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Intrinsic structural differences in the N-terminal segment of pulmonary surfactant protein SP-C from different species

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Abstract

Predictive studies suggest that the known sequences of the N-terminal segment of surfactant protein SP-C from animal species have an intrinsic tendency to form β-turns, but there are important differences on the probable location of these motifs in different SP-C species. Our hypothesis is that intrinsic structural determinants of the sequence of the N-terminal region of SP-C could define conformation, acylation and perhaps surface properties of the mature protein. To test this hypothesis we have synthesized peptides corresponding to the 13-residue N-terminal sequence of porcine and canine SP-C, and studied their structural behaviour in solution and in phospholipid bilayers and monolayers. In these peptides, leucine at position 1 of both sequences has been replaced by tryptophan in order to allow their study by fluorescence spectroscopy. Far-u.v. circular dichroism spectra of the peptides in aqueous and organic solutions and in phospholipid micelles or vesicles are consistent with predicted conformational differences between the porcine and the canine sequences. Both families of peptides showed changes in their fluorescence emission spectra in the presence of phospholipids that were consistent with spontaneous lipid/peptide interactions. Both canine and porcine peptides were able to form monolayers at air–liquid interfaces, the canine peptides occupying lower area/molecule and being compressible to higher pressures than the porcine sequences. The peptides also shifted the isotherms and perturbed the packing of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) monolayers, the effects being always higher in anionic than in zwitterionic lipids, and also substantially higher in films containing canine peptide in comparison to porcine peptide. Acylation of cysteines at the N-terminal end of SP-C may modulate these intrinsic conformational features and the changes induced could be important for the development of its surface activity. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Lipid–protein interactions; Monolayer; Bilayer; Beta-turn; Circular dichroism; Synthetic peptide; Surface activity; Protein acylation

Abbreviations: ACM, acetamidomethyl; DPPC, 1,2-dipalmitoylphosphatidylcholine; DPPG, 1,2-dipamitoylphosphatidylglycerol; DTNB, dithio-bis-nitrobenzoic acid; EDT, ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; LPC, 1-palmitoyl-2-*lyso*-phosphatidylcholine; MBHA, methylbenzhydrylamine; PC, 1,2-diacylphosphatidylcholine from egg yolk; PG, 1,2-diacylphosphatidylglycerol from egg yolk; Pmc, 2,2,5,7,8-penthamethyl chroman-6 sulfonyl; PyBop, (benzotriazolyl)*N*-oxy-pyrrolidinium phosphonium hexafluorophosphate; SP-C, surfactant protein C; Tboc, terbutyloxycarbonyl; TFA, trifluoroacetic acid; TRT, Trityl

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1. Introduction

Air-breathing animals have developed a specialized system to physically stabilize the air–liquid interface of their respiratory epithelium. Pulmonary surfactant is a lipid–protein complex, the main but not exclusive function of which is to reduce the surface tension at the interface and facilitate respiratory mechanics (for updated reviews see Batenburg and Haagsman, 1998; Griese, 1999). Although phospholipids, especially dipalmitoylphosphatidylcholine (DPPC), play the major biophysical role in surfactants, certain hydrophobic small polypeptides promote transfer of DPPC into the interface and modulate physical properties of the surfactant under the dynamic conditions imposed by the respiratory cycle (Perez-Gil and Keough, 1998). Surfactant-associated lipopeptide SP-C is specifically synthesised by the type II pneumocytes, at the mammalian lung alveoli, as a precursor of 191 amino acids (Weaver, 1998). After a series of processing steps mature SP-C, consisting of 35 residues, is assembled into surfactant bilayers which are stored in the lamellar bodies of pneumocytes. The sequence of SP-C is highly conserved among the species studied so far (Johansson et al., 1991; Perez-Gil and Keough, 1994). Although the specific role of SP-C in vivo is not well understood, numerous studies in vitro suggest that SP-C promotes formation of surface active films and facilitates stabilization of those films when they are subjected to dynamic compression/expansion cycles (Oosterlaken-Dijksterhuis et al., 1991; Perez-Gil et al., 1992a; Creuwels et al., 1993; Johansson, 1998).

The structure of SP-C has been determined in organic solution, where it is soluble due to its high hydrophobicity (Johansson et al., 1994). It is composed of a regular hydrophobic alpha-helix of 23 residues and a more polar 10-residue N-terminal segment which has no defined conformation in chloroform/methanol or in detergent micelles (Johansson et al., 1995). The alpha-helical portion of SP-C adopts a transmembrane orientation in phospholipid bilayers (Pastrana et al., 1991; Vandenbussche et al., 1992) with the N-terminal segment the only region probably available to sustain interactions with proteins or with other bilayers or monolayers. SP-C from different species possess two cysteine residues at the N-terminal region except for those from dog and mink which have only one. In all the species with no exception the cysteines are quantitatively palmitoylated (Curstedt et al., 1990; Johansson et al., 1991). Acyl groups and positive charges in the N-terminal region have been found to be essential for the surface active properties of SP-C (Creuwels et al., 1993, 1995; Wang et al., 1996; Flach et al., 1999).

