The α-helical neck region of human lung surfactant protein D is essential for the binding of the carbohydrate recognition domains to lipopolysaccharides and phospholipids

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We have expressed the carbohydrate recognition domains (CRDs) of human lung surfactant protein D (SP-D) in *Escherichia coli* as a trimeric structure held together by the α-helical neck region of the molecule. The DNA sequence coding for the neck-region peptide and the CRD of SP-D was subcloned and expressed as a fusