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Canine hydrophobic surfactant polypeptide SP-C

A lipopeptide with one thioester-linked palmitoyl group

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The amino acid sequence and the posttranslational modification of the hydrophobic surfactant polypeptide SP-C from canine, rabbit and bovine lungs were established by direct sequence analysis and plasma-desorption time-of-flight mass spectrometry. The results reveal that canine SP-C has only one cysteine residue which, however, is palmitoylated, like the two Cys residues in other characterized SP-C molecules. In addition, canine SP-C is N-terminally truncated, with only 34 amino acid residues in its longest form. Thus, SP-C molecules can apparently vary to some extent in the N-terminal lipid-modified part, whereas the extremely hydrophobic middle and C-terminal parts are well conserved.

Hydrophobic surfactant polypeptide; Structural analysis; Thioester; Palmitoylation

1. INTRODUCTION

Pulmonary surfactant, which is a mixture of phospholipids and specific proteins in small amounts, is required for normal respiration, by reducing the surface tension at the air/liquid interface of the alveoli [1]. In premature infants a decreased content of surfactant is associated with respiratory distress syndrome. This serious disease can be effectively treated by airway instillation of surfactant preparations containing phospholipids and hydrophobic proteins [2]. The proteins, called SP-B and SP-C [3], seem to facilitate rapid spreading of the surface active phospholipids [4–6].

Most of the native SP-B is a homodimer [7] with the two 79-residue polypeptides [8] disulfide linked [9]. SP-C from human and porcine lungs has 35 residues in its longest form [10–11] with two juxtapositioned Cys residues palmitoylated [7]. It has been reported [12] that canine SP-C may lack Cys, suggesting the existence of SP-C molecules lacking palmitoyl groups. We therefore isolated SP-C from dog and other species and determined amino acid sequences and extents of palmitoylation. The results show that rabbit and bovine SP-C have two thioester-linked palmitoyl groups like the human and porcine molecules, while canine SP-C has one Cys, but this indeed is stoichiometrically palmitoylated.

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2. MATERIALS AND METHODS

2.1. *Preparation of SP-C*

Pulmonary phospholipids were isolated [5] from canine, bovine and rabbit lungs. SP-C was separated from the phospholipids by chromatography on Sephadex LH-60 in chloroform/methanol, 1:1 (v/v), containing 5% 0.1 M HCl [5]. Maximal concentrations of phospholipids in the SP-C fraction were determined by analyses of phosphorus [13].

2.2. *Structural analysis*

SP-C was deacylated and reduced at 37°C for 2 h in chloroform/methanol, 1:2 (v/v), containing 60 mM trimethylamine and 1 mM dithioerythritol [7]. After addition of [^{14}C]iodoacetate (3 mM final concentration), and incubation for another 2 h at 37°C, the carboxymethylated polypeptides were analyzed by degradations in an ABI 470A sequencer with on-line HPLC for PTH identification [10]. Amino acid compositions were determined with an LKB Alpha Plus analyzer after hydrolysis in 6 M HCl/0.5% phenol at 110°C for 24 h, or at 150°C for 72 h [10]. For C-terminal determination, the polypeptide was treated with anhydrous hydrazine in evacuated tubes for 6 h at 110°C [8]. Total fatty acids were released by treatment with KOH, methylated and analyzed by capillary gas-liquid chromatography [7].

2.3. *Mass spectrometry*

1–5 nmol SP-C in 5 μl 0.1% trifluoroacetic acid/ethanol, 1:1 (v/v), was applied on a nitrocellulose-coated sample foil, spin dried and analyzed in a Bioion 20 ^{252}Cf plasma-desorption time-of-flight mass spectrometer [7]. Deacylation of SP-C was then performed by treatment of the sample adsorbed on the nitrocellulose-coated foil with an aqueous solution, containing 80 mM dithiothreitol and 80 mM trimethylamine, in a humid chamber for one hour at room temperature. Finally, the nitrocellulose-foil was spin-dried, washed with 0.1% trifluoroacetic acid, spin-dried again and analyzed by mass spectrometry.

