Superficial disposition of the N-terminal region of the surfactant protein SP-C and the absence of specific SP-B–SP-C interactions in phospholipid bilayers

Inés PLASENCIA, Antonio CRUZ, Cristina CASALS and Jesús PÉREZ-GIL[1]

Departamento de Bioquímica, Facultad de Biología, Universidad Complutense, 28040 Madrid, Spain

A dansylated form of porcine surfactant-associated protein C (Dns-SP-C), bearing a single dansyl group at its N-terminal end, has been used to characterize the lipid–protein and protein–protein interactions of SP-C reconstituted in phospholipid bilayers, using fluorescence spectroscopy. The fluorescence emission spectrum of Dns-SP-C in phospholipid bilayers is similar to the spectrum of dansyl-phosphatidylethanolamine, and indicates that the N-terminal end of the protein is located at the surface of the membranes and is exposed to the aqueous environment. In membranes containing phosphatidylglycerol (PG), the fluorescence of Dns-SP-C shows a 3-fold increase with respect to the fluorescence of phosphatidylcholine (PC), suggesting that electrostatic lipid–protein interactions induce important effects on the structure and disposition of the N-terminal segment of the protein in these membranes. This effect saturates above 20 % PG molar content in the bilayers. The parameters for the interaction of Dns-SP-C with PC or PG have been estimated from the changes induced in the fluorescence emission spectrum of the protein. The protein had similar K_d values for its interaction with the different phospholipids tested, of the order of a few micromolar. Cooling of Dns-SP-C-containing dipalmitoyl PC bilayers to temperatures below the phase transition of the phospholipid produced a progressive blue-shift of the fluorescence emission of the protein. This effect is interpreted as a consequence of the transfer of the N-terminal segment of the protein into less polar environments that originate during protein lateral segregation. This suggests that conformation and interactions of the N-terminal segment of SP-C could be important in regulating the lateral distribution of the protein in surfactant bilayers and monolayers. Potential SP-B–SP-C interactions have been explored by analysing fluorescence resonance energy transfer (RET) from the single tryptophan in porcine SP-B to dansyl in Dns-SP-C. RET has been detected in samples where native SP-B and Dns-SP-C were concurrently reconstituted in PC or PG bilayers. However, the analysis of the dependence of RET on the protein density excluded specific SP-B–Dns-SP-C associations.

Key words: pulmonary surfactant, lipid–protein interactions, membrane protein, resonance energy transfer.

INTRODUCTION

Pulmonary surfactant lipopeptide SP-C is the only surfactant-associated protein specifically expressed in the lung tissue, and for which there is no known homologous protein [1]. This hydrophobic polypeptide has been considered, since its identification, to be essential in promoting the biophysical properties of pulmonary surfactant, which is a surface-active material synthesized by the alveolar epithelium and secreted into the aqueous lining of the lungs, where it reduces drastically the surface tension of liquid at the air–liquid interface. Such activity contributes to stabilize the respiratory surface during the successive compression–expansion processes of the breathing cycles [2–4]. The main surface-active principle in surfactant, dipalmitoyl phosphatidylcholine (DPPC), is unable, by itself, to traverse rapidly enough through the alveolar aqueous phase to adsorb at the interface. There, DPPC forms a monolayer as the ultimate structure that produces surface tensions close to zero under compression. Surfactant protein SP-C and the other hydrophobic surfactant-associated protein, SP-B, promote rapid interfacial adsorption of phospholipids to open air–liquid interfaces [5] and an efficient insertion of phospholipids into pre-existing films [6,7]. The presence of SP-C at the interfacial monolayer has been implicated in the process of selective depuration of the film during compression which leads to an enrichment in DPPC [8,9]. Furthermore, SP-C exhibits the ability to form and sustain interface-associated reservoirs of surface-active material, which are considered essential to replenish the bioactive films after compression, and therefore contributes to maintain the biophysical activity of surfactant during respiratory dynamics [10–12]. Paradoxically, and in spite of all these surfactant-related biophysical activities shown by SP-C *in vitro*, recent controversial results challenge an essential role for SP-C *in vitro*. Lack of mature SP-C did not cause any apparent respiratory dysfunction in SP-C knock-out mice [13], but Nogee and co-workers have described cases of familial interstitial lung disease originating from inherited deficiencies in the SP-C gene [14].

SP-C comprises a C-terminal highly regular α-helix, containing almost exclusively hydrophobic β-branched residues, and a ten-residue N-terminal segment that is cationic with one or two cysteines which are stoichiometrically palmitoylated [15–17]. When reconstituted in phospholipid bilayers, the α-helix of SP-C, which matches almost perfectly the thickness of a DPPC bilayer in the fluid liquid-crystalline phase, adopts a transmembrane disposition [18]. The protein is segregated from DPPC at temperatures below the gel-to-fluid phase transition temperature of the lipid [19], probably as a consequence of hydrophobic mismatch between the α-helix and the thicker DPPC gel

Abbreviations used: SP-C, surfactant-associated protein C; Dns-SP-C, dansyl-labelled SP-C; Dns-ITC, dansyl isothiocyanate; Dns-PE, N-dansyl-phosphatidylethanolamine; PC, phosphatidylcholine from egg yolk; PG, phosphatidylglycerol from egg yolk; DPPC, dipalmitoyl PC; DPPG, dipalmitoyl PG; MLV, multilamellar vesicles; RET, resonance energy transfer.

[1] To whom correspondence should be addressed (e-mail perejil@solea.quim.ucm.es).

phase. The effect of the protein on the thermotropic properties of DPPC and/or dipalmitoyl phosphatidylglycerol (DPPG) bilayers have been extensively characterized by techniques such as calorimetry [20–22], infrared [23], fluorescence spectroscopy [24], deuterium NMR [25] and ESR [26]. SP-C produces a marked broadening and a decrease in the enthalpy associated with the gel-to-fluid phase transition of DPPC or DPPG bilayers. The interaction of the protein with the lipids reduces the mobility of the lipid molecules in the fluid phase and perturbs their packing, an effect that, in principle, should facilitate transfer of phospholipids during bilayer–monolayer transitions. However, the effects of SP-C on the physical properties of surfactant phospholipid bilayers do not seem to be dramatically different from those of other transmembrane proteins and peptides, and certainly do not offer clues about the role of the protein in the dynamic behaviour of surfactant at the alveolar spaces. One reason may be that the general features of the lipid–protein interactions of SP-C, such as those analysed in the studies mentioned above, are dominated by the properties of the hydrophobic transmembrane α-helix. Some of the dynamic properties of surfactant phospholipids might be modulated by the behaviour of the N-terminal segment of SP-C, considering that this segment could be the only part of the molecule protruding from bilayers and monolayers. Modifications introduced into the structure of SP-C at this segment, such as deacylation [10,27–29] or charge-neutralization [30], produce significant effects on the SP-C-mediated biophysical surfactant activities.

