Secondary structure and limited proteolysis give experimental evidence that the precursor of pulmonary surfactant protein B contains three saposin-like domains

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Abstract The 42 kDa precursor of surfactant protein B generates the 79 residue mature SP-B polypeptide, which belongs to the family of saposin-like proteins and has unique functional roles in pulmonary surfactant. From sequence comparisons it has been suggested that proSP-B, in addition to SP-B, contains two saposin-like domains, but their existence has until now not been experimentally verified. The 381 residue human proSP-B was now fused to an N-terminal poly-His tag, expressed in *Escherichia coli*, and purified from inclusion bodies by resolubilisation with 2.5% (w/v) SDS and, after removal of SDS, subsequent metal affinity chromatography. Recombinant proSP-B thus obtained exhibits about 35% α-helical structure in sodium phosphate buffer and is proteolytically cleaved preferentially between the three saposin-like domains. These results experimentally support that proSP contains, in addition to SP-B, two further saposin-like domains.

Key words: Surfactant protein; Recombinant proSP-B; Saposin-like protein; Secondary structure; Limited proteolysis

1. Introduction

Lung surfactant is a complex mixture of phospholipids and proteins. The main function of this system is to reduce the surface tension at the alveolar air/liquid interface. Four different surfactant-associated proteins have been purified. The larger surfactant proteins A (SP-A) and D (SP-D) are hydrophilic, while surfactant protein B (SP-B) and C (SP-C) are small and insoluble in water, see [1]. SP-B and SP-C probably have unique functional roles in the formation of a surface tension-reducing lipid monolayer at the alveolar air/liquid interface. These two hydrophobic proteins are unrelated in structure, but both the two mature proteins are formed by proteolytic cleavage from larger precursors. SP-B, with an M_r of 17 400 (unreduced) and 8700 (reduced), emanates from a 42 000 Da precursor [2,3], while SP-C with an M_r of 4200 is produced from a 22 000 Da precursor [4,5]. The cDNA coding for the precursor of human SP-B (proSP-B) has been cloned and sequenced and codes for a protein of 381 amino acid residues [3]. The mature SP-B polypeptide chain consists of 79 residues, forms disulphide-dependent homodimers, and displays about 45% α-helical secondary structure [6,7]. Each SP-B monomer contains three intramolecular disulphide bridges that link Cys-8–Cys-77, Cys-11–Cys-71 and Cys-35–Cys-46 [6].

From sequence alignments with saposins (which promote enzymatic degradation of sphingolipids in lysosomes), proSP-B has been proposed to contain three tandem repeats of about 90 residues [8], where mature SP-B corresponds to the second of these repeats (residues 201–279 in proSP-B). In these three so-called saposin-like repeats 17 positions are conserved, including the six half-cystine residues involved in intramolecular disulphide bonds. Furthermore, SP-B, amoebapores (which are pore-forming polypeptides from *Entamoeba histolytica*), parts of acid sphingomyelinase and acyloxyacylhydrolase, the saposins and NK-lysin (which is an antibacterial and tumourolytic polypeptide from Natural Killer cells), exhibit 17–24% pairwise residue identities [9]. Moreover, the intramolecular disulphide patterns in SP-B, NK-lysin, and saposins B and C are identical [6,10,11]. From the similarities between SP-B and the other members of the saposin-like family, it has been suggested that they exhibit a four-helix topology [9], and molecular modelling of NK-lysin and amoebapore has likewise yielded a four-helix structure [12].

We have expressed human proSP-B in *E. coli*, and characterised the recombinant protein in terms of overall secondary structure and susceptibility to limited proteolysis with trypsin. This shows that proSP-B exhibits about 35% α-helical structure and is preferentially cleaved between the three proposed saposin-like domains.