3. RESULTS

3.1. *Primary structure of canine SP-C*

The primary structure of canine SP-C was established by sequencer degradation of the [^{14}C]carboxymethylated polypeptide. N-terminal heterogeneity was detected, corresponding to two chains with different starting points, Gly-1 and Ile-2 (Fig. 1) in the relative proportions of 3:5. The two chains otherwise had identical structures and could be followed to the C-terminal Leu (Fig. 1). No other forms of the polypeptide were detected. The C-terminal end was confirmed by recovery of free leucine after hydrazinolysis. One Cys was clearly detected in the N-terminal segment, at position 4 (numbering according to the longest form; Fig. 1). It was identified both as Cys(Cm) phenylthiohydantoin and from its ^{14}C-radioactivity. Some residues in the extremely hydrophobic segment were difficult to assign, especially those at positions 22 and 25 (Val/Leu and Val/Ile, respectively, as deduced from the direct sequence analysis). However, the mass values of canine SP-C (Table I) are only compatible with the Val alternatives for those positions, and the total composition after prolonged hydrolysis (72 h at 150°C, to stoichiometrically recover the aliphatic residues [10]) also supports the Val assignments. Consequently, the amino acid sequence of canine SP-C is concluded to be as shown in Fig. 1, in agreement with all data combined.

3.2. *Canine SP-C is a lipopeptide with one palmitoylated cysteine residue*

The mass spectrometrical analyses of native canine SP-C showed molecular ion regions at *m/z* 3805–3809 and *m/z* 3753–3759 (Fig. 2 and Table I). After treatment of the sample with dithiothreitol and trimethylamine, the molecular ions decreased about 240 mass units, giving ions at *m/z* 3570 and *m/z* 3512, respectively (Fig. 2B and Table I). These results fit with one palmitoyl group covalently linked to canine SP-C. In addition the data confirm that the polypeptide has a truncated form, lacking the N-terminal Gly, and that the longest form is a 34-residue polypeptide. The ions observed are adducts with alkali metal ions (mainly sodium), as noticed before for the human and porcine molecules [7]. Treatment of SP-C with KOH released fatty acids, of which more than 70 mol% was found to be palmitic acid. According to phosphorus determinations, only a minor amount of the fatty acids can originate from phospholipids, and the molar ratio between fatty acids and polypeptide is calculated to be 0.8–1.3. Combined, all results clearly show that native canine SP-C is a lipopeptide with one palmitoyl group covalently linked to the polypeptide chain. Treatment of the native molecule with dithiothreitol and trimethylamine, which does not hydrolyse hydroxyl esters or amide bonds [7], cleaves the linkage (Fig. 2B) and establishes the palmitoyl group to be thioester-linked to the Cys residue.