In the present study we have used a dansylated form of SP-C, Dns-SP-C, to gain information about parameters governing the interaction of the N-terminal segment of SP-C with phospholipid bilayers of surfactant phospholipids. Dns-SP-C bears a single dansyl group at its N-terminal end and conserves its two cysteine residues palmitoylated. The modified protein conserves the secondary structure of native SP-C, as well as its ability to promote interfacial adsorption of DPPC suspensions and to influence the thermotropic behaviour of DPPC bilayers [31]. The dansylated derivative has been also used to find out whether SP-C interacts with SP-B in phospholipid bilayers, through the analysis of fluorescence resonance energy transfer (RET) from the single SP-B tryptophan residue to the SP-C dansyl fluorophore.

MATERIALS AND METHODS

Materials

Chloroform and methanol were HPLC-grade solvents from Scharlau (Barcelona, Spain). The lipids dipalmitoyl phosphatidylcholine (DPPC), egg yolk phosphatidylcholine (PC) and egg yolk phosphatidylglycerol (PG) were from Avanti Polar Lipids (Birmingham, AL, U.S.A.). 1,2-di[1-^{14}C]palmitoyl L-3-phosphatidylcholine (112 Ci/mol) was from Amersham International (Little Chalfont, Bucks, U.K.). The fluorescent reagent dansyl isothiocyanate (Dns-ITC) and N-dansyl-phosphatidylethanolamine (Dns-PE) were purchased from Molecular Probes (Eugene, OR, U.S.A.). Dansyl-labelled amino acid N-dansyl-L-leucine (Dns-Leu) was from Sigma (St. Louis, MO, U.S.A.). Sephadex LH-20 and LH-60 chromatography gels were from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Protein preparation

Surfactant proteins SP-B and SP-C were isolated from porcine lungs by a modification of the method described by Curstedt et al. [32], which has been discussed elsewhere [33]. The proteins were routinely checked for purity by SDS/PAGE, and for residual phospholipid contamination by phosphorus determination. Quantification of isolated proteins was carried out by amino acid analysis. After isolation, SP-B and SP-C were stored as chloroform/methanol (2:1, v/v) solutions at -20 °C until required.

Labelling of SP-C with the derivative Dns-ITC was carried out as described previously [31]. Briefly, the apparent pH of a solution containing approx. 1 mg of pure SP-C in chloroform/methanol (2:1, v/v) was adjusted to 7–7.2 by the addition of an appropriate volume of a methanolic 50 mM Tris/HCl solution. This SP-C solution was incubated at 4 °C overnight in the presence of Dns-ITC (0.6 mg/ml, final concentration). The reaction was stopped by the addition of HCl until the apparent pH decreased to 2. The unreacted probe was removed by Sephadex LH-20 chromatography. The elution profile was monitored by measuring the absorbance of the fractions at 240 and 330 nm. Quantification of the amount of bound label was performed after amino acid analysis by spectroscopic determination [molar absorption coefficient of dansyl determined in chloroform/methanol (2:1, v/v), $\epsilon_{330} = 9.23 \times 10^3$ $M^{-1} \cdot cm^{-1}$] and was estimated to be 0.6–0.8 mol of dansyl/mol of protein. The state of acylation of native and derivatized SP-C was checked by matrix-assisted laser-desorption ionization (MALDI) MS [34].

Lipid/protein reconstitution

SP-C and Dns-SP-C were reconstituted in phospholipid vesicles by two different methods [35,36]. To prepare most samples, lipids (DPPC, PC or PG) were mixed with the appropriate amount of protein in chloroform/methanol (2:1, v/v) and the mixture was dried under a N_2 stream and then under vacuum overnight. A trace of 1,2-di[1-^{14}C]palmitoyl-L-3-phosphatidylcholine (20000 d.p.m./mg of phospholipid) was also included in these samples to allow for a rapid evaluation of the final concentration of phospholipid once the reconstitution process was finished. The resulting dried films were then hydrated by the addition of 1 ml of 50 mM Hepes buffer (pH 7), containing 150 mM NaCl, and incubated for 1 h, with eventual vortex-mixing, at a temperature above the phase-transition temperature of the corresponding phospholipid. This treatment typically produces multilamellar vesicles (MLV). To obtain large unilamellar vesicles with a mean size of 100 nm, the MLV suspension was extruded ten times through a polycarbonate membrane of 100 nm pore size in a high pressure extruder (Lipex Biomembranes Inc., Vancouver, BC, Canada) thermostatted at a temperature above the lipid-phase transition temperature. An aliquot of each final vesicle suspension was then added to a vial containing 10 ml of Biogreen scintillation cocktail (Scharlau, Barcelona, Spain) and counted for radioactivity in a Beckman LS 3801 scintillation counter. Samples were finally diluted to equivalent phospholipid concentrations in order to make fluorescence spectra from different samples directly comparable. As an alternative reconstitution method, lipid and lipid/protein vesicles were formed by the injection of a minimal volume of lipid or lipid/protein organic solution into aqueous buffer. Stock solutions of SP-C or Dns-SP-C (1.2 mg/ml) were prepared as methanol-enriched solutions by evaporation under a N_2 stream of the original stocks in chloroform/methanol (2:1, v/v). To avoid irreversible precipitation of the protein, which cannot be resolubilized in methanol [33], additional volumes of methanol were continuously added to the sample during evaporation. This step was repeated several times to reduce the amount of chloroform in the sample,

which was estimated to be lower than 1 % in the final solution. Vesicles were formed by injection of up to 10 μl of lipid or lipid/protein methanolic mixture into 1 ml of 50 mM Hepes buffer (pH 7), containing 150 mM NaCl, thermostatted at a temperature above the phase-transition temperature of the phospholipid and undergoing vigorous vortex-mixing.