The main objective of the present work was to explore intrinsic structural determinants of the N-terminal segment of SP-C that could be identified by detailed comparison of the sequence of the protein from different species. Structural traits have been experimentally assessed by analysing the conformational behaviour of synthetic peptides designed from the N-terminal region of SP-C, in solution, bilayers and monolayers.

2. Materials and methods

2.1. Peptides and lipids

The peptides studied in this work were synthesised by Fmoc chemistry, as a C-terminal carboxamide. The peptide chain assembly was performed on an automated multiple peptide synthesiser AMS 422 (Abimed, Langelfeld, Switzerland). Rink amide-MBHA resin was used as polymeric support (0.41 $\text{mmol} \times \text{g}^{-1}$). Fivefold excess amino acid derivatives were added at the coupling steps. PyBop was used as the activating agent and the side chain protecting groups were BOC for Trp, PMC for Arg, and ACM and TRT for protected and free cysteines, respectively. Peptides were excised by TFA treatment, using the mixture TFA/H_2O/thioanisol/phenol/EDT 82.5:5:5:5:2.5. Afterwards, they were precipitated and washed by cold ether, and lyophilized. Purity of the products of synthesis was checked by MALDI-TOF mass spectrometry analysis and N-terminal Edman amino acid sequencing. Quantitation of free thiol groups in the peptides was achieved by reaction with dithio-bis-nitrobenzoic acid (DTNB) (Lukas and Bennett, 1980).

The lipids used, 1-palmitoyl-2-lyso-phosphatidylcholine (LPC), 1,2-dipalmitoylphosphatidylcholine (DPPC), 1,2-dipalmitoylphosphatidylglycerol (DPPG) and 1,2-diacylphosphatidylcholine (PC) and 1,2-diacylphosphatidylglycerol (PG) from egg yolk, were all purchased from Avanti Polar Lipids (Alabaster, AL).

Lipid/peptide samples were prepared by dif-

ferent methods depending on the experiment. For the circular dichroism experiments, lipid/peptide suspensions were prepared by hydrating dry lipid/peptide films formed after evaporation under a N_2 stream of organic lipid/peptide mixtures. The dry films were then hydrated for 1 h in 50 mM Hepes buffer at either room temperature (LPC) or 50°C (DPPC) with occasional vortexing, and the suspensions were subjected to sonication in a Branson UP 200S tip sonifier. To prepare the samples for fluorescence spectroscopy small aliquots, typically 5–10 μl, of a concentrated 1 mg/ml methanolic solution of the peptides were injected into suspensions of lipid vesicles in 50 mM Hepes buffer, containing 150 mM NaCl, pH 7.0, which had been prepared by extrusion through 0.1 μm (pore diameter) polycarbonate membranes (Nucleopore, Costar, Cambridge, MA, USA) in an Extruder (Lipex, Biomembranes Inc., Vancouver).

2.2. Circular dichroism and fluorescence spectroscopies

CD spectra were obtained as previously described (Ruano et al., 2000) on a Jasco J-715 spectropolarimeter fitted with a 150 W xenon lamp. Thermostated quartz cells of 0.1 cm optical path were used to record spectra at a scanning speed of 50 nm/min. Four scans were accumulated and averaged for each spectrum, which were then corrected by subtracting the appropriate blanks, subjected to noise-reduction analysis and presented as molar ellipticities. Estimation of secondary structure content from the CD spectra was performed after deconvolution of the spectra into four simple components — α-helix, β-sheet, turns and random coil — according to the convex constraint algorithm (Perczel et al., 1992).

Tryptophan fluorescence emission spectra of peptides were recorded using 275 nm as the excitation wavelength in a Perkin-Elmer MPF-44E spectrofluorimeter operated in the ratio mode (Cruz et al., 1998). Spectra were recorded at 25°C, using a scanning speed of 1 nm/s. Samples were in thermostated cells with a 0.2-cm optical path. The slit widths were 7 and 5 nm for the excitation and emission beams, respectively.

