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Hydrophobic 3.7 kDa surfactant polypeptide: structural characterization of the human and bovine forms

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The human and bovine forms of the hydrophobic 3.7 kDa surfactant polypeptide have been structurally analyzed. The polypeptide is essentially inert to enzymatic proteolysis, and methods for analysis include peptide handling in organic solvents and fragment generation by limited acid hydrolysis. The molecule exhibits N-terminal trimming, and the relative abundance of the different starting positions varies both among species and between adult and fetal forms of the surfactant polypeptide. The bovine major form is one residue shorter than the mature 35-residue human molecule. Comparison of the porcine, human and bovine polypeptides reveals a conserved hydrophobic middle/C-terminal segment and a variable hydrophilic N-terminal part.

Surfactant; Hydrophobic polypeptide; Primary structure; N-terminal trimming; Species variation

1. INTRODUCTION

Pulmonary surfactant is essential for normal lung function by reducing the surface tension at the air/liquid interface [1]. Its major components are phospholipids, especially 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, hydrophilic proteins (28–36 kDa) and hydrophobic proteins (3–18 kDa) [2]. Replacement therapy with different surfactant preparations in preterm neonates has shown that the phospholipids and the hydrophobic proteins are the essential components of exogenous surfactants [3–7]. In previous studies, we have isolated and characterized the two major types of hydrophobic low-molecular-mass protein from porcine lung [8–10]. Both proteins exhibit some N-terminal heterogeneity, with forms starting at different positions; the two polypeptides consist in their longest forms of 79 and 35 residues, respectively [9,10], corresponding to molecular masses of 8.7 and 3.7 kDa. Recombination of either of these polypeptides with synthetic phospholipids yields preparations that are surface-active both in vitro and in experiments on premature newborn rabbits [8,10].

The 35-residue polypeptide, which is the major hydrophobic protein in pulmonary surfactant, has unusual solubility properties, is resistant to enzymatic cleavage, and is to some extent inert even to chemical degradation [10]. N-terminal amino acid sequence analysis of the human [11] and bovine [12–14] forms of this polypeptide and the nucleotide sequence of a corresponding human cDNA structure [15] have recently been reported but, except for the structure of the porcine form [10], information on the size, C-terminal end, or all amino acid replacements has not been given. In the present study, we characterize the structure, including the C-terminal end, of the major human and bovine forms of the hydrophobic surfactant polypeptide.

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Table 1

Relative occurrence of the differently truncated forms found in adult and fetal human hydrophobic 3.7 kDa surfactant polypeptide

N-terminal structure	Occurrence (%)	
	Adult	Fetal
F G[a] I P C C ⟶	60	30
G[a] I P C C ⟶	20	40
I P C C ⟶	20	30

[a] Arg detected (10–25%) together with Gly at position 2

2. MATERIALS AND METHODS

2.1. *Protein preparations*

Bronchoalveolar lavage fluid was obtained from healthy adults by instillation of sterile saline (5 × 50 ml), and subsequent gentle aspiration (approved by ethical committee). The fluid was filtered and centrifuged, 400 × *g* at 4°C for 5 min [16]. Amniotic fluid was obtained from full-term pregnancies and was filtered before protein isolation.

Portions of 300 ml amniotic fluid or bronchoalveolar lavage fluid were mixed with 400 ml methanol. After addition of 800 ml chloroform, the lower phase was filtered and evaporated to dryness. The extract was separated by reverse-phase chromatography on Lipidex-5000 in a system of ethylene chloride/methanol, 1:4 (by vol.). The hydrophobic proteins in the phospholipid fraction were separated into two fractions by Sephadex LH-60 chromatography [8] and the protein in the second fraction was submitted to sequence analysis. The corresponding protein from bovine and human lung tissue was isolated as described [8–10].

2.2. *Structural analysis*

The pure polypeptides were reduced, [^{14}C]carboxymethylated and separated from excess reagents [8,9]. Limited acid hydrolysis was performed with 12 M HCl for 24 h at ambient temperature. The hydrophilic and hydrophobic fragments produced were separated using a chloroform/methanol/water (8:4:3, by vol.) system [10]. Hydrophilic peptides recovered in the methanol/water phase were separated by HPLC on an Ultropac TSK C18 column with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Peptides were subjected to sequence analysis in an Applied Biosystems 470A gas-phase instrument and a Beckman 890D liquid-phase instrument. Phenylthiohydantoin derivatives were detected by HPLC using a Hewlett-Packard 1090 instrument or an Applied Biosystems 120A on-line system.

Amino acid compositions were obtained after hydrolysis in evacuated tubes with 6 M HCl/0.5% phenol for 24 h at 110°C and for 72 h at 150°C [10]. Hydrazinolysis was performed with anhydrous hydrazine for 6 h at 110°C. Amino acids were determined with a Beckman 121M instrument.