Fluorescence spectroscopy of Dns-SP-C

Fluorescence emission spectra of Dns-SP-C in organic solvents or lipid environments were recorded in a PerkinElmer MPF-44E spectrofluorimeter operated in the ratio mode [35]. Spectra were obtained at 25 °C from samples in organic solvents, and at 45 °C for lipid–protein complexes, in thermostatted cells of 0.2 cm optical pathlength. The slits widths were 5 nm for the excitation and emission beams, the scanning speed was 1 nm/s and the excitation wavelength used was 330 nm. Titration of the interaction of Dns-SP-C with different phospholipids was carried out by recording the emission fluorescence of dansyl in samples reconstituted independently by the injection method described above at different protein-to-lipid ratios. When the fluorescence of Dns-SP-C was compared after reconstitution in different lipids, a final aliquot of 10 μl of a 10 % (v/v) Triton X-100 solution was added to each sample to check that protein fluorescence in all the samples was comparable once the environment was similar.

Spectra were corrected for the scatter contribution of lipid dispersions by subtracting the corresponding control spectra obtained in the absence of protein. In addition, an inner-filter correction was applied to the emission spectra according to the equation

$$F_c = F_m \cdot 10^{(A_{em}+A_{ex})/2}$$

where F_c is the corrected fluorescence intensity, F_m is the measured fluorescence intensity after correction for scattering, and A_{ex} and A_{em} are the absorbances measured at the excitation and emission wavelengths respectively [37]. Absorption spectra of the samples were recorded using a Beckman DU-640 spectrophotometer.

RET experiments

Fluorescence energy transfer from the SP-B tryptophan as the donor to the dansyl extrinsic probe in Dns-SP-C as the acceptor, was measured in two ways. In the first type of experiments, the lipid/donor ratio was held constant while increasing the amount of acceptor molecules. In a second set of experiments, the donor to acceptor ratio was held constant and the total protein to lipid ratio was changed. To calculate the effective energy transfer in each experiment, the following samples were prepared and measured: phospholipid vesicles containing only SP-B (D), vesicles containing both SP-B and Dns-SP-C (DA) and vesicles containing only Dns-SP-C (A). As a control, fluorescence from the DA samples was compared with that of samples containing SP-B and native non-fluorescent SP-C. The efficiency of energy transfer (E) was estimated using the equation

$$E = (1 - Q_{DA}/Q_D) = 1 - [(F_{DA} - F_A)/F_D]$$

where Q_{DA} and Q_D are the quantum yields of DA and D samples, and F_{DA}, F_A and F_D are the measured fluorescence of the DA, A and D samples at the emission wavelength of the donor (330 nm) [19,35].

All the results presented in the Figures of this work are representative of three repeated experiments. Three different batches of Dns-SP-C, prepared independently from SP-C isolated from different lungs, were characterized and the results were qualitatively and quantitatively comparable.

RESULTS

Figure 1(A) compares the fluorescence emission spectra of Dns-SP-C reconstituted in PC bilayers with that of the fluorophore attached to the amino acid leucine (which is the N-terminal residue in the sequence of porcine SP-C) free in water, and with the spectra of a dansylated phospholipid, Dns-PE, in which the

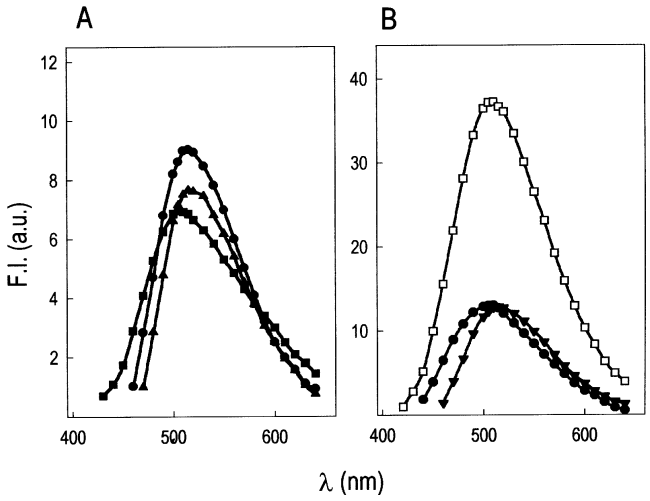

Figure 1 Fluorescence emission spectrum of the dansyl group in different environments

(**A**) Fluorescence emission spectra for Dns-SP-C (●) reconstituted in bilayers of egg yolk PC, in 50 mM Hepes buffer (pH 7) containing 150 mM NaCl, compared with the spectra for Dns-Leu (■) in the same buffer or Dns-PE (▲), also included in the PC bilayers. (**B**) Fluorescence emission spectrum for Dns-SP-C reconstituted in bilayers of DPPC (●), egg yolk PC (▼) or egg yolk PG (□), at a 5:1 lipid to protein weight ratio. All the spectra were obtained at a fluorophore concentration of 12 μM. Fluorescence (F.I.) was measured at 45 °C upon excitation at 330 nm and is presented in arbitrary units (a.u.).

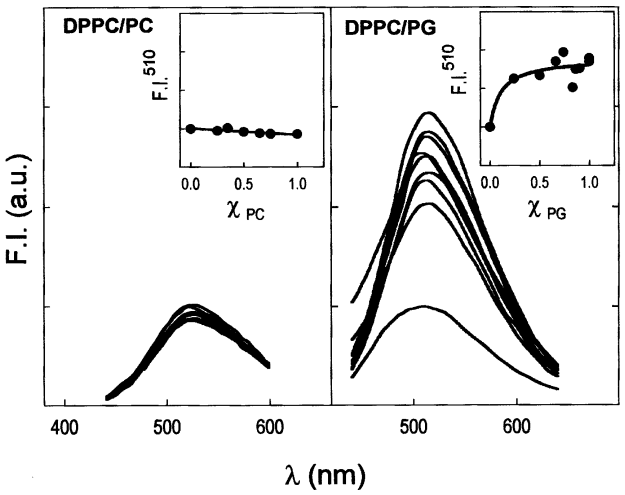

Figure 2 Fluorescence emission spectrum of Dns-SP-C reconstituted in bilayers of DPPC in the absence or presence of different proportions of PC (left panel) or PG (right panel)

Inserts: the fluorescence emission of Dns-SP-C at 510 nm is plotted against the molar fraction (χ) of PC or PG in the bilayers. All the samples contained 15 μg of Dns-SP-C reconstituted in the corresponding phospholipids at a 5:1 lipid to protein weight ratio. The excitation wavelength was 330 nm and the fluorescence (F. I.) measured at 45 °C is presented in arbitrary units (a.u.).

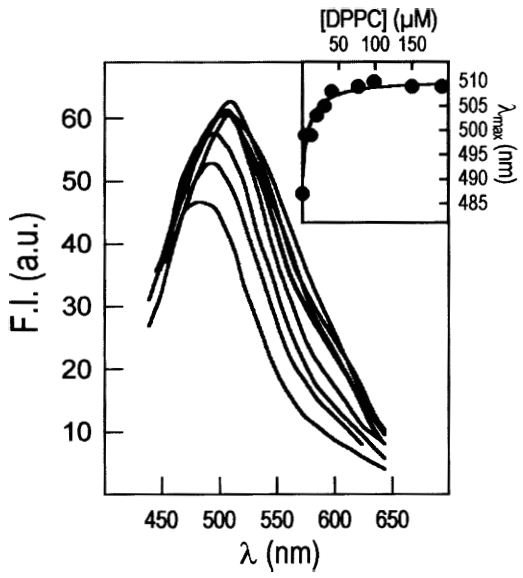

Figure 3 Effect of the lipid–protein interaction on the fluorescence emission spectrum of Dns-SP-C

(**A**) Fluorescence emission spectrum of Dns-SP-C (20 μg/ml) injected in the absence and in the presence of 9, 19, 28, 37, 94 and 187 μM DPPC (lower to upper spectra respectively) in 50 mM Hepes buffer (pH 7) containing 150 mM NaCl. The excitation wavelength was 330 nm and the fluorescence (F. I.) measured at 45 °C is presented in arbitrary units (a.u.). Insert: the wavelength of the fluorescence emission maximum of each sample is plotted against the lipid concentration.

same fluorophore is attached to the amine at the phospholipid headgroup, also in PC bilayers. The spectra of Dns-SP-C and Dns-PE are rather similar, with emission maxima at 515 nm, and are red-shifted with respect to the spectrum of the dansyl in aqueous bulk phase. This feature is probably due to the particular ionic environment of the highly hydrated phospholipid-headgroup region of the bilayers, as has been described for other fluorescent probes [38].

Figure 1(B) compares the emission fluorescence spectra of Dns-SP-C reconstituted in bilayers of DPPC, PC or PG from egg yolk. The emission spectrum of Dns-SP-C is similar in DPPC or PC bilayers, but shows a significantly higher intensity in membranes of PG. In Figure 2 the fluorescence emission of Dns-SP-C in DPPC has been titrated in the presence of increasing molar percentages of either PC or PG. Presence of approx. 15–20 % of PG, but not of PC, in DPPC bilayers produced close to the maximal increase in the fluorescence of dansylated protein.

The wavelength of the emission maximum of Dns-SP-C reconstituted in the different phospholipids was always in the range between that of free dansyl in buffer, 505 nm, and that of Dns-PE in the same phospholipid, 515 nm. This fact strongly suggests that in all the phospholipids the fluorophore in Dns-SP-C is orientated toward the polar, aqueous phase and hardly penetrates into the non-polar regions of the membranes. Interestingly, while the fluorescence emission maximum of Dns-PE was always detected at the same wavelength in the different phospholipid vesicles assayed, with maxima at 515 nm, Dns-SP-C had emission maxima at different wavelengths depending on the host phospholipid, at 510 nm in DPPC and egg PG bilayers, and at 515 nm in egg PC membranes. The addition of an aliquot of Triton X-100 to different samples produced fluorescence spectra of Dns-SP-C which all had maxima at 515 nm and similar fluorescence emission intensity values, no matter the lipid present (results not shown). These experiments suggest that the differences in fluorescent properties of Dns-SP-C in different membranes originates from differences in conformation/disposition of the N-terminal segment of the protein.

When a sample of Dns-SP-C was transferred into buffer in the absence of phospholipids, the fluorescence spectrum of dansyl showed a significant blue-shift, with emission maximum at 485 nm. This is probably a consequence of the formation of protein aggregates in which the dansyl group would be embedded in the hydrophobic protein matrix. The changes in the fluorescence spectra of Dns-SP-C in the absence and in the

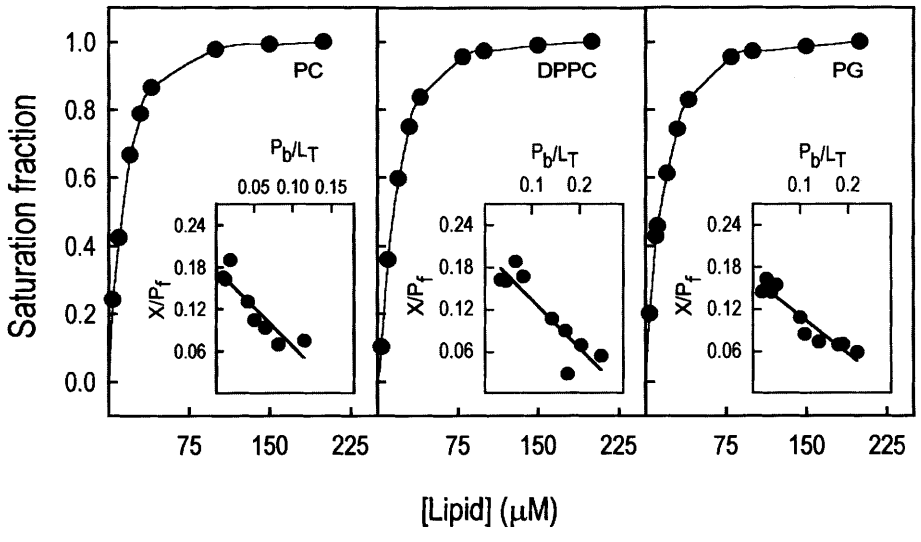

Figure 4 Lipid–protein binding curves for Dns-SP-C reconstituted in phospholipids

Dependence of the saturation fraction of the interaction of Dns-SP-C with egg yolk PC, DPPC or egg yolk PG, calculated from the change observed in the fluorescence emission spectrum, on the phospholipid concentration. Inserts: Scatchard plots of data from the binding isotherms, where $X = P_b/L_T$, P_b is the concentration of lipid-bound protein at a given phospholipid concentration, L_T, and P_f is the concentration of free protein.

Table 1 Apparent parameters for the interaction of Dns-SP-C with different phospholipids

Phospholipid	n	K_d (μM)
Egg PC	5.6 ± 0.2	5.6 ± 0.1
DPPC	3.1 ± 0.1	5.0 ± 0.2
Egg PG	3.2 ± 0.1	6.0 ± 0.2

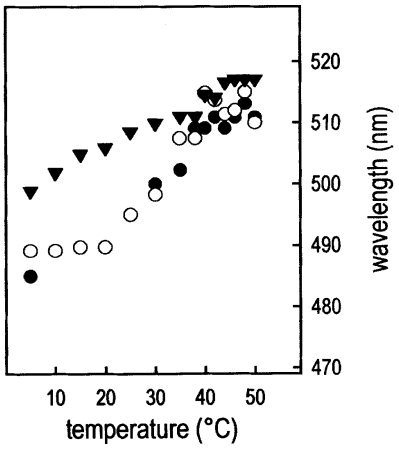

Figure 5 Effect of the temperature on the wavelength of emission maximum of Dns-SP-C or Dns-PE in DPPC bilayers

DPPC/Dns-SP-C or DPPC/Dns-PE membranes were reconstituted in this experiment by hydrating dry lipid or lipid/protein films. Density of Dns-SP-C (○, ●) and Dns-PE (▼) was 20% lipid/protein and 5% lipid/lipid weight ratio respectively. The effect of temperature on the fluorescence of Dns-SP-C was measured upon heating from 5 to 50 °C (●) or upon cooling from 50 to 5 °C (○).

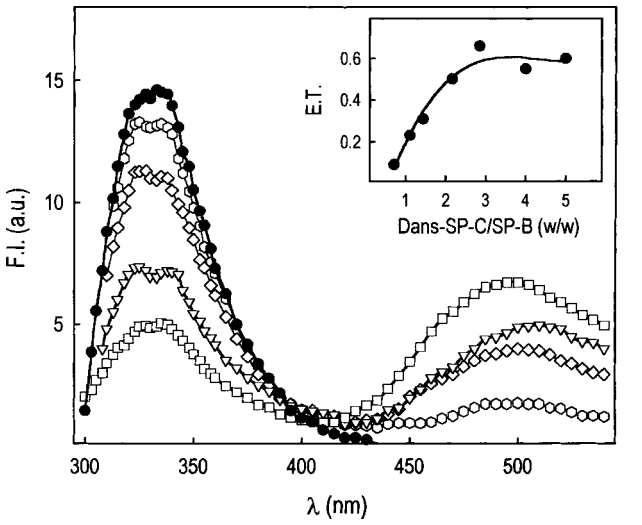

Figure 6 Fluorescence RET from SP-B to Dns-SP-C in phospholipid bilayers

Fluorescence emission spectrum of porcine SP-B (10 μg/ml) reconstituted in egg yolk PC bilayers (1:10 lipid/protein weight ratio) in the absence (●) or in the presence of Dns-SP-C at 0.7 (○), 1.1 (◇), 1.5 (▽), or 2.1 (□) Dns-SP-C/SP-B weight ratios. Insert: effect of Dns-SP-C to SP-B protein/protein ratio on the efficiency of energy transfer (E.T.) from SP-B to Dns-SP-C, calculated as described in the Materials and methods section.

presence of increasing amounts of phospholipids can therefore be used to estimate some parameters of the lipid–protein interaction. Figure 3 shows fluorescence emission spectra at 45 °C of Dns-SP-C injected into buffer in the absence and in the presence of increasing amounts of DPPC. A progressive shift of the emission maximum from 485 to 510 nm can be followed as the lipid/protein ratio increases, associated with an increase in the intensity of fluorescence emission. Comparison of the spectra of Dns-SP-C in the absence and in the presence of the different amounts of phospholipids allowed the estimation of the apparent saturation fraction at each lipid/protein ratio, for the different phospholipids assayed (Figure 4). The resulting binding curves were fitted by classical Scatchard plots to estimate apparent parameters characterizing the association of SP-C with phospholipids, as described previously [35]. Table 1 summarizes the values estimated for the parameters n, the number of lipid molecules with which each protein interacts, and K_d, the apparent dissociation constant for the formation of lipid–protein complexes once lipid and protein can encounter each other in bulk aqueous solution. Similar values of K_d of approx. 5–6 μM were determined for the association of Dns-SP-C with PC, DPPC or PG. The stoichiometry, n, of the lipid–protein association was three to five for all the lipids. This parameter indicates that once three to five lipid molecules are associated with each SP-C molecule, the fluorescent properties of the attached dansyl do not further change at higher lipid/protein ratios.

We have also tested the effect of lateral distribution on the fluorescence properties of the protein reconstituted in phospholipid bilayers, analysing the effect of temperature on the wavelength of emission of Dns-SP-C reconstituted in DPPC bilayers (Figure 5). It has been reported that SP-C is excluded from the gel phase of DPPC. Therefore, cooling of SP-C-containing DPPC bilayers below the phase-transition temperature leads to protein self-aggregation [19]. Figure 5 shows that Dns-SP-C exhibits fluorescence emission maximum at 510 nm at temperatures above 40 °C. The emission maximum, however, progressively shifts to shorter wavelengths as the temperature decreases. Below 20 °C, a temperature at which the protein is completely segregated from the DPPC gel phase [19], the protein exhibits the fluorescence emission maximum at 485 nm. Segregation of protein domains therefore induces transfer of the N-terminal segment of the protein to a markedly less polar environment, probably defined by protein–protein interactions. The fluorescence of Dns-PE also shifted 10–15 nm to shorter wavelengths upon cooling of the bilayers, which is probably associated with progressive exclusion of the bulky dansyl-bearing phospholipid molecules from the ordered gel phase of DPPC. However, the extent of the shift and the dependence of the shift on temperature were markedly different to the behaviour shown by Dns-SP-C, reflecting the different forces inducing translational dynamics in lipid and protein molecules.

The dansylated form of SP-C has been also used to explore the possible occurrence of SP-B–SP-C interactions in phospholipid bilayers that could be of relevance for the structure of surfactant complexes. Porcine SP-B has a single tryptophan residue in its sequence whose spectroscopic characteristics make it a perfect donor for carrying out RET experiments between SP-B (donor) and the dansyl group in Dns-SP-C (acceptor). The large spectral overlap between tryptophan emission of SP-B and dansyl adsorption of Dns-SP-C should allow radiationless transfer of excitation energy between the two fluorophores, which is critically dependent on the distance between them, if SP-B–Dns-SP-C complexes are formed. Figure 6 shows the fluorescence emission

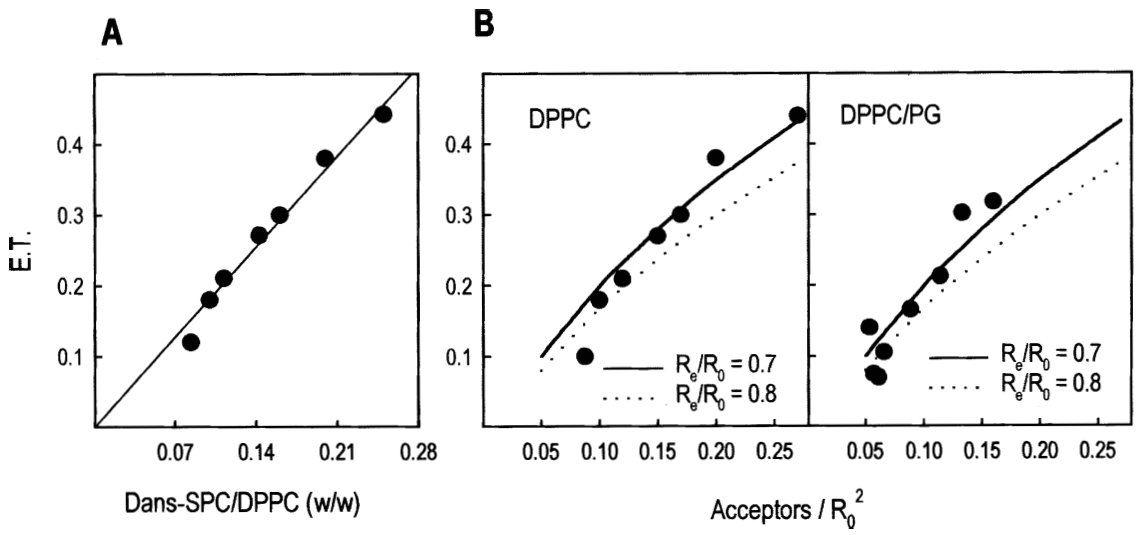

Figure 7 Effect of protein density on the efficiency of RET from SP-B to Dns-SP-C in phospholipid bilayers

(**A**) Effect of the total protein/lipid ratio, presented as the Dns-SP-C/lipid weight ratio at a constant Dns-SP-C/SP-B 2:1 (w/w) ratio, on the efficiency of energy transfer from SP-B to Dns-SP-C in egg yolk PC bilayers. Closed circles are the experimental points and the line is the linear regression of the data, indicating that energy transfer efficiency tends towards zero at low protein density in the membranes. (**B**) Efficiency of energy transfer versus surface density of Dns-SP-C (expressed as acceptors/R_0^2, taking $R_0 = 20$ Å for the tryptophan/dansyl couple) in DPPC (left panel) or DPPC/egg yolk PG 1:1 (mol/mol; right panel) bilayers, at 45 °C. The Dns-SP-C/SP-B ratio was kept constant at 2:1 (w/w). Closed circles are the experimental data and the curves represent the theoretical energy transfer calculated for the R_e/R_0 values indicated by using eqn 17 of Wolber and Hudson [41].

spectrum of SP-B in PC bilayers, in the absence and in the presence of increasing amounts of Dns-SP-C, obtained at an excitation wavelength of 280 nm. In these spectra the fluorescence emission intensity of similar samples containing Dns-SP-C but not SP-B has been subtracted, and therefore, the emission observed in Figure 6 at 450–550 nm can only be produced after the transfer of energy from the SP-B tryptophan. In the presence of increasing amounts of Dns-SP-C, the intensity of the emission spectrum of SP-B progressively lowers while a fluorescence peak with maximum emission at 500–510 nm, becomes progressively more intense. Such spectral features indicate molecular proximity between the tryptophan in SP-B and the dansyl in SP-C, on the distance scale specified by R_0. The R_0 parameter, which is specific for each donor/acceptor couple and defines the molecular distance permitting 50 % of RET between the two fluorophores, has been estimated for the couple tryptophan/dansyl to be around 17–20 Å (where 1 Å = 0.1 nm) [39,40]. The extent of RET increases as the amount of Dns-SP-C increases for a given proportion of SP-B in the bilayers. The insert in Figure 6 shows that the maximal energy transfer from SP-B to Dns-SP-C at SP-B/lipid ratio of 1:10 (w/w) was reached above a 2.5:1 Dns-SP-C/SP-B (w/w) ratio, which corresponds to a 5:1 molar ratio.

