics of spin label electron spin resonance (ESR) spectroscopy make it a tool that is particularly well adapted to the study of lipid−protein interactions [see Knowles and Marsh (1991) for a review]. In particular, it has been used previously to demonstrate a direct motional restriction of the lipid chains upon their interaction with membrane-associated proteins. This method employs lipid probes with nitroxide spin labels at different positions along the acyl chain that can be introduced into lipid−protein recombinants to quantitate lipid−protein interactions in terms of lipid−protein stoichiometry and lipid specificity. This approach has provided information on several lipid−protein systems, such as those containing cytochrome oxidase (Knowles et al., 1981), apocytochrome *c* (Jordi et al., 1989), reconstituted myelin proteins (Sankaram et al., 1989a,b; Horváth et al., 1990), and bacteriophage M13 coat protein (Peelen et al., 1992).

The goal of the present study was to apply ESR spectroscopy to determine the specific features of the lipid−protein interactions with the hydrophobic surfactant proteins SP-B and SP-C. These were isolated from porcine lungs and reconstituted in the bilayers of the relevant surfactant lipids, mainly DPPC. Here we present results on the lipid−protein interactions, lipid selectivity, effects on the bilayer acyl chain flexibility gradient, and effects on the bilayer surface polarity. These studies give new insights into the properties of the surfactant lipid−protein system, some of which are directly related to the special function of this tensoactive material.

MATERIALS AND METHODS

Materials. Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were obtained from Avanti Polar Lipids (Birmingham, AL). Spin-labeled phospholipids, with the nitroxide group at different positions on the *sn*-2 acyl chain, were synthesized as described in Marsh and Watts (1982). The spin labels were stored at −20 °C in chloroform/methanol (2/1, v/v) solutions at a concentration of 1 mg/mL. Sephadex LH-20 and LH-60 chromatographic gels were from Pharmacia (Stockholm, Sweden). Chloroform and methanol were HPLC-grade solvents from Merck (Darmstadt, Germany).

Isolation of Hydrophobic Surfactant-Associated Proteins. Surfactant proteins SP-B and SP-C were isolated from pig lungs by minor modifications of the method of Cursted et al. (1987), as described elsewhere (Pérez-Gil et al., 1993). The purity of the SP-B and SP-C fractions was checked by electrophoresis, determination of the amino acid composition, and N-terminal sequencing analysis. The proteins were quantitated routinely by amino acid analysis. Analysis of SP-C acylation as previously described (Pérez-Gil et al., 1992b) showed that the protein had most of its two cysteine residues palmitoylated after the isolation procedure. Both proteins were assayed for biophysical activity, as described previously (Pérez-Gil et al., 1992a), and were active in promoting the interfacial adsorption of a mixture of DPPC and egg PG (7/3, w/w), when present at a level of 5% protein in lipid (w/w).

Reconstitution of Samples. Appropriate amounts of DPPC or DPPG and the selected spin-labeled probe (1 mol %) were dissolved in chloroform/methanol (2/1, v/v) and mixed with the desired amount of protein (stored in chloroform/methanol (2/1, v/v) solutions). Solvent was then evaporated under a N_2 stream, and the samples were dried under vacuum in a dessicator overnight. Lipid and protein−lipid samples were hydrated in 100 μL of buffer (50 mM HEPES, 150 mM NaCl, and 5 mM EDTA, pH 7) at 50°C for 1 h, with occasional vortex mixing. The reconstituted material was then pelleted by centrifugation at 3000 rpm, in a bench centrifuge, into 100-μL capillary tubes for ESR spectroscopy. This procedure yielded multilamellar aggregates, as checked by negative staining electron microscopy.

After the ESR experiments, the samples were again suspended in the preceding buffer, and aliquots were taken to quantify protein and lipids in order to determine the lipid/protein ratio of the recombinants.

To check the conformation of the proteins once reconstituted in lipids, far-UV circular dichroism spectra of the lipid−protein samples were recorded on a Jobin Yvon Mark III dichrograph fitted with a 250-W xenon lamp. Both proteins, SP-B and SP-C, gave circular dichroism spectra that were consistent with a mainly α-helical secondary structure, in both DPPC and DPPG bilayers. SP-B was estimated to possess around 45% α-helix, while SP-C was calculated to have 70% of its structure in an α-helical conformation.

pH Titration of Samples with a Spin-Labeled Stearic Probe. Lipid or lipid−protein material containing 1 mol % of the 5-SASL stearic acid spin probe was suspended in 100 μL of a buffer containing 10 mM citrate, 10 mM Tris, 10 mM borate, 5 mM EDTA, and 150 mM NaCl, at the desired pH, as described earlier. After the sample was pelleted, the pH was checked in the supernatant that was removed from the ESR capillary.