2.3. Peptide and lipid/peptide monolayers

Surface pressure measurements were performed on a Longmuir through (Applied Imaging, Dukesway Team Valley, Gateshead, UK) which employed a continuous Teflon ribbon barrier to minimise film leakage at high surface pressures (Perez-Gil et al., 1992b). Monolayers of pure peptides, pure lipids or lipid/peptide binary systems were formed by spreading 25–50 μl of concentrated peptide or lipid/peptide solutions in chloroform/methanol — 3:1 (vol/vol) on top of 5 mM Tris 150 mM NaCl pH 7.0 subphases prepared in deionized, doubly-distilled water, at 21–23°C. Films were equilibrated for 30 min before starting compression to allow for solvent evaporation. The total area of the interface was 500 cm^2 and the monolayer was compressed at 9 cm^2/s, while monitoring the changes in surface tension by a Wilhelmy dipping plate attached to a force transducer.

3. Results

The amino acid sequence of SP-C from different species is highly conserved (Johansson et al., 1991; Perez-Gil and Keough, 1994), especially with respect to the highly hydrophobic valine-rich C-terminal moiety. The N-terminal half of the molecule presents relatively more numerous variations in sequence, although certain traits such as presence of basic residues, prolines and palmitoylated cysteines are well maintained. We were interested in exploring conformational tendencies defined by the sequence of the protein in this region. In this regard, the sequence of SP-C from different species has been analyzed to determine its probability of forming β-turns, according to the rules proposed by Chou and Fasman (1978) (Fig. 1). The 10-residue N-terminal segment of the molecule presents a high probability of forming β-turns in all species analyzed. This is not surprising considering the high occurrence of prolines at those positions of the SP-C sequences. Considering all the possible sequential tetrapeptides (necessary to form β-turns) that could be defined in the sequence of the protein, two of them have substantial probability to form turns in all the species, those comprising residues 3–6 and residues 6–9. The tetrapeptide 3–6 has the highest probability to form a turn in all the species analysed except in canine SP-C, where the probability of a turn to be formed by residues 6–9 is the highest. The sequence of mink SP-C is very

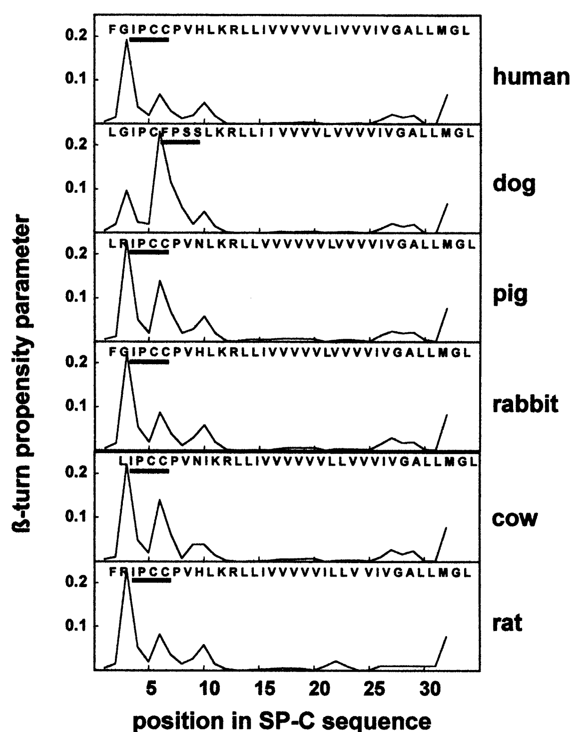

Fig. 1. Theoretical propensity of the sequence of SP-C from the indicated animal species to form β-turns, calculated according to Chou and Fasman (1978). The β-turn parameter is plotted against the position in the sequence where the tetrapeptide forming the turn would start. Bold lines indicate the position in the sequence where the most probable turn would be formed in each protein.

similar in its 10 N-terminal residues to the canine protein and has also the maximal probability of forming a β-turn at position 6–9 (not shown). This is remarkable considering that SP-C from dog and mink are the only ones having one instead of two palmitic chains, attached to their single cysteine residues at position 5. This analysis suggests that the primary sequence of the N-terminal segment of SP-C could impose certain conformational constraints that may differ with acylation. Previous structural characterization of whole SP-C from different sources could never define a particular conformation for the N-terminal tail of the protein (Perez-Gil et al., 1993; Johansson et al., 1994; Cruz et al., 1995; Johansson et al., 1995), in part due to dominant contributions of the ordered α-helical region and in part to intrinsic flexibility of that N-terminal segment in the solvents studied. To approach a more detailed characterization of the intrinsic conformational properties of this particular region of SP-C in different environments, we have designed and synthesised some short peptides mimicking the sequence of porcine and canine SP-C, two examples with different predicted turn propensities.

Fig. 2 summarizes the sequences of four 13-residue peptides synthesised taking as a model the sequence of porcine and canine SP-C. The N-terminal leucine residues in the native sequences have been replaced in all the synthetic peptides by tryptophan, with the purpose of using the tryptophan as an intrinsic probe to characterize structure and lipid–protein interactions by fluorescence spectroscopy. Likewise, the C-terminal carboxyl of the peptides has been amidated to better mimic the interactions occurring at that region of native SP-C. Two peptides were made based in the porcine SP-C sequence, corresponding to the native version without palmitoylation of the cysteines (peptide pSP-C13$_{L1W}$) and to a variant with the two thiols blocked by an acetamidomethyl group (peptide pSP-C13$_{L1W}$b). Mass spectrometry analysis of this peptide revealed that it consisted of a mixture of two forms differing in the mass of a single blocking group. Quantitation of free thiol groups by reaction with DTNB confirmed that peptide pSP-C13$_{L1W}$b consisted of a mixture of 70% of the form with completely blocked cysteines and 30% of the form having one free and one blocked cysteine. This blocked peptide had less peptide oligomerization by disulfide formation than the non-blocked peptide. Canine peptides, with a single non-palmitoylated cysteine, were never detected as dimers by mass spectrometry and were analysed as the non-blocked versions. We have designed a version of the canine sequence having arginine instead of lysine in position 11 because we expect to use this peptide — with a single amine group of the N-terminal end — in the future, for introduction of certain probes by site-directed chemical modification. In our experiments, the peptide with Arg instead of Lys behaved always similarly to the one with lysine.

The secondary structures of the synthesised peptides have been explored in different solvents by analyzing their far-u.v. CD spectra (see Fig. 3). In the solvents studied, all the peptides showed CD spectra dominated by the contribution of a negative ellipticity band at approximately 202–204 nm, indicative of a substantially disordered conformation. Conformational characterisation of the

	1 5 10
porcine SP-C	NH_2L R I P C C P V N L K R L L..............
pSP-C13$_{L1W}$	NH_2W R I P C C P V N L K R L$_{CONH_2}$
pSP-C13$_{L1W}$b	NH_2W R I P O O P V N L K R L$_{CONH_2}$
canine SP-C	NH_2L G I P C F P S S L K R L L............
cSP-C13$_{L1W}$	NH_2W G I P C F P S S L K R L$_{CONH_2}$
cSP-C13$_{L1W,K11R}$	NH_2W G I P C F P S S L R R L$_{CONH_2}$

Fig. 2. Amino acid sequences of the N-terminal region of porcine and canine SP-C and the four synthetic peptides studied in this work.

short peptides, such as those analysed here, by spectroscopic techniques is difficult, because they have high intrinsic conformational flexibility. Weak structural determinants in the spectra may be indicative of either a low percentage of ordered secondary structure in the peptide or, more probably, a low concentration of the structured population of the peptide. Still the CD spectra presented in Fig. 3 suggest significant conformational differences between the porcine and the canine peptides. The CD spectrum of the two porcine peptides shows a shoulder at 222 nm in all the solvents assayed, including methanol, TFE and TFE/water mixtures. This shoulder suggests that there is some α-helical component in their structure. In contrast, the CD spectra of canine peptides have much lower ellipticity in the range of 220–225 nm and they even show a maximum centred at approximately 224 nm, indicating secondary structure with low α-helical content. Such spectroscopic differences were maintained by the peptides when they were included in lipid suspensions, either in LPC micelles or DPPC vesicles (Fig. 4). As summarized in Table 1, the CD spectra of porcine peptides had an ellipticity ratio 222/202 of approximately 0.4–0.5 in all the environments while those of canine peptides were mostly lower than 0.3. This parameter would be sensitive to differences in secondary structure, especially α-helical content. A lower amount of α-helix would be the expected consequence of the different position of the β-turn in the canine sequences. A β-turn in positions 3–6 of porcine sequence would still allow formation of some α-helix in residues 10–13, perhaps a single α-helical turn. Determination by NMR of the structure of a 17-residue peptide from the N-terminal moiety of porcine SP-C in lipid micelles also indicated the presence of α-helix starting in residue 10 (Johansson et al. 1995). In contrast, the position of the β-turn in the canine peptides at residues 6–9 probably impairs stabilization of

Table 1
Parameters of the far-u.v. circular dichroism spectra of peptides from the N-terminal segment of porcine and canine SP-C, in aqueous or organic solution and in phospholipid micelles or vesicles

Peptide	Environment	$\lambda_{min\ ellipticity}$ (nm)	Min. ellipticity (deg cm^2 dmol)	Ellipticity ratio (222:202)
PSP-C13$_{L1W}$	Methanol	204	−11 310	0.59
	100% TFE	204	−10 890	0.51
PSP-C13$_{L1W}$b	Methanol	204	−18 480	0.39
	100% TFE	204	−13 490	0.52
	Buffer	201	−10 440	0.30
	LPC	200	−11 600	0.36
	DPPC	203	−9950	0.39
cSP-C13$_{L1W}$	Methanol	202	−10 940	0.08
	100% TFE	202	−11 150	0.25
CSP-C13$_{L1W,K11R}$	Methanol	202	−14 580	0.05
	100% TFE	203	−11 660	0.33
	Buffer	198	−14 090	0.11
	LPC	201	−10 380	0.14
	DPPC	203	−9530	0.27