To find out whether the molecular proximity that gives rise to the observed energy transfer could be a consequence merely of high protein density in the membranes rather than an effect of real SP-B–Dns-SP-C complexes, we have performed experiments using different protein/lipid ratios. Figure 7(A) shows that the apparent efficiency of energy transfer from SP-B to Dns-SP-C, at a constant Dns-SP-C/SP-B 2.5:1 (w/w) ratio, decreases as the total lipid/protein ratio increases, tending to disappear at high lipid/protein ratios. Similar results were also obtained in DPPC:PG (1:1, w/w) bilayers (results not shown), indicating that the special features of the N-terminal segment of Dns-SP-C in PG-containing bilayers are not apparently involved in specific interactions with SP-B. The energy transfer data obtained in both DPPC and DPPC/PG bilayers could be reasonably well-fitted to the behaviour predicted by Wolber and Hudson [41] for the energy transfer expected when donors and acceptors are randomly dispersed in a bilayer. Figure 7(B) plots the apparent efficiency of energy transfer against the surface density of acceptors, expressed as acceptors/R_0^2 and taking R_0 as 20 Å, for samples containing a constant Dns-SP-C/SP-B ratio of 2.5:1 (w/w), in DPPC or DPPC/PG 1:1 (w/w) bilayers, at 45 °C. The experimental data in these plots could be fitted to a theoretical curve that defines the energy transfer as a function of the number of acceptors/R_0^2 and the ratio R_e/R_0, where R_e is the distance of closest approach between donor and acceptor, for an R_e/R_0 of approx. 0.7. This means that R_e for the closest possible approach between SP-B and Dns-SP-C would be around 14 Å, which is similar to the value calculated by others to model non-specific energy transfer between small proteins, such as SP-C, randomly distributed in a bilayer [19]. The conclusion is therefore that selective SP-B–SP-C interactions should be excluded in both DPPC and DPPC/PG bilayers, at least under the conditions used in the experiments presented in this study.

DISCUSSION

We have previously shown that the dansylated form of SP-C used in this work maintains its acylation state and secondary structure as native SP-C, as well as the ability to promote interfacial phospholipid adsorption and to perturb the thermotropic properties of DPPC bilayers [31]. The analysis of the fluorescent properties of Dns-SP-C in phospholipid bilayers can, therefore, provide information on lipid–protein and protein–protein interactions potentially occurring with native SP-C. Furthermore, conclusions about environmental effects on the fluorescence of dansyl in Dns-SP-C can be extrapolated and assigned to the specific local environment of the N-terminal segment of the native protein. Dansyl derivatives have been widely used to monitor and characterize interaction and insertion of proteins and peptides into membrane environments [42–45].

The quantum yield and the wavelength of fluorescence emission maximum of dansyl are both very much affected by the polarity of the environment. Immersion of a protein dansyl-bearing segment into non-polar membrane regions can be followed by the concomitant changes in the fluorescence emission spectrum of dansyl. The fluorescence emission spectrum of a dansylated phospholipase A_2, for instance, shifted its wavelength of emission maximum from 516 to 484 nm and its fluorescence was ten times more intense upon interaction of the protein with n-hexadecylphosphocholine [46]. In chloroform/methanol solutions, the maximum emission fluorescence of Dns-SP-C occurs at the same wavelength as that of free Dns-ITC, suggesting that the SP-C-attached fluorophore is completely exposed to the solvent. This feature could be expected considering that in the NMR structure determined for SP-C in chloroform solution [17], the N-terminal segment of the protein seemed to be flexible and conformationally disordered.

The absence of significant blue-shifts in the wavelength of emission of dansyl upon reconstitution of Dns-SP-C in membranes, in contrast with the features observed in other membrane-interacting proteins [46], strongly suggests that the dansyl group in Dns-SP-C is situated at the polar region of the membranes, exposed to the aqueous environment. We therefore speculate that the free N-terminal end of native SP-C could also be located at the interface of surfactant membranes, exposed to the aqueous medium. One could argue that modification of the free N-terminal amine could modify the interaction properties of that segment with the membranes, due, for instance, to the removal of a net positive charge at neutral pH. This is true, but the study of porcine SP-C is particularly useful in this matter. The sequence of SP-C from pig possesses a cationic arginine residue at position 2 which is absent, for instance, in the human, canine or bovine proteins. While all these proteins have positive charge close to the N-termini in their native state, just the porcine sequence maintains a cationic end after dansylation. The fluorescence intensity and the wavelength of emission maximum of Dns-SP-C are sensitive to the lipid composition of membranes. In contrast, the fluorescence of Dns-PE is very similar in all the bilayers tested. Differences in the fluorescent behaviour of Dns-SP-C in the different membranes (Figure 1) should therefore be attributed to the properties of the N-terminal segment of SP-C in the different lipids and not to differential properties of the fluorophore by itself.

The higher fluorescence emission intensity of Dns-SP-C in PG-containing membranes, compared with that observed in zwitterionic bilayers, is probably caused by a large shielding of dansyl from the quenching effects of the solvent, as a consequence of electrostatic lipid–protein interactions, especially through Arg^2, which is next to the dansyl. This effect was saturated above 20–30 mol% PG in the bilayers. The presence of sufficient anionic phospholipid molecules to saturate electrostatic interactions with the positive charges of the N-terminal segment of SP-C could then define the conformation and disposition of this region of the protein in phospholipid bilayers. Previous studies already showed that the overall conformation of the protein could be different in anionic and in zwitterionic membranes [35]. The PG fraction present in natural surfactant may be therefore important to finely tune the structure of the N-terminal segment of SP-C, whatever its function is.

Our results also show that Dns-SP-C is a useful tool to analyse the parameters governing the lateral distribution of the protein in phospholipid environments. Segregation of protein domains, a process which is critically dependent on the properties and lipid composition of bilayers, and that is thought to occur in monolayers at high compression rates [8,34], leads to a change in the disposition of the N-terminal segment of the protein. The conformation of the N-terminal segment of SP-C and its specific lipid–protein interactions could have important effects on the lateral distribution of the protein in surfactant environments. The loss of the ability of SP-C to insert phospholipids into interfacial monolayers when the positive charges of the protein are blocked [30] could be explained by the failure of the N-terminal segment of the protein to adopt the functionally competent conformation. On the other hand, electrostatic interactions of the N-terminal segment of SP-C with anionic phospholipids in surfactant are also likely to contribute to the sorting and segregation of phospholipid species, which indirectly leads to the selective enrichment of the interfacial film in DPPC [8,20].

Table 2 Parameters of the lipid–protein interaction estimated for different membrane proteins

LPC, lysophosphatidylcholine; DOPC, dioleoyl phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PS, phosphatidylserine.

		Parameters			
Protein	Lipid	K_d (μM)	n	Method	References
Myelin proteolipid	LPC	4.5	2.3	Fluorescence	[53]
PhoE protein signal peptide	DOPG	0.1–0.4	–	Monolayers	[48]
Cytochrome *c* oxidase subunit IV signal peptide	POPG	26	5	Fluorescence	[49]
M13 procoat protein	POPC	10	–	Fluorescence	[51]
Spectrin	PC	0.5	–	Centrifugation	[50]
Cecropin P1	PC/PS	8	–	Fluorescence	[60]

It has been proposed recently that SP-C has an essential role in promoting formation of surface-associated surfactant reservoirs from which the surface-active monolayer would be replenished by bioactive molecules during successive breathing cycles [10,12,47]. A model has been proposed in which SP-C would act as a sort of 'bridge' between monolayer and bilayers, or between bilayers, with the α-helix embedded in a bilayer and the palmitoylated N-terminal segment inserted by the palmitic chains in a neighbouring bilayer or monolayer [12,47]. The conformation of the N-terminal segment in the presence of PG may be essential to mediate this activity. Empirical evidence for this model is not available yet, and Dns-SP-C could be a good tool in designing experiments to confirm it.

The intensity of fluorescence emission and the wavelength of emission maximum of Dns-SP-C change within a certain range of the protein/lipid ratio. Injection of Dns-SP-C, in the absence of lipids or in the presence of low concentrations of lipids, into aqueous buffer probably leads to the formation of protein aggregates. In this environment dansyl has a lower fluorescence emission intensity and a shorter emission maximum wavelength than it shows when Dns-SP-C is co-injected with higher concentrations of lipids. This effect has been used to titrate the interaction of SP-C with different phospholipids, and the resulting lipid–protein binding curve has been fitted to a simple model previously applied to SP-B under similar conditions [35]. The association between protein and lipid molecules, which occurs instead of the protein self-association found in the absence of lipids, can be described by two parameters: K_d, the apparent dissociation constant of the protein–lipid interaction, and n, the number of phospholipid molecules saturating the measured effect. Table 2 allows comparison of the values of these parameters determined for the interaction of Dns-SP-C with PC, DPPC and PG (summarized in Table 1) with those determined for other proteins or peptides structurally similar to SP-C, such as certain

signal peptides [48,49], single transmembrane segments [50,51] or myelin proteolipids [52,53]. The values of K_d are in the few-micromolar range in all cases, which probably represents the apparent affinity of a single hydrophobic α-helical stretch for being surrounded by phospholipid molecules. The stoichiometry determined for the number of phospholipid molecules saturating the interaction with Dns-SP-C, calculated at approx. 5–6, is rather low compared with the phospholipid/SP-C stoichiometry determined by other techniques [21,22,26], or with the stoichiometry expected for the maximum number of phospholipids surrounding a single transmembrane α-helix [54]. This is probably because the stoichiometry determined in the present experiments just reflects the number of phospholipid molecules per molecule of protein producing the maximum effect in the dansyl fluorescence. In the presence of five phospholipid molecules per molecule of Dns-SP-C, the lipid–protein interaction might not be completely saturated, but most of the dansyl groups might have already shifted their location from the interior of protein aggregates to the environment of the membrane interfacial region. Complete protein dissociation could perhaps occur only at higher lipid/protein ratios. On the other hand, the similarity in values of K_d and n for the interaction of Dns-SP-C with PC and PG membranes is remarkable. It suggests that there are small differences in the mode of interaction of SP-C with zwitterionic or anionic bilayers, which is probably dominated, as suggested above, by the hydrophobic component of the interaction. The fact that the curve for the interaction of Dns-SP-C with PG can be fitted to a simple model that does not consider the electrostatic component of the interaction, suggests that selective electrostatic interactions are not participating primarily in the association of the protein with the phospholipids in our model system. Previous studies using ESR spectroscopy and phospholipid spin probes with different headgroups already excluded selective interaction of SP-C with PG over PC at physiological ionic strength [26].

An advantage of using dansyl as an extrinsic fluorescence probe in proteins is that its absorption spectrum largely overlaps with the fluorescence emission spectrum of tryptophan. This feature permits detection of protein–protein interactions by the possible occurrence of radiationless fluorescence energy transfer from tryptophan to dansyl, provided the two fluorophores are close enough on the molecular scale [55–58]. Little is known about protein–protein interactions occurring in surfactant complexes. We have explored the possible existence of interactions between SP-B and Dns-SP-C in membranes by analysing the occurrence of fluorescence energy transfer. This possibility has been explored in zwitterionic but also in anionic membranes, where the N-terminal segment of SP-C has the special conformation/disposition discussed above. Although molecular proximity between SP-B and Dns-SP-C has been detected in our samples under certain circumstances, the fluorescence energy transfer between tryptophan and dansyl fluorophores is highly dependent on the SP-B and SP-C amounts in the membrane, excluding the formation of specific SP-B–Dns-SP-C complexes. Possible SP-B–SP-C interactions have also been explored previously in monolayers [9,59]. Taneva and Keough concluded that SP-B and SP-C exhibited completely independent behaviour in DPPC, DPPG or DPPC:DPPG films in the absence of calcium, but they could form a sort of complex in the simultaneous presence of anionic phospholipids and the cation. Such an SP-B–SP-C complex might have been mediated directly by calcium or perhaps through protein segregation upon interaction of the anionic phospholipid headgroups with the cation. Unfortunately, the large scattering effects that calcium causes in anionic phospholipid vesicle suspensions prevent the application of fluorescence spectroscopy to analyse SP-B–Dns-SP-C interactions in the presence of calcium. Thus we cannot exclude that specific SP-B–SP-C interactions could occur in the presence of this cation.

We thank Alicia Prieto at Centro de Investigaciones Biológicas (Consejo Superior de Investigaciones Científicas) for carrying out the MALDI-MS analysis of samples and Dr Derek Marsh (Max-Planck Institut für Biophysikalische Chemie, Göttingen, Germany) for suggestions and critical reading of the manuscript. This work was supported by grants from Dirección General de Educación Superior e Investigación Científica (BIO2000-0929 and PB98-0769), Communidad Autónoma de Madrid (08.3/0008/2000) and the European Commission (QLK2-CT-2000-00325). I. P. is the recipient of a Formación del Personal Investigador grant and A.C. is the recipient of a post-doctoral grant, both from Comunidad Autónoma de Madrid.

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Received 12 March 2001/2 July 2001; accepted 28 August 2001