ESR Spectroscopy. ESR spectra were recorded on a Varian E-12 Century Line 9-GHz spectrometer equipped with a nitrogen gas flow temperature regulation system. The sealed capillaries (1-mm outer diameter) were placed in a quartz tube containing light silicone oil for thermal stability.

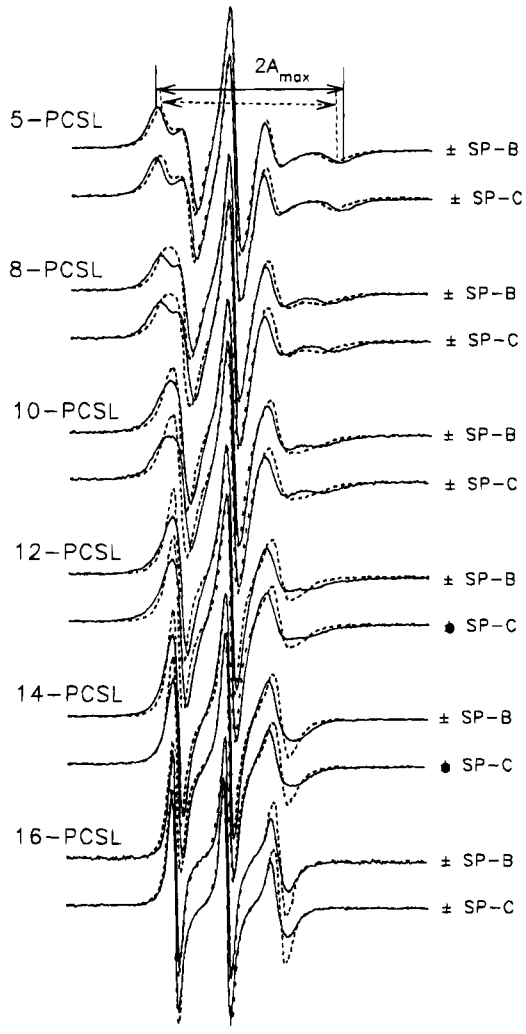

FIGURE 1: ESR spectra of phosphatidylcholine spin label positional isomers, *n*-PCSL, in DPPC dispersions (buffer: 50 mM HEPES, 150 mM NaCl, and 5 mM EDTA, pH 7.0) in the presence (solid lines) and absence (dotted lines) of porcine SP-B or SP-C. The protein/lipid ratio was 20% (w/w) in both cases. Total spectral width was 100 G and temperature was 45 °C.

Temperature was measured with a fine-wire thermocouple positioned in the silicone oil at the top of the microwave cavity.

Spectra were collected digitally on an IBM PC computer with Labmaster interface. Instrumental settings were as follows: 10-mW microwave power, 1.25-G modulation amplitude, 100-kHz modulation frequency, 0.25-s time constant, 4-min scan time, 100-G scan range, and 3245-G center field. Several scans, typically 3−5, were accumulated to improve the signal-to-noise ratio.

RESULTS AND DISCUSSION

Spin Label ESR Spectra of Reconstituted Lipid−Protein Systems. The effect of the surfactant hydrophobic proteins, SP-B and SP-C, on the chain motion and packing of bilayers of DPPC was studied by using different positional isomers of the phosphatidylcholine spin probes (*n*-PCSL). Figure 1 shows the ESR spectra of phosphatidylcholine spin probes labeled at different positions of the *sn*-2 chain in bilayers of DPPC, with and without the surfactant proteins SP-B and SP-C, and recorded at a temperature in the fluid lipid phase (45 °C). The spectra have outer hyperfine splittings and hyperfine anisotropies that are larger in the presence than in the absence of either SP-B or SP-C. This would indicate that both proteins cause a reduction in the acyl chain mobility of the DPPC molecules. This effect is somewhat greater for SP-C than for SP-B at the same protein (w/w) contents. Significantly, all of the ESR spectra of the different lipid spin label positional isomers essentially consist of a single component. There is no evidence for a second, more motionally restricted component such as is seen with large integral proteins for labels close to the terminal methyl end of the chain (Marsh, 1985), even up to surfactant protein contents higher than 60% (w/w).

This latter finding is an interesting result that may be attributed, on the one hand, to a more peripheral mode of association of SP-B (will be discussed later) and, on the other hand, to the relatively small size of the proteins, particularly SP-C. In the case of SP-B, this suggests that the protein does not have an appreciable part that penetrates deeply into the bilayer, in contrast to what is found with certain larger peripheral proteins, e.g., the myelin basic protein (Sankaram et al., 1989a), which has structural features similar to those of surfactant protein SP-B (Pérez-Gil & Keough, 1994). In the case of SP-C, a single transmembrane α-helix is expected to directly contact approximately 10 lipids in its monomeric form (Marsh, 1993). However, it is not known whether a single transmembrane helix provides a sufficiently large hydrophobic surface specifically to immobilize the lipid chains, as is done by larger integral membrane proteins. The present results possibly indicate that it does not. A motionally restricted lipid component is observed for the M13 bacteriophage coat protein in its α-helical form, but in this case the transmembrane monomers are aggregated (Peelen et al., 1992). A possible aggregation of SP-C in the bilayer can be discounted because, as recently reported (Horowitz et al., 1993), self-association of the protein occurs only in the lipid gel phase and not in the fluid phase. The present results indicate that the maximal effect of both proteins, SP-B and SP-C, occurs in the fluid lipid phase, where it remains monomeric. For gramicidin (which, however, is not an α-helical peptide), a clearly resolved, motionally restricted component is not readily observed, at least at low concentrations (Ge & Freed, 1993). Our results are, as far as we know, one of the first clear examples of the lipid−protein interactions with a single, monomeric, transmembrane α-helical peptide. The possible extrapolation of these results to other protein transmembrane segments should be checked, however, because the particular behavior of SP-C could be specific and related to its unusual amino acid composition and structure (Johansson et al., 1994).

Stoichiometry and Extent of Interaction. The dependence of the outer hyperfine splitting, $2A_{max}$, of the 5-PCSL spin label in DPPC on protein content, for samples of both DPPC/SP-B and DPPC/SP-C, is shown in Figure 2. The maximum saturating effects on the spectral splitting were induced at levels of about 20% (w/w) SP-B or higher and about 30% (w/w) SP-C or higher. At higher protein concentrations, the effect on $2A_{max}$ is maintained, as indicated by the plateau in Figure 2. This shows that, even beyond saturation, additional protein molecules do not reduce the hydrophobic surface area accessible to lipid chains disproportionately, as would occur, for instance, with extensive protein aggregation. The maximal increase in $2A_{max}$ was also somewhat higher for SP-C than for SP-B, suggesting that SP-C caused a greater

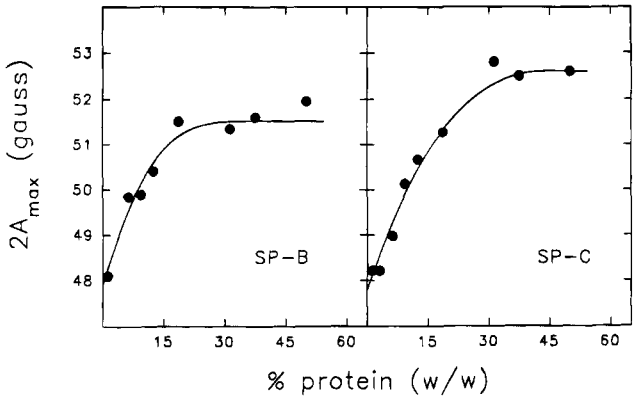

FIGURE 2: Dependence of the outer hyperfine splitting, $2A_{max}$, for the 5-PCSL phosphatidylcholine spin label in DPPC dispersions at 45 °C on the weight percent of protein present in samples, containing either SP-B or SP-C.

restriction in lipid mobility than did SP-B. This result is consistent, at least partially, with SP-B acting as a peripheral protein in surfactant bilayers (Morrow et al., 1993), while SP-C is thought to insert as a transmembrane peptide (Simatos et al., 1990).

By assuming that the protein/lipid ratio that gives rise to the maximal effect in the outer splittings in Figure 2 is related to the saturation "binding" of DPPC by the proteins, each monomer of SP-C would be associated with 18 lipid molecules, while each SP-B monomer would associate with around 50 molecules of DPPC. These values agree well with previous data reported in the literature, where the lipid–protein interactions have been evaluated by calorimetric techniques for both SP-C (Simatos et al., 1990) and, very recently, SP-B (Shiffer et al., 1993). The value of 18 lipid molecules possibly surrounding each monomer of SP-C lies at the upper end of the lipid/protein stoichiometry expected for hexagonal lipid packing around a single transmembrane α-helix (Bradrick et al., 1989). This would predict 12–18 lipids/helix and suggests that additional interstitial lipids may contribute to the optimal packing of SP-C with its accompanying boundary layer.

The lipid/protein stoichiometry calculated for SP-B in this and other studies is unusually high, taking into account the small size of SP-B compared with the stoichiometry proposed for other membrane proteins. Myelin basic protein, for instance, a peripheral protein of myelin sheath, associates with around 36–38 mol of acidic lipids per mole of protein (Sankaram et al., 1989a), in spite of the fact that it is twice as large as SP-B. SP-B has been proposed to adopt an extended conformation in the bilayer, with several helical amphipathic motifs (Waring et al., 1989; Cochran & Revak, 1991; Vandenbussche et al., 1992b). The relatively high lipid/SP-B molar stoichiometry would agree with such an extended protein structure at the bilayer surface.