2. Materials and methods

2.1. Preparation of human proSP-B cDNA and construction of the expression vector

Polymerase chain reaction (PCR) primers were synthesised based on the human proSP-B cDNA sequence [3]; 5′-ATA CAT ATG GCT GAG TCA CAC CTG CTG-3′ and 5′-ATA GGA TCC TCA AAG GTC GGG GCT GTG GAT AC-3′, which correspond to nucleotides 1–21 and 1136–1162 of human proSP-B, and contain an *Nde*I and *Bam*HI cleavage site, respectively. PCR was carried out with a Robo-Cycler (Stratagene) for 30 cycles consisting of denaturation at 95°C for 2 min, annealing at 51°C for 2 min and extension at 72°C for 2 min. Reaction mixtures (total volume 100 μl) contained reaction buffer (New England Bio Labs), 10 μM of each primer, 2 mM dNTPs, 1 unit of Vent polymerase (New England Bio Labs) and 1 ng of adult human lung cDNA (Clontech). The resulting product was inserted into the pCRII vector by use of the TA Cloning kit (Invitrogen), and sequenced by the dideoxy chain-termination method [13] using the Sequenase kit (United States Biochemicals). The pCRII insert was liberated by *Nde*I and *Bam*HI cleavage and religated into the pET15b vector (Novagen), which gives an N-terminal poly-His tag fused to the recombinant protein. The ligation product was transferred into *E. coli* JM 109 cells for selection of plasmid-carrying clones. Finally, pET15b vector with inserted proSP-B DNA was transferred to *E. coli* BL21

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Abbreviations: DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria Bertani; rproSP-B, recombinant precursor of pulmonary surfactant protein B; SP, surfactant protein

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DE3 cells, which contain an integrated copy of the T7 DNA polymerase gene under control of the *lac* UV5 promoter.

2.2. Expression and purification of recombinant proSP-B (rproSP-B)

A single colony of *E. coli* BL21 DE3 transformed with pET15b/proSP-B vector was grown overnight at 37°C in 25 ml LB medium containing 50 μg/ml ampicillin. A 15 ml aliquot of the culture was inoculated into 500 ml of LB medium containing 50 μg/ml ampicillin and was grown to an OD_{600nm} of 1.2–1.5. Induction was then performed by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM. The culture was grown for an additional 3 h at 37°C, and harvested by centrifugation at 5000 rpm for 15 min. The bacterial pellet was suspended in 18 ml buffer A (20 mM Tris-HCl, 100 mM NaCl, pH 8.0) and sonicated 10 times at 60 Hz for 30 s on ice with an interval of 30 s. After centrifugation at 15 000 rpm for 15 min, the supernatant (supernatant 1) was saved and the pellet was resuspended in 15 ml buffer A containing 2.5% (w/v) SDS, sonicated as described above, and incubated at 37°C for 30 min. After another centrifugation at 15 000 rpm for 15 min, the supernatant was divided into two aliquots, which were dialyzed at room temperature against 20 mM Tris-HCl, pH 8.0, in the presence (supernatant 2) or absence (supernatant 3) of 2.5% (w/v) SDS, respectively. Supernatants 1–3 were separately loaded onto a Talon Metal Affinity column (5 ml bed volume, Clontech) pre-equilibrated with buffer A. The columns were washed with 20 bed volumes of buffer A, and 20 volumes of buffer A containing 5 mM imidazole. The bound proteins were finally eluted with 3 bed volumes of buffer A containing 100 mM imidazole. The eluates were dialyzed at room temperature against 50 mM sodium phosphate buffer, pH 6.0, or 20 mM Tris-HCl, pH 8.0, and stored at 8°C.

2.3. Limited proteolysis

Limited trypsin digestion of rproSP-B in 20 mM Tris-HCl, pH 8.0 (1:500, w/w, protease to substrate ratio) was carried out at 37°C for 24 h. After proteolysis, the product was lyophilised, dissolved in a small volume of sample buffer (62 mM Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerin, 5% mercaptoethanol, 0.02% bromophenol blue), and separated by SDS/PAGE in 15% gels. Protein bands were electrotransferred from the gel to a PVDF membrane (Millipore) for amino acid sequence analysis with an Applied Biosystems 470 A gas-phase sequencer equipped with on-line HPLC for identification of released phenylthiohydantoin derivatives.

2.4. Circular dichroism (CD) spectroscopy

Freshly prepared rproSP-B in 50 mM phosphate buffer at pH 8.0, and the same sample after 4 months storage at 8°C, were analysed. Reduction was performed by incubation with 5 mM DTT for 5.5 h at 37°C. CD spectra were recorded in a J-720 spectropolarimeter (Jasco) at 22°C. The scan speed was 20 nm/min (184–260 nm), the response time 2 s, and the resolution 2 data points/nm. The spectra shown are the averages of two scans. The concentration of rproSP-B was determined by amino acid analysis. θ is in units of $deg \times cm^2 \times dmol^{-1}$. Content of α-helical secondary structure was estimated from the molar ellipticities at 208 and 222 nm [14].

2.5. Immunodetection

Recombinant proSP-B (0.3 μg) before or after reduction (5 mM DTT, 4 h at 37°C) was spotted onto nitrocellulose membranes (Amersham) and the membranes were blocked for 1 h with 5% milk powder in phosphate-buffered saline containing 2.5% Tween 20 (TPBS). The membranes were then washed 3 times for 10 min in TPBS containing 0.25% Tween 20 and subsequently probed overnight at room temperature with a monoclonal anti-human SP-B antibody (HS-2 [15], kindly provided by Prof. Y. Suzuki, Kyoto, Japan). After further 3×10 min washes in TPBS, anti-mouse IgG antibodies conjugated with alkaline phosphatase were added. After washing, binding of the secondary antibody was detected by the colour development after addition of bromo-chloro-indolyl-phosphate (2 mg) and nitro-blue tetrasolium (4 mg) in 100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.0.

3. Results

3.1. Expression of proSP-B in E. coli and purification of the recombinant protein

A 1.2 kbp fragment was PCR-amplified from human lung cDNA by the use of probes corresponding to the 5′ and 3′ ends of proSP-B. 435 bp of the 5′ end and 360 bp of the 3′ end of the PCR product were sequenced by the dideoxy chain-termination method, and were found to be identical to the corresponding regions reported for the proSP-B cDNA sequence (data not shown). The fragment was purified by agarose electrophoresis and ligated into the expression vector PET15b. The vector containing inserted DNA was transfected into *E. coli* strain BL21 DE3, and rproSP-B was produced after addition of IPTG.

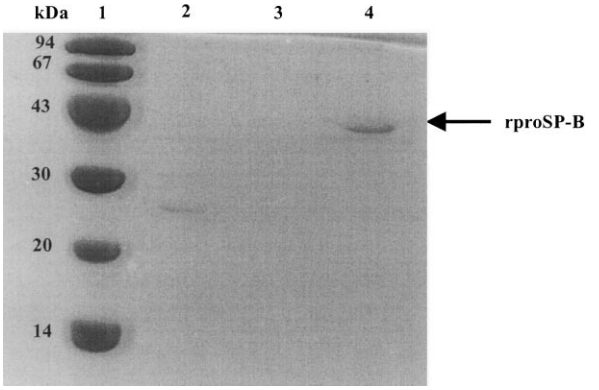

Fig. 1. Purification of rproSP-B. Proteins eluted from the metal affinity column with 100 mM imidazole were analysed by SDS/PAGE in a 15% acrylamide gel. Lane 1: molecular weight markers: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa); lane 2: supernatant after cell lysis (supernatant 1); lane 3: supernatant after resolubilisation of first 15 000 rpm pellet with 2.5% SDS and dialysis in the presence of SDS (supernatant 2); lane 4: same as lane 3 but dialysed in the absence of SDS (supernatant 3). See Section 2 for details.

No rproSP-B could be purified from the soluble fraction after cell lysis (Fig. 1, lane 2). Instead recombinant proSP-B was purified from the insoluble inclusion bodies that precipitated after centrifugation of the lysed bacterial cells. Recombinant proSP-B in such inclusion bodies can be purified to apparent homogeneity after resolubilisation using 2.5% SDS. Removal of SDS by dialysis after resolubilisation does not cause detectable precipitation, and significantly increases the yield of rproSP-B upon affinity chromatography (Fig. 1, lanes 3 and 4). The identity of the purified protein was confirmed by amino acid analysis and protein sequence determination. Purified rproSP-B was soluble in sodium phosphate buffer and remained in solution at least for several months. The maximal yield obtained was approximately 1.5 mg rproSP-B/l cell culture, as determined by amino acid analysis. Occasionally lower yield of recombinant protein (0.2–0.5 mg per l cell culture) was obtained. Recombinant proSP-B analysed by SDS/PAGE under reducing conditions gives an estimated molecular mass of 43 kDa (Fig. 1), which is in agreement with the expected mass of 44.4 kDa of the His-tag/proSP-B fusion protein.