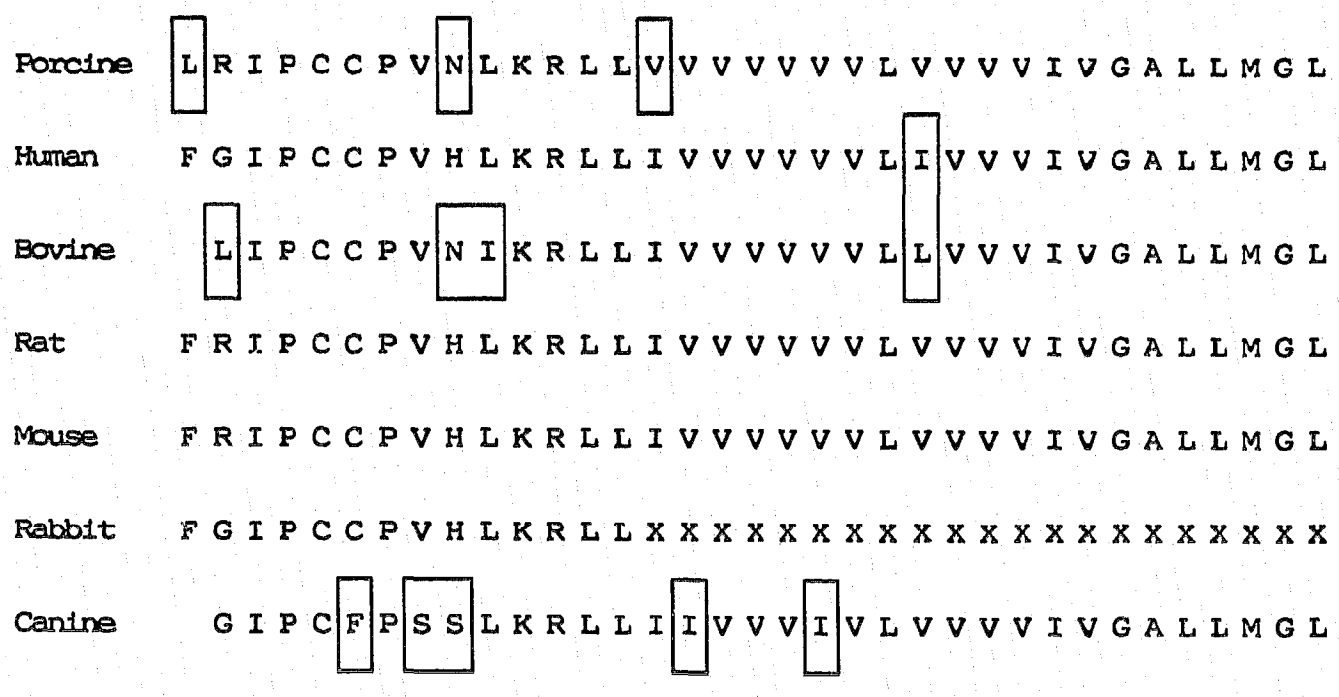

Fig. 1. Alignment of the amino acid sequences of the longest forms of porcine, human, bovine, rat, mouse, rabbit and canine SP-C. Residues differing from the most abundant alternative(s) have been boxed. Except for rat and mouse SP-C, for which the extents of acylation have not been determined, the Cys residues are all palmitoylated. The mass data indicate that rabbit SP-C has 35 residues in its longest form (cf. Table I). Structures from this work and [10–12,14].

Table I

Calculated molecular masses (M_r) and ions ($[M + Na]^+$) vs. observed ions for various forms of SP-C. Intact denotes the longest molecules with thioester-linked palmitoyl groups (Pam). Values for rabbit SP-C cannot be calculated from the partial structure available (cf. Fig. 1)

SP-C	Calculated M_r	Calculated $[M + Na]^+$ m/z	Observed m/z
Canine			
Intact	3782	3805	3805 and 3809
Minus Gly (truncated)	3725	3748	3753 and 3759
Minus Pam	3544	3567	3570
Minus Gly and Pam	3487	3510	3512
Bovine			
Intact	4058	4081	4076
Minus Leu (truncated)	3945	3968	3962
Minus Pam	3820	3843	3830
Minus Leu and Pam	3707	3730	3724
Minus 2 Pam	3582	3605	3597
Rabbit			
Intact			4181
Minus Pam			3947
Minus 2 Pam			3709

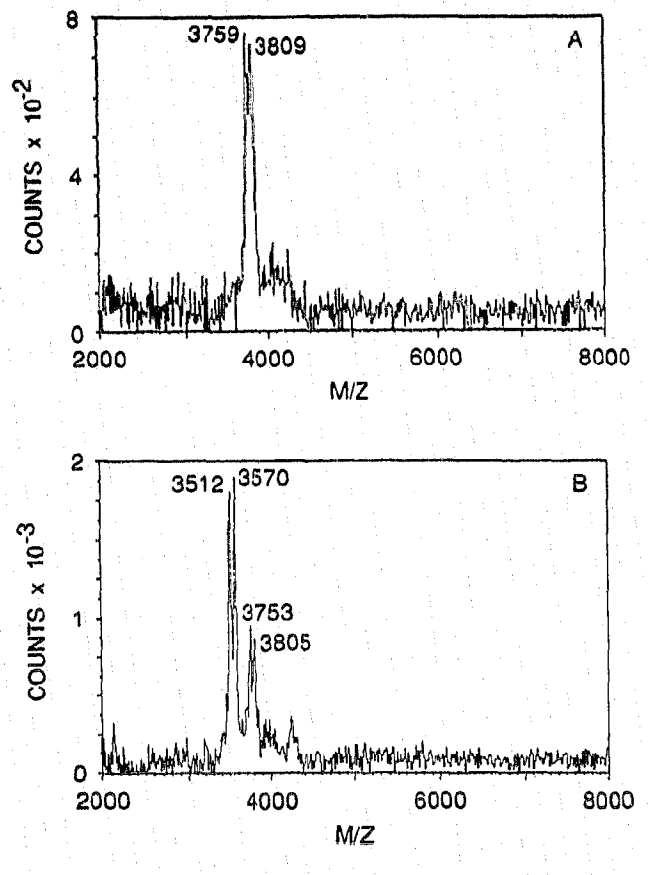

Fig. 2. Plasma-desorption time-of-flight mass spectra of canine SP-C, native (A) and after deacylation with dithiothreitol and trimethylamine (B).

3.3. *Structure of bovine and rabbit SP-C*

The primary structure of the N-terminal region of rabbit SP-C was determined by sequencer degradation (Fig. 1). In addition to the longest form having an N-terminal Phe, truncated forms starting with Gly-2 and Ile-3 were detected, constituting about 60% and 30% of the longest form, respectively. The molecule contained two adjacent Cys residues in agreement with the structures determined for human [11], porcine [10], bovine [11], rat [12] and mouse [14] SP-C. Mass spectrometry of native samples and samples treated with dithiothreitol and trimethylamine confirmed that both bovine and rabbit SP-C contained two palmitoylated Cys residues (Table I).

4. DISCUSSION

4.1. *Primary structures of different SP-C molecules*

SP-C molecules contain two Cys residues in all species examined, except dog where the second Cys is replaced by Phe (Fig. 1). The sequence deduced now for the canine polypeptide differs at one position from that previously published [12], by having only one of the two Cys residues replaced by Phe. Thus, canine SP-C is now established to be a Cys-containing polypeptide. Canine SP-C has two juxtapositioned Ser residues which are not present in any other species thus far examined (Fig. 1).

As noticed before [11], the N-terminal part of SP-C is variable between species, due to amino acid exchanges and different truncations. However, the N-terminal parts of human and rabbit SP-C are identical. It is not known whether the truncations are due to low specificity of the enzyme(s) liberating SP-C from pro SP-C, or to later aminopeptidase-like activities. The middle/C-terminal segment is conserved between the species ([11]; Fig.1). No C-terminal truncation has been detected.

4.2. *Functional implications of palmitoylation*

The functional significance of palmitoylation in general is disputed [15]. The structure of canine SP-C with a single Cys indicates that one thioester-linked palmitoyl group is sufficient for the function of SP-C in the pulmonary surfactant system. Still, modification of this Cys appears essential, here as in other species, since all SP-C's investigated have all Cys residues acylated. This may indicate that the palmitoylation is of importance. Judging from the present results, stoichiometric modification rather than absolute palmitoyl content is the critical factor.

One functional role of the palmitoylation might be merely to protect the free -SH group(s) and thereby to prevent disulfide-dependent dimerization. On the other hand, the function might be to give a hydrophobic center, and the Phe residue of canine SP-C, replacing

one of the palmitoylated Cys residues of other SP-C's, might then partly mimic the hydrophobic properties of a palmitoyl group. Alternatively, the palmitoylation of SP-C could have a regulatory function. However, since no significant amount of depalmitoylated SP-C has been demonstrated, a regulatory function via palmitoylation/depalmitoylation of SP-C seems unlikely. Finally, the palmitoylation could well occur already in pro SP-C, especially since there is no conserved amino acid sequence around the palmitoylation site that could be associated in mature SP-C with a recognition signal for the enzyme(s) responsible for the palmitoylation. Irrespective of functional significance, a physical link between lipid components and the SP-C polypeptide via cysteine palmitoylation is now established also for canine SP-C and therefore appears to be a general phenomenon.

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