Chain Flexibility Gradient. As can be seen in Figure 1, the ESR spectra of 5-, 8-, 10-, 12-, 14-, and 16-PCSL spin-labeled probes in DPPC bilayers are all affected by the presence of either SP-B or SP-C. The effect of the proteins extends as far as probes with the spin label located deep in the bilayer, close to the chain methyl termini. This is illustrated in Figure 3, where the outer hyperfine splittings of the PC spin label positional isomers are plotted versus chain position in the absence and presence of 20% (w/w) of either SP-B or SP-C. Samples with 20% (w/w) SP-B (45/1

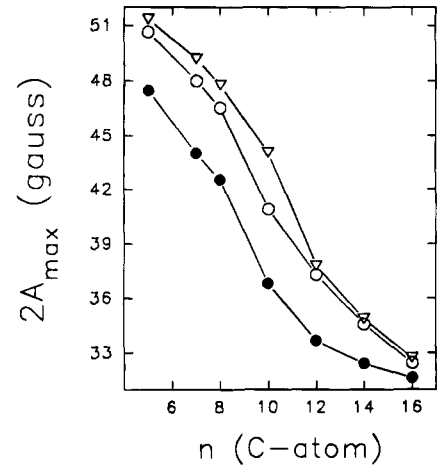

FIGURE 3: Outer hyperfine splittings ($2A_{max}$) at 45 °C as a function of nitroxide position, *n*, in the *sn*-2 chain for the *n*-PCSL phosphatidylcholine positional isomers in DPPC dispersions: (○) in the presence of 20% (w/w) porcine SP-B; (▽) in the presence of 30% (w/w) porcine SP-C; (●) in the absence of protein.

lipid/protein molar ratio) or 20% SP-C (22/1 lipid/protein molar ratio) were chosen for most of the experiments because these protein contents are close to saturation, according to Figure 2, and therefore showed the maximal effects. From the regularity of the concentration dependence, it can be assumed that the effects detected in the ESR spectra at higher concentrations are qualitatively similar to those of direct lipid–protein interactions at lower, physiological protein concentrations. Effective order parameters (S_{eff}) calculated from the total spectral anisotropy, rather than simply the outer splittings (cf. Sankaram et al., 1989b), also show a differential profile similar to those in Figure 3.

The characteristic chain flexibility gradient seen in Figure 3 for bilayers of DPPC alone is also obtained for those containing SP-B or SP-C. The motional restriction induced by the surfactant proteins extends more or less uniformly throughout the length of the lipid chains. This could be caused either by an increase in the lipid packing density induced by a surface interaction of the protein with the lipid headgroups or by motional restriction of the acyl chains arising directly from the interaction with parts of the protein embedded in deeper regions of the bilayer. Although the effects, for all of the positional isomers, were always higher for SP-C than for SP-B, the behavior of both proteins was not different enough, from a qualitative point of view, to reflect the different possible modes of association of SP-B and SP-C with the bilayer. The absence of clearly different effects of SP-C, compared with SP-B, could be related to the absence from SP-C/lipid complexes of a motionally restricted lipid population that would be expected if SP-C resembled integral membrane proteins (cf. above).

Phase Transition Behavior. The effect of the presence of SP-B and SP-C on the gel to liquid-crystalline phase transition of DPPC and DPPG bilayers is illustrated in Figure 4. The outer splittings of the ESR spectra from the 5-position spin label are plotted versus temperature for the pure lipids and lipid–protein systems containing 20% (w/w) of either SP-B or SP-C. Both pure DPPC and DPPG dispersions showed phase transition temperatures of around 40–41°C, as deduced from the temperature dependence of $2A_{max}$. The presence of SP-B or SP-C caused a broadening in the transition width without shifting the temperature of the

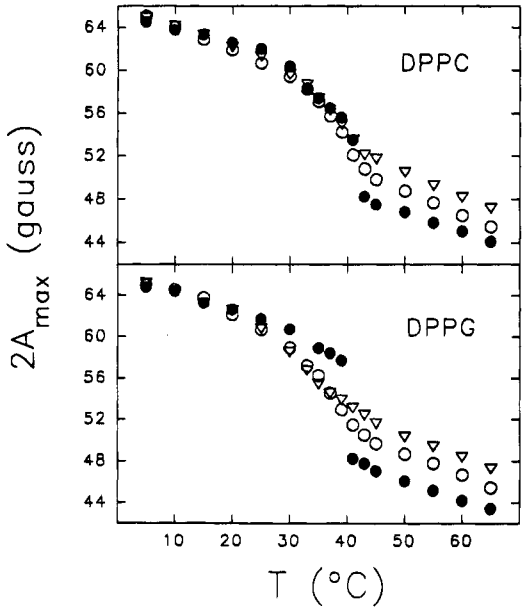

FIGURE 4: Temperature dependence of the maximum outer hyperfine splitting, $2A_{max}$, of the 5-PCSL phosphatidylcholine spin label in DPPC dispersions (upper panel) and 5-PGSL phosphatidylglycerol spin label in DPPG dispersions (lower panel): in the absence of protein (●) and in the presence of 20% (w/w) SP-B (○) or 20% (w/w) SP-C (▽).