3. RESULTS

3.1. *Human polypeptides*

Direct degradations of intact material of adult (bronchoalveolar lavage fluid) and fetal (amniotic fluid) origin gave identical results upon analysis of the N-terminal 30 residues. However, both the adult and the fetal polypeptide showed a complex pattern of N-terminal heterogeneity, with peptide chains starting at positions 1, 2 and 3 (all numbers according to the longest form; table 1). At position 2, results suggest the occurrence of both Gly and Arg. The further analysis to establish the complete structure of the human low-molecular-mass surfactant protein was performed with the material from the amniotic fluid.

The peptides isolated from the lipophilic phase

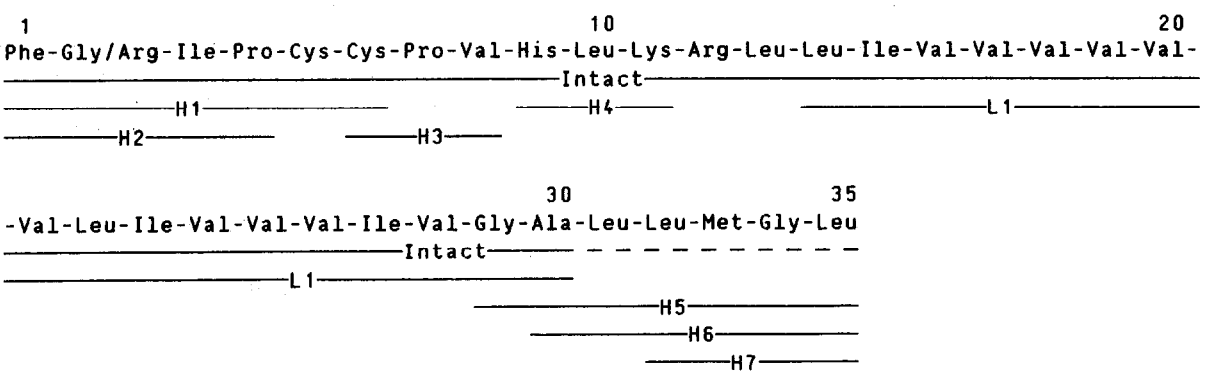

Fig.1. Amino acid sequence of human hydrophobic 3.7 kDa surfactant polypeptide and fragments analyzed. Only the mature, full-length sequence is shown, but truncated forms starting at positions 2 and 3 were found (cf. section 3). At position 2, both Gly and Arg occur. Intact, results from degradations of uncleaved material. H1–H7 and L1, peptides generated by limited acid hydrolysis and separated into a hydrophilic (H1–H7) and lipophilic (L1) phase. Continuous lines, peptide regions determined by sequence analysis; dashed line, region supported by total composition.

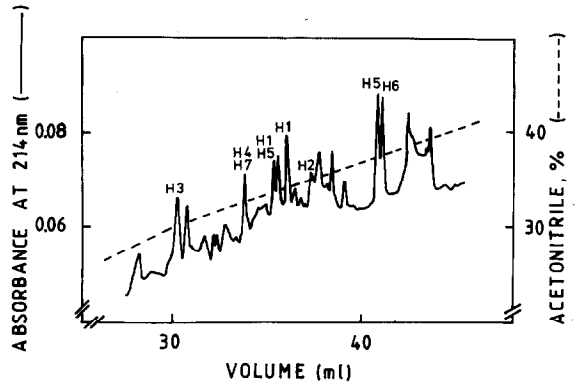

Fig.2. Elution profile of peptides produced by limited acid hydrolysis and recovered in the water/methanol phase of the chloroform/methanol/water system. Peptide designations as in fig.1. The peptides were separated by high-performance liquid chromatography on an Ultropac TSK C18 column (flow 1 ml/min) with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid.

of the chloroform/methanol/water system after limited acid hydrolysis of the intact polypeptide consisted of fragments from the extremely hydrophobic middle third of the molecule, mixed with uncleaved material. Direct degradation of the fragments in the mixture established the sequence for positions 14–30 (fig.1). The peptides in the methanol/water phase were purified by HPLC (fig.2). Sequence analysis of these peptides established the structure of the N- and C-terminal fragments (fig.1). The C-terminal end was also confirmed by recovery of free leucine after hydrazinolysis of intact material, both from lung tissue and amniotic fluid. Thus, the human hydrophobic 3.7 kDa surfactant polypeptide has 35 residues in its longest form and the amino acid sequence is given in fig.1. These results are in agreement with the total composition from acid hydrolysis, provided extensive hydrolysis is carried out to liberate all residues [10] of this extremely hydrophobic polypeptide.