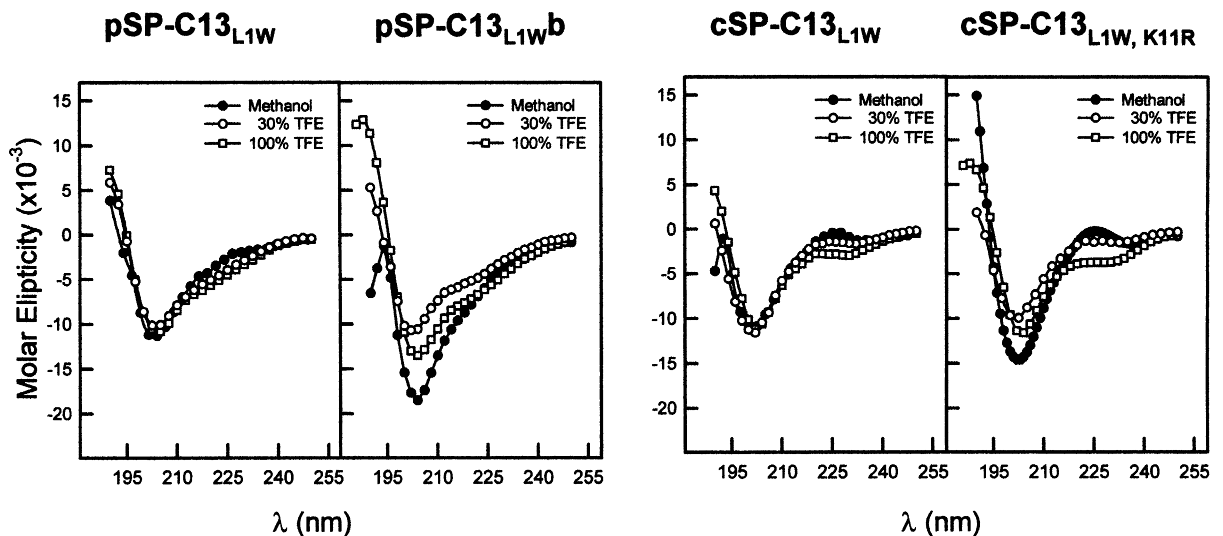

Fig. 3. Far-u.v. circular dichroism spectra, at 25°C, of porcine and canine peptides in methanol (closed circles), TFE (open squares) and TFE/water mixtures (open circles).

that limited amount of α-helix at the C-terminal end of the sequences.

In spite of the intrinsic conformational differences, both porcine and canine peptides have the ability to interact with phospholipid bilayers as detected by concomitant changes in the fluorescence properties of tryptophan (Fig. 5). The tryptophan fluorescence spectra of all the porcine and canine peptides in solution had similar features, with maximal emission at approximately 347 nm in methanol and 350–355 nm in buffer, indicating similar solvent exposure of the N-terminal fluorophore. However, in the presence of phospholipid vesicles of PC or negatively-charged PG all the peptides showed significant changes in their fluorescence spectra including both higher emis-

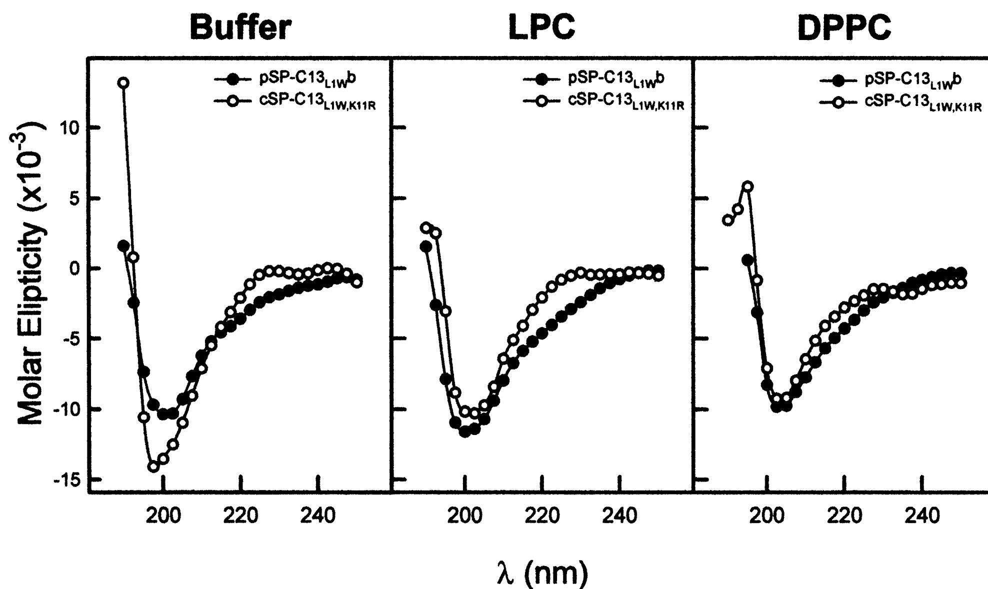

Fig. 4. Far-u.v. circular dichroism spectra of porcine (closed circles) and canine (open circles) peptides in buffer and included in LPC micelles or in vesicles of DPPC.

sion intensity and shift of the maximum fluorescence to shorter emission wavelengths. These effects are indicative of at least partial insertion into and shielding of the N-terminal tryptophan of the peptides in the membrane environment. All the peptides showed stronger effects in the presence of anionic bilayers suggesting that electrostatic interactions between the cationic peptides and negatively-charged lipids could be important in the interaction and insertion of the N-terminal segment of SP-C with surfactant bilayers or monolayers.

A different conformation of the peptides may also lead to differences in disposition or orientation at the air–liquid interface when the peptides are part of surfactant lipid–protein monolayers. Fig. 6 shows the surface behaviour of pure peptide or lipid–peptide monolayers subjected to compression. Both porcine and canine peptides are able to form stable monolayers on saline subphases, a consequence of their intrinsic amphipathicity. Monolayers of the porcine peptides collapse at approximately 19 mN/m, while canine peptide monolayers could sustain pressures a bit higher than 25 mN/m. The limiting areas per residue of the porcine peptides, calculated from the extrapolated intercept of the isotherm with the abscissa, were approximately 43 Å^2, higher than that of the canine peptides, which were approximately 38 Å^2. These differences indicate again a different conformation or disposition of the two kinds of peptides, the canine one taking less space and being more stable at the interface. The possibility that additional peptide–peptide

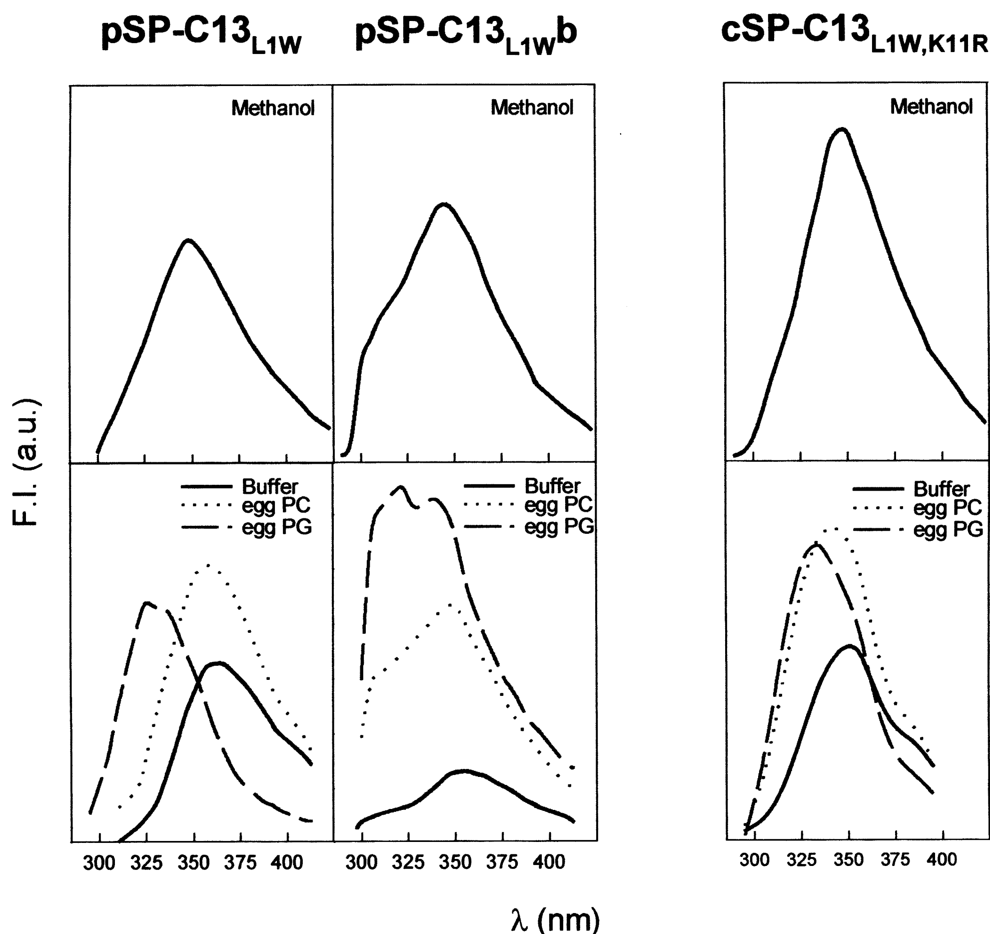

Fig. 5. Fluorescence emission spectra of porcine (left) and canine (right) peptides in methanolic (upper panels) or aqueous (lower panels, bold line) solution and included in vesicles made of PC or PG (lower panels, dashed and dotted lines). Excitation wavelength was 275 nm and emission is presented in arbitrary units.