transition midpoint very much. These results are consistent with scanning calorimetric data from others (Simatos et al., 1990; Shiffer et al., 1993). The effect on the transition of both proteins is somewhat greater in DPPG than in DPPC bilayers, particularly in the region immediately below the phase transition. SP-C always showed stronger effects on the transition than did SP-B, in both lipids.

Both proteins caused a decrease in the transition amplitude by increasing the splittings of the ESR spectra in the fluid phase of the lipids, apparently without modifying the spectral ESR parameters of the lipids at lower temperatures in the gel phase. Similarly, a slight ordering of the chains by SP-C in DPPC/DPPG bilayers in the fluid phase, without an effect on the gel phase, was found by FTIR spectroscopy (Pastrana et al., 1991). In contrast, studies of fluorescence anisotropy have reported that SP-B induced ordering at the surface of DPPC/DPPG (7/1, w/w) bilayers in the gel phase, but not in the fluid phase (Baatz et al., 1990). These studies were conducted only at low protein/lipid ratios, however (up to 2% w/w).

In all of the experiments, it can be seen that the upper transition boundary never extends higher than 45 °C, and therefore, the spectra given in Figure 1 and the values of A_{max} presented in Figures 2 and 3 all correspond to parameters of the fluid liquid-crystalline state of the lipids and lipid−protein complexes.

Spin-Broadening Effects. The ESR spectra from several of the PC spin label positional isomers showed significant broadening from spin−spin interactions at temperatures around 10 °C and below. This is illustrated in Figure 5 by spectra in the DPPC gel phase of 12-PCSL, which shows one of the most pronounced broadenings. The spin−spin broadening arises from partial exclusion of the lipid spin probe from the tightly packed DPPC gel phase. In the presence of 20% (w/w) of either SP-B (Figure 5, solid lines) or SP-C (Figure 5, dashed lines), the spin−spin broadening

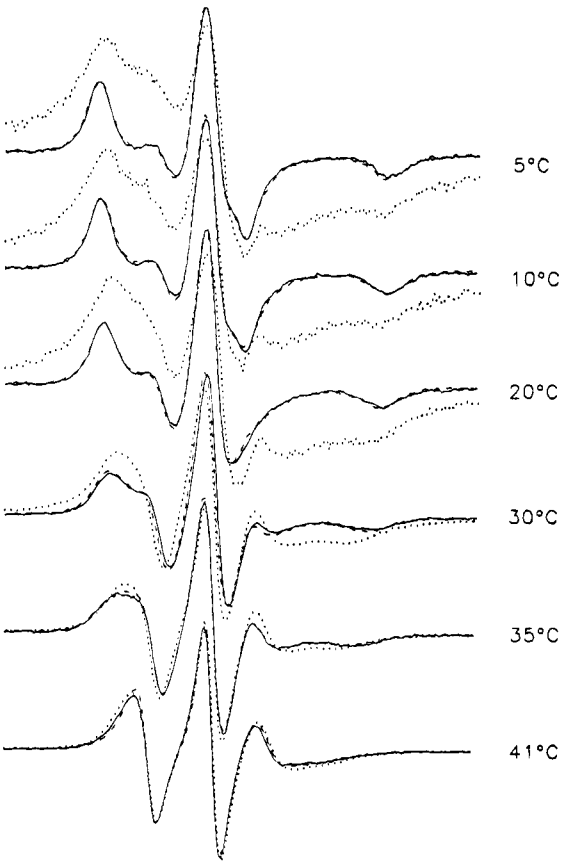

FIGURE 5: ESR spectra of the 12-PCSL phosphatidylcholine spin label at different temperatures in the gel phase of DPPC dispersions, in the absence of protein (dotted line) and in the presence of 20% (w/w) SP-B (solid line) or 20% (w/w) SP-C (dashed line). Total scan width, 100 G.

is completely abolished. This dramatic effect on the lipid mixing properties is not accompanied by significant changes in the dynamics of the lipid chains, as registered by the outer hyperfine splittings of the spin label. Similar qualitative results were observed in samples with protein contents as low as 2−3% (w/w) of either SP-B or SP-C (around 450/1 and 220/1 lipid/protein molar ratios, respectively), and the effect was already saturated at approximately 5% (w/w) (data not shown). This demonstrates that SP-B and SP-C greatly improve the solubility of the lipid probes, and perhaps of other components, in the gel phase.