3.2. *Bovine polypeptide*

Degradation of the intact material established positions 1–33 (fig.3). Sequence analysis of two hydrophilic peptides, generated by limited acid hydrolysis and separated by HPLC as for the human polypeptide, gave the N- and C-terminal regions (positions 1–10 and 29–34). Free leucine was recovered upon hydrazinolysis of intact protein. This defines the structure of the 3.7 kDa bovine hydrophobic surfactant polypeptide as a 34-residue molecule (fig.3). This structure is also in agreement with results from amino acid analysis after extensive acid hydrolysis. The bovine polypeptide exhibits N-terminal trimming with starts at either of the first two positions, with a relative occurrence of 85 and 15% (long and short form, respectively).

4. DISCUSSION

4.1. *Sequence analysis and N-terminal heterogeneities*

The direct analyses of the intact material were complicated by multiple starting points and the extremely hydrophobic middle third of the molecule. The fetal and adult human polypeptides (as well as the porcine polypeptide [8,10]) have starting points at positions 1–3, whereas the native, mature bovine polypeptide has only two starting points, and lacks a residue corresponding to position 1 (fig.3; or to position 2, wher the gap alternatively could be positioned) in the other forms. The relative abundance of the different forms varies both among the three species and between the adult and fetal human polypeptides (table 1).

Regarding the human polypeptide, evidence suggests a polymorphism at position 2 (fig.1). Although peptides with either Gly or Arg at this position could not be purified after limited acid hydrolysis, the recoveries of these residues are con-

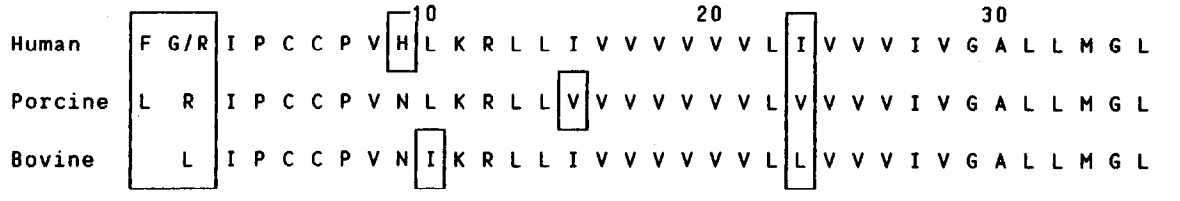

Fig.3. Comparison of the structures of human, porcine and bovine 3.7 kDa hydrophobic surfactant polypeptide. Residue exchanges are boxed.

sidered likely to represent a polymorphism since all degradations of intact material and N-terminal peptides (fig.1) yielded both these residues at position 2. Their relative abundance is similar in the adult and fetal polypeptide (table 1); in both cases forms with Arg appear to be slightly more common (25% versus 10%) in the full-length molecules starting at Phe than in the truncated forms.

The total compositions of both the human and bovine polypeptides can only be correctly obtained after non-conventional, extensive acid hydrolysis as for the porcine protein [10]. After hydrolysis for 3 days at 150°C, liberated hydrophobic residues are in excellent agreement with the results from the sequence analysis.

4.2. *Structural comparison of the human, porcine and bovine hydrophobic 3.7 kDa surfactant polypeptides*

With the present report, it is possible to compare the 3.7 kDa polypeptides of human, porcine, and bovine origin. Previous reports on the structures of the human and bovine polypeptides, deduced from analyses at the protein level have been incomplete [11–14], and a human cDNA sequence did not establish the exact size of the mature, native, and biologically active molecule [15]. However, the major form of the human polypeptide now defined, with Gly at position 2, is identical to a segment of the structure deduced from the cDNA reported.

Alignment of the human, porcine and bovine polypeptides reveals identical residues at 83% of all positions (fig.3). The first two residues (in the numbering system of the human and porcine forms) show no consistent pattern between the three species and at position 9 an exchange affecting the net charge is found. The hydrophobic region starting at position 13 is conserved in properties, revealing only single branched-chain residue exchanges, and exhibiting identical 12-residue C-terminal segments in all three polypeptides. Interestingly, the residue at position 23 is different among the polypeptides, but is in all cases an aliphatic, branched-chain residue. Thus, the 3.7 kDa surfactant polypeptide has a general structure of two distinct parts [10], one hydrophilic N-terminal segment and one extremely hydrophobic, middle/C-terminal part, as expected for a structure with surfactant properties. The more variable N-terminal part, with size differences, residue exchanges, multiple starting points, and possibly residue polymorphism, suggests less constraints on the N-terminal segment and emphasizes the importance of the middle/C-terminal, hydrophobic part for the function of the surfactant polypeptide.

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