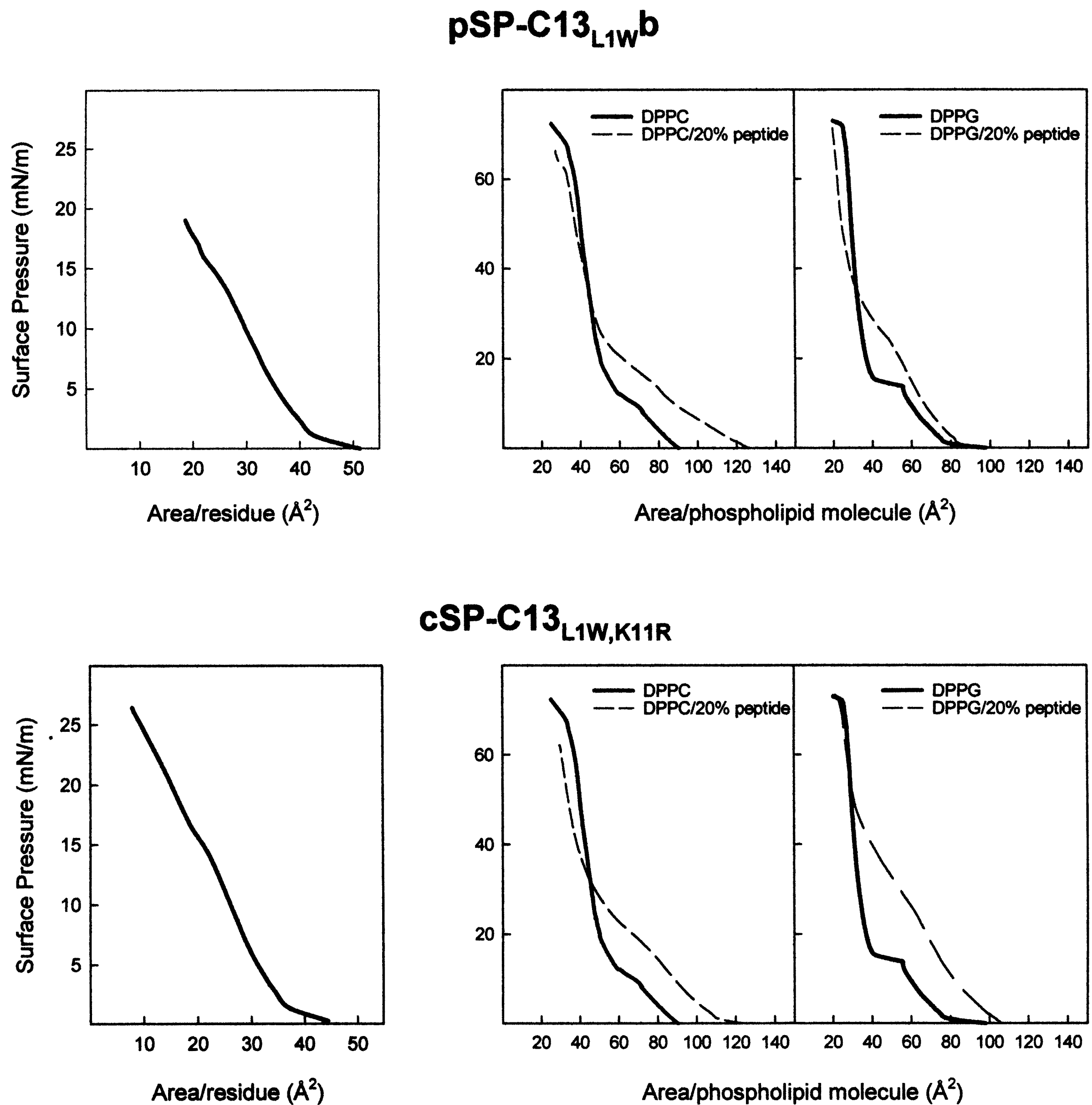

Fig. 6. *Left*. Compression isotherms of porcine (upper panel) and canine (lower panel) pure peptide monolayers. *Right*. Compression isotherms of DPPC or DPPG monolayers in the absence (bold lines) or presence (dashed lines) of 20 wt.% of porcine (upper panels) or canine (lower panels) peptides. All the monolayers were spread on buffered saline subphases Tris 5mM NaCl 150 mM pH 7, and compressed at 22 ± 1°C.