Studies using epifluorescence microscopy with spread monolayers of DPPC also concluded that SP-C improves the solubility of a fluorescent lipid probe in the gel phase domains (Pérez-Gil et al., 1992b). The fact that spin−spin interactions are completely abolished at protein/lipid ratios as low as 2−3% (w/w), on the order of the content of SP-B and SP-C in surfactant, supports the idea that such effects could have important implications for the functional role of the hydrophobic proteins in lung surfactant. Although surfactant is synthesized and secreted to the hypophase as a much more complex lipid mixture, it is assumed that the interfacial monolayer is transformed during compression to render an almost pure DPPC monolayer (Pastranarios et al., 1994). Under such conditions, at physiological temperatures and very high surface pressures, the lipid surfactant monolayer is the only known lipid system working in vivo in the gel phase. The presence of SP-B and SP-C in the monolayer could then confer new properties on the lipid system,

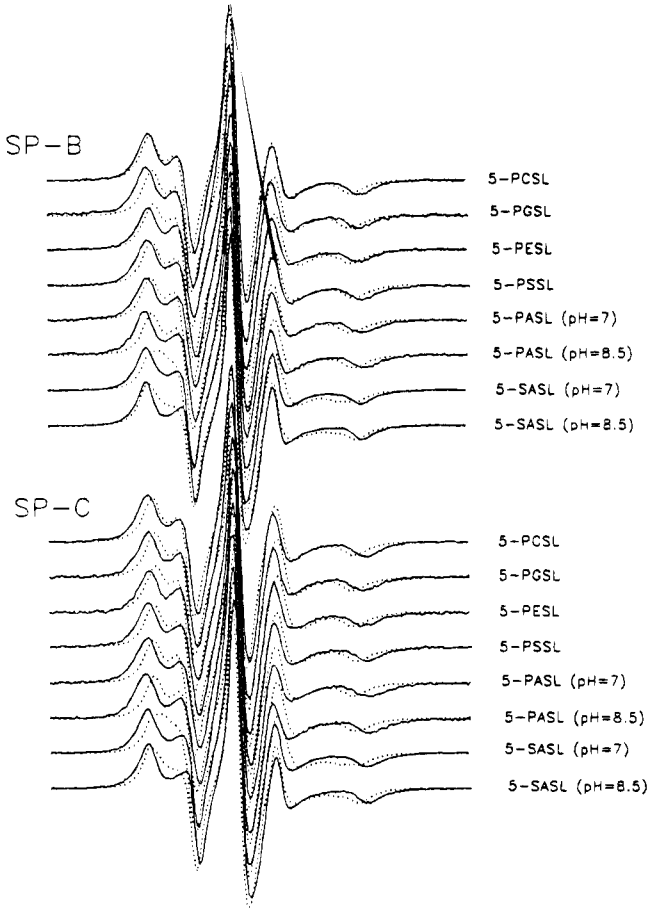

FIGURE 6: ESR spectra of different spin-labeled lipids in DPPC dispersions in the absence of protein (dotted line) and in the presence of 20% (w/w) SP-B (upper set) or 20% (w/w) SP-C (lower set), at 45 °C. Samples with phosphatidic acid spin label, 5-PASL, and stearic acid spin label, 5-SASL, were assayed at two different pH's, as indicated. Total scan width, 100 G.

increasing both the flexibility and dynamics of the otherwise rigid gel phase of DPPC.

One of the properties that a functional surfactant necessarily must possess to work properly in vivo is the ability to spread rapidly at the air–water alveolar interface and to respread after each compression cycle so as to regenerate a functional surfactant monolayer. Hydrophobic surfactant proteins have been proposed efficiently to improve such properties, when included in simple lipid mixtures (e.g., Pérez-Gil et al., 1992a). The present data are consistent with such a modulatory effect of SP-B and SP-C on the properties of the DPPC gel phase.

Phospholipid Selectivity. ESR spectra of several phospholipids spin-labeled at the fifth position in the *sn*-2 chain and incorporated into bilayers of DPPC, with and without surfactant proteins SP-B or SP-C, are given in Figure 6. Included are the spectra of spin-labeled phosphatidylcholine (5-PCSL), phosphatidylglycerol (5-PGSL), phosphatidylethanolamine (5-PESL), phosphatidylserine (5-PSSL), phosphatidic acid (5-PASL), and stearic acid (5-SASL), at 45 °C in the presence of 20% (w/w) SP-B or SP-C and the absence of protein. ESR spectra of 5-PASL and 5-SASL are also given at two pH values to investigate different ionization states of the headgroup of the spin-labeled lipid.

The spectra of all lipid labels display a larger outer hyperfine splitting in the presence of the proteins than in their absence, indicating a decrease in the mobility of the acyl chains of all of the different lipids due to the presence of the proteins. The extent of the motional perturbation was different for the different lipid species and also varied for each protein. The effects of the two hydrophobic surfactant proteins on the different lipids are compared in Table 1, which presents the increases in the outer hyperfine splitting ($2\Delta A_{max}$) of the various C5 spin labels induced by the presence of either SP-B or SP-C. Data at low (0 mM NaCl) and around physiological (150 mM NaCl) ionic strength are presented to assess the possible electrostatic components of the apparent lipid selectivity. At low ionic strength, SP-B showed greater effects on the mobility of all of the negatively charged spin-labeled phospholipids than the zwitterionic lipids. This may be expected because SP-B is a positively charged protein, with many basic residues in its primary sequence. At low ionic strength, the order of selectivity for SP-B, based on the data in Table 1 (cf. Sankaram et al., 1989b,c), follows the sequence

$$PA^{2-} > PG^{-} > PA^{-} > PS^{-} > PE^{\pm} > PC^{\pm} > SA^{-}$$

which also indicates a preference of SP-B for phospholipids over that for stearic acid, in spite of the negative charge of the latter. The largest effect was for phosphatidylglycerol and the two charged forms of phosphatidic acid. These selectivities for negatively charged phospholipids were decreased at higher ionic strength, with the exception of the effect of SP-B on phosphatidylglycerol. At the physiological ionic strength of 150 mM NaCl, the attenuated selectivity sequence of SP-B is in the following order:

$$PG^{-} > PA^{2-} = PE^{\pm} \geq PA^{-} = PC^{\pm} > PS^{-} = SA^{-}$$

Under these conditions, only the surfactant-specific, negatively charged lipid phosphatidylglycerol expressed a clear selectivity for SP-B. This is consistent with the idea that SP-B interacts specifically with the phosphatidylglycerol fraction of surfactant, as previously proposed by others (Baatz et al., 1990; Cochrane & Revak, 1991).

The presence of SP-B together with phosphatidylglycerol markedly enhances the tensoactive properties in surfactant preparations (e.g., Pérez-Gil et al., 1992a). This suggests that specific SP-B/PG interactions could play an important role in the dynamic architecture of lung surfactant. Particularly interesting is the requirement of SP-B and PG for the

Table 1: Increases Induced by SP-B and SP-C in the Outer Hyperfine Splitting ($2\Delta A_{max}$) of Different Phospholipids Spin-Labeled at the Fifth Position of the *sn*-2 Chain Incorporated in Bilayers of DPPC, at 45 °C, in the Absence and Presence of 150 mM NaCl

	$2\Delta A_{max}$ (G)			
	+SP-B[a]		+SP-C[a]	
	0 mM NaCl	150 mM NaCl	0 mM NaCl	150 mM NaCl
5-PCSL	2.3	2.7	4.2	3.2
5-PGSL	3.8	3.8	3.8	2.8
5-PSSL	3.1	2.3	3.9	2.7
5-PESL	2.8	3.1	3.6	2.8
5-PASL (pH 7)	3.6	2.8	5.4	3.5
5-PASL (pH 8.5)	4.1	3.2	4.7	
5-SASL (pH 7)	2.5	2.4	2.9	2.8
5-SASL (pH 8.5)	1.4	1.5	2.5	1.8

[a] The content of protein in lipid was 20% (w/w) for both SP-B and SP-C samples.

Table 2: Increases Induced by SP-B and SP-C in the Outer Hyperfine Splitting ($2\Delta A_{max}$) of Different Phospholipids Spin-Labeled at the Fifth Position of the *sn*-2 Chain Incorporated in Bilayers of DPPG, at 45 °C, at Low Ionic Strength

	$2\Delta A_{max}$ (G)	
	+SP-B[a]	+SP-C[a]
5-PCSL	3.0	4.3
5-PGSL	2.6	4.6

[a] Protein content was 20% (w/w) in DPPG, in both SP-B and SP-C samples.

assembly of tubular myelin, a complex lattice-like, membrane-based structure that is thought to be an intermediate in the movement of surfactant from the hypophase to the interfacial monolayer (Suzuki et al., 1989; Williams et al., 1991).

The possibility that SP-C exhibits a selectivity of interaction with particular phospholipids has not been studied previously. The data given in Table 1 indicate that the pattern of phospholipid preference shown by SP-C is different from that observed for SP-B. With two exceptions, SP-C has rather similar effects on the chain mobility of all the spin probes studied. At low ionic strength, SP-C shows a selectivity for phosphatidic acid in its different charged forms. On the other hand, as also found for SP-B, SP-C has the weakest effect on the stearic acid probe. Under these conditions, the sequence of selectivity for SP-C is

$$PA^- > PA^{2-} > PC^{\pm} \geq PS^- = PG^- = PE^{\pm} > SA^-$$

The preference exhibited by SP-C for PA is very much diminished when the ionic strength is increased to physiological values. This indicates that the selectivity is primarily electrostatic in origin. In contrast to the situation with SP-B, no preferential interaction is found with SP-C for the unique negatively charged lipid component of lung surfactant, namely, phosphatidylglycerol. The efficacy of SP-C therefore most probably lies in its hydrophobic properties, rather than in specific interactions with the lipid polar groups.