3.2. Secondary and quaternary structure of rproSP-B

The overall secondary structure of rproSP-B was studied by CD spectroscopy. Recombinant proSP-B in sodium phosphate buffer exhibits a far-UV CD spectrum indicative of a protein with a significant content of secondary structure, and which is similar to the spectrum of SP-B in dodecylphosphocholine micelles (Fig. 2). The minima at 208 and 222 nm and

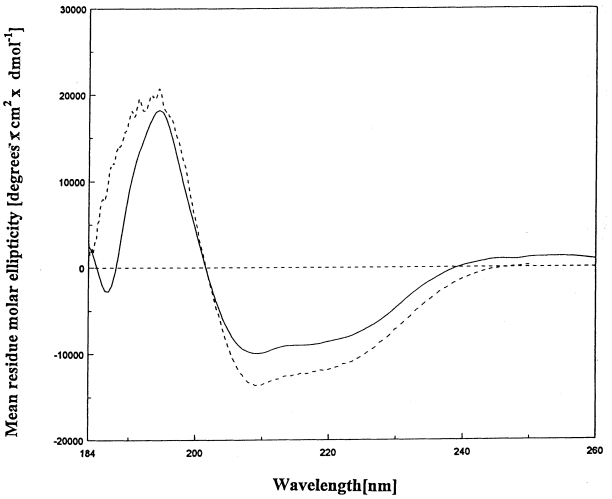

Fig. 2. Circular dichroism spectroscopy of recombinant proSP-B. CD spectra of rproSP-B in 50 mM phosphate buffer, pH 8.0 (solid line), and SP-B in 10 mM dodecylphosphocholine/50 mM phosphate buffer, pH 6.0 (dashed line).

the maximum at about 195 nm further indicate a substantial amount of α-helical structure. About 35% of helical content is estimated from the molar ellipticities of rproSP-B at 208 and 222 nm. Recombinant proSP-B stored for 4 months or treated with DTT (5 mM, 5.5 h at 37°C) exhibited CD spectra similar to those of the freshly prepared protein (not shown).

A monoclonal anti-human SP-B antibody (HS-2, [15]) reacted with rproSP-B, both in the absence and presence of DTT, while no cross-reactivity was found with trypsin-digested rproSP-B (data not shown).

SDS/PAGE of rproSP-B under non-reducing conditions does not give detectable bands at about 43 kDa, but the protein forms high molecular mass oligomers, which enter the gel with difficulty. This oligomerisation was confirmed by gel filtration on Superose 12 in the absence of reducing agents which did not show elution of any protein corresponding to monomeric proSP-B. It is therefore concluded that rproSP-B produced in *E. coli* consists of disulphide-dependent oligomeric forms. Also after reduction and reoxidation, rproSP-B is not monomeric.

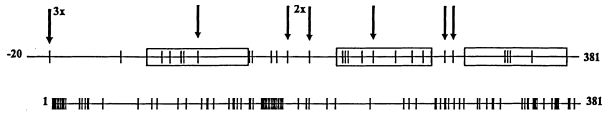

Fig. 3. Summary of results from proteolytic cleavage of rproSP-B with trypsin. In the upper line the three tandem saposin-like domains are boxed. The boxes cover the region from the first to the last (sixth) Cys that forms intramolecular disulphides in each domain. Domain 1 covers residues 69–143, domain 2 residues 208–277 and domain 3 residues 299–375. Mature SP-B corresponds to domain 2. Each vertical bar indicates Arg or Lys in the amino acid sequence. The arrows show the identified trypsin cleavage sites. In the lower line the sequence similarities between canine, rabbit, rat and human proSP-B [3,26–28] are summarised. Each vertical bar represents a position of variability (defined as different residues in two or more of the sequences).

3.3. Limited proteolysis of rproSP-B

CD spectroscopy of rproSP-B thus indicated a similar helical content as in SP-B. However, the helical contents estimated for the two proteins may not be directly comparable (see below). We therefore next wanted to find out whether the proposed conformation of proSP-B, containing three saposin-like domains, could be experimentally verified. If three saposin-like domains are present in rproSP-B, limited proteolysis, which is known to cleave preferentially at exposed areas [16], should give cleavages in the regions outside the saposin-like domains more frequently than within the domains, since each saposin-like domain is expected to be tightly folded due to the presence of three intra-chain disulphide bridges. Significantly, limited proteolysis of rproSP-B with trypsin produced 11 major bands which were resolved by SDS/PAGE. These bands were electrotransferred from the gel to PVDF membranes and subjected to sequence analysis. The amino acid sequences, determined for 6 to 19 cycles, in all but one case unambiguously assigned the cleavage position (Fig. 3).