interactions could contribute to the lower apparent surface area and the additional stability of canine compared with porcine peptides at the air–liquid interface cannot be discarded, but those properties would also point to intrinsic structural differences between the two structures. Both families of peptides expand the compression isotherms of monolayers of either DPPC or DPPG (Fig. 6) indicating either penetration of the polypeptides into the monolayer or peptide-induced perturbation of the phospholipids. At given pressures both peptide-free and peptide-containing monolayer isotherms converge, indicating pressure-driven peptide squeeze-out. As observed in purely peptidic films, canine peptides were squeezed from the interface at higher pressures than porcine peptides. Both peptides had larger perturbing effects on the phospholipid and higher surface

stability in negatively-charged as compared to zwitterionic monolayers This also suggests that electrostatic peptide–lipid interactions are important in lipid/peptide interfacial complexes.

4. Discussion

Current models on the structure of SP-C in surfactant phospholipid bilayers and monolayers suppose that the N-terminal portion of the protein interacts with the lipids due to the palmitoylation of cysteines in that region (Johansson, 1998). However, evidence for the three-dimensional structure of native SP-C in lipid environments is lacking. Several authors have reported data on the overall secondary structure of the protein in lipids (Pastrana et al., 1991; Vandenbussche et al., 1992; Perez-Gil et al., 1993; Cruz et al., 1995), and the detailed three-dimensional structure of the protein in organic solvent (Johansson et al., 1994, 1995). These data have allowed a detailed characterization of the structure and dynamics of the remarkably regular hydrophobic α-helix in SP-C but little if any detail about conformation and disposition of the N-terminal segment of the protein. Although the highly hydrophobic helical region of the protein is probably essential to function, some of the functional features reported for the protein must reside in the structural properties and lipid–protein interactions of its N-terminal segment. Interaction of SP-C with phospholipid bilayers has been reported to include an electrostatic component (Shiffer et al., 1993; Perez-Gil et al., 1995) and to result in perturbation of conformation of the phospholipid headgroup region of the bilayers (Morrow et al., 1993). These effects are mediated by polar interactions between phospholipid headgroups and the polar charged and non-charged residues of the N-terminal tail of SP-C. Acylation and positive charges, both occurring in the N-terminal segment of the protein, seem to be also critical for the surface activity of SP-C, including the formation of monolayers and maintenance of low surface tension during dynamic compression–expansion cycles of surfactant films (Creuwels et al., 1993, 1995; Wang et al., 1996; Flach et al., 1999).

The data presented in this work indicate that apart from certain intrinsic structural differences among species, the N-terminal portions of SP-C of all the species may have enough intrinsic lipid affinity to interact and associate with phospholipid bilayers and monolayers, even without being acylated. Acylation, therefore, seems not to be required for membrane-interaction of the SP-C N-terminal segment. It will, no doubt, strengthen such interactions. The data available today on sequence and acylation level of SP-C from different species induce to us to speculate that differences in acylation could be related to intrinsic differences in conformational properties at the N-terminal segment of SP-C. It has been proposed that palmitoylation occurs posttranslationally in the endoplasmic reticulum, soon after the synthesis of a protein (Resh, 1999). Differences in local conformation and/or in E.R. membrane association of pre-SP-C might be important in defining the timing and extent of palmitoylation and, ultimately, the bioactive conformation of native SP-C once processed. The canine sequence apparently has higher effect in perturbing phospholipid packing in monolayers and higher stability at the interface than the porcine sequence. These superior surface properties could be due to an intrinsically higher hydrophobicity or to a more favourable conformation. The conformation and interfacial stability of the canine sequence could permit the function of the protein with lower extent of acylation. Acylation of SP-C N-terminal sequences in vivo could be important in modulating both hydrophobicity and conformation at that region of the protein. In this sense, comparative studies of non-acylated and acylated versions of the peptides would provide data on the local effects of acylation on protein structure. Several studies have recently suggested that acyl chains in SP-C may be important for the formation of a surface-associated surfactant reservoir from which the interfacial films would be replenished of surface active molecules lost during compression (Gustafsson et al., 2000; Kramer et al., 2000). Insertion of SP-C acyl chains into the interfacial monolayer would sustain attachment of SP-C-containing surfactant bilayers to the monolayer. The N-terminal segment of SP-C is able by itself to interact with bilayers and monolayers, contributing to those monolayer–bilayer contacts. The presence of acyl chains in the protein could still be essential to keep association of SP-C and SP-C-containing bilayers to the highly compressed monolayers at end-expiration.

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