Table 2 presents data on the effects of SP-B and SP-C on the 5-PCSL and 5-PGSL spin labels in bilayers of DPPG at low ionic strength. The effects of both proteins were similar on both 5-PCSL and 5-PGSL lipid probes in DPPG. This table shows that there is not a clear preference for PG over PC in the effects caused by either SP-B or SP-C when DPPG is the host matrix. The fact that SP-B does not express the selectivity for PG over PC spin probes in host bilayers of DPPG, even at low ionic strength, is expected because the protein is then saturated with the unlabeled host PG lipids.

pH Titration of Stearic Acid. Although both proteins, SP-B and SP-C, caused smaller perturbations in the chain mobility of spin-labeled stearic acid, suggesting that the hydrophobic surfactant proteins interact more strongly with phospholipid species than with free fatty acids, it is of interest to investigate the effects on the interfacial ionization equilibrium of the fatty acid. The shift in interfacial pK_a of the fatty acid carboxyl group depends on the polarity in the region of the lipid headgroups, the extent of electrostatic neutralization of the lipid polar groups, and the energetics of the lipid–protein interaction (Sankaram et al., 1989b, 1990). Therefore, pH titration is able to yield valuable information on the lipid–protein interaction, particularly the aspects associated with the membrane surface.

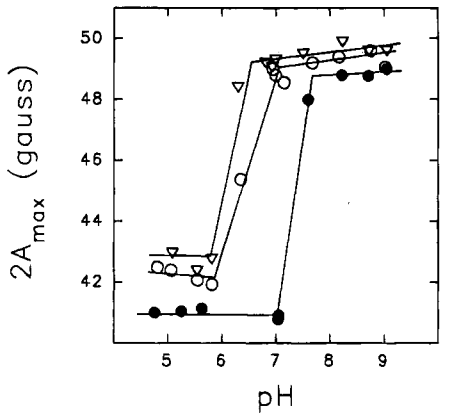

FIGURE 7: pH dependence of the outer hyperfine splitting constant, $2A_{max}$, of a stearic acid spin probe, 5-SASL, in DPPC dispersions at 60 °C: in the absence of protein (●) and in the presence of 20% (w/w) SP-B (○) or 20% (w/w) SP-C (▽).

The dependence of the outer hyperfine splittings ($2A_{max}$) on pH for 5-SASL in DPPC dispersions, in the presence and absence of either SP-B or SP-C, is given in Figure 7. The measurements were made at 60 °C because the difference in the splitting constants between the two ionization states of stearic acid was larger at this temperature. The effect of the proteins on the titration behavior of 5-SASL was qualitatively similar at 45 °C (data not shown). The intrinsic pK_a of stearic acid in aqueous solution is $pK_a^0 \approx 5.0$ (Ptak et al., 1980). The sharp increase in A_{max} with increasing pH seen in Figure 7 allows one to calculate an interfacial pK_a of 7.2 for the stearic acid ionization in bilayers of DPPC, where the shift in pK_a arises from the reduced polarity of the bilayer surface relative to bulk solution. The presence of 20% (w/w) SP-B shifts this interfacial pK_a to a value of 6.5, and even further to 6.1 in the presence of 20% (w/w) SP-C. The results in Figure 7 and Table 1 show that both proteins increase the outer hyperfine splittings of both the protonated and ionized forms of the stearic acid label, compared with the absence of protein. This effect is somewhat greater for the protonated than for the charged form of the fatty acid, suggesting that both surfactant proteins express a preference for the protonated over the ionized form of stearic acid (cf. Sankaram et al., 1990). This selectivity would result in a shift in pK_a to higher pH, which is the opposite direction to that observed. Therefore, the results of Figure 7 provide clear evidence for an additional contribution to the shift in pK_a that arises from an increase in the surface polarity of the lipid bilayer, in the direction of that of bulk water, in the presence of the surfactant proteins. Such a change in polarity of the lipid surface might be expected to reduce the surface tension of a lipid monolayer and, hence, to enhance its tensoactive properties.

In summary, the present studies with spin-labeled lipids have revealed new aspects of the lipid interactions with the surfactant proteins SP-B and SP-C. Some of these, for example, the specificity of interaction with phosphatidylglycerol and the effects on lipid mixing properties and the interfacial polarity, may be correlated with the specific function of the pulmonary surfactant.

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