4. Discussion

ProSP-B fused to an N-terminal poly-His tag was expressed in *E. coli* and purified from inclusion bodies by metal affinity chromatography after resolubilisation with 2.5% (w/v) SDS. This yields approximately 1.5 mg rproSP-B/l cell culture. ProSP-B has previously been expressed in CHO cells [17], mammary gland of transgenic mice [18] and *E. coli* [19]. In the latter case the protein expressed was purified and refolded simultaneously by solubilisation with guanidinium-hydrochloride and subsequent removal of the denaturant after immobilisation of the His-tagged protein on a metal affinity column [18]. With our experimental setup removal of SDS prior to affinity chromatography greatly increases the yield of rproSP-B (Fig. 1). Thus, it can be concluded that SDS is required for solubilisation of aggregated/denatured rproSP-B and that the protein refolds into a conformation which is soluble in aqueous buffer upon gradual removal of the denaturant. ProSP-B expressed in type II cells and in a pulmonary adenocarcinoma cell line is apparently monomeric also in the absence of reducing agents [20,21]. In contrast, proSP-B expressed in *E. coli* apparently forms higher order disulphide-dependent oligomers. This is likely caused by the abundance of Cys residues in proSP-B and inadvertent disulphide formation (25 Cys in human proSP-B, of which 6 are located outside the saposin-like domains). Further, it should be pointed out that SP-B purified from alveolar surfactant and native proSP-B also differ in quaternary structure. Thus, SP-B purified from alveolar surfactant exists mainly as a homodimer [1,22], while native proSP-B is a monomer [20,21]. It remains to be established whether the covalent dimerisation occurs at the level of proSP-B or SP-B. Anyway, in spite of the difference in quaternary structure between recombinant and endogenous proSP-B, the secondary structure and domain organisation of rproSP-B was now found to be as expected from sequence alignments [8].

The CD spectrum of rproSP-B indicates that it contains about 35% α-helix (Fig. 2). Different saposin-like proteins have been found to contain between 26 and 53% helices, see [9]. SP-B contains about 45% helices as determined by Fourier transform infrared and CD spectroscopy [7,9]. The present CD data are thus compatible with the idea [8] that rproSP-

B is composed of three saposin-like domains. It should however be pointed out that experimentally determined secondary structure contents of different saposin proteins may not be directly comparable for two reasons. First, proSP-B contains significant regions located outside the saposin-like domains (Fig. 3), the secondary structure of which of course influences the CD spectrum of rproSP-B. Second, because of its hydrophobic nature, SP-B can only be studied in detergent micelles or in the presence of organic solvents, while rproSP-B was analysed in aqueous solution. However, for NK-lysin CD spectra have been recorded both in aqueous solution and in the presence of DPC micelles, and no significant differences in secondary structure could then be observed [9].

Limited proteolysis of rproSP-B indicates that the three saposin-like domains are located as indicated in Fig. 3. A clear correlation between sites of limited proteolysis and segmental mobility has been documented for several proteins [16]. Similarly, the limited proteolysis of proSP-B shows preferential cleavage in those segments which are expected to exhibit high flexibility if the polypeptide is folded into three saposin-like domains. Eight of the ten cleavage sites identified are located outside of the proposed saposin-like domains. Although the remaining two cleavage sites were found within the saposin-like domains, they are situated in the same region in domains 1 and 2, where domain 2 corresponds to SP-B (Fig. 3). This region is located between half-cystine residues 3 and 4 in the respective domains. These half-cystines form a disulphide bridge in SP-B, and probably also in domain 1, and the peptide bond where the two cleavages occur is expected to be located in the last turn of a helix which precedes a loop that lacks ordered structure [9]. Likewise, in the recently determined NMR structure of NK-lysin, the position corresponding to the bond now cleaved precedes the last residue of helix 2 [23]. Taken together, the present CD and proteolysis data lend experimental support to an arrangement of proSP-B into three saposin-like domains.

Of the three saposin-like domains present in proSP-B, only SP-B has been isolated. It is an open question whether the remaining two saposin-like domains in proSP-B are processed to yield unique entities or if the sole function of proSP-B is to give rise to SP-B. Sequence alignments of canine, rabbit, rat and human proSP-B show that the first and second (i.e. SP-B) domains exhibit a high degree of conservation, while the third domain is little conserved (Fig. 3). Notably, the proSP-B N-terminal part, but not the C-terminal region, is required for its processing and intracellular targeting [24,25]. The conservation of the first saposin-like domain may indicate that it is particularly important for proSP-B functions, or that it serves a role also after processing of proSP-B to SP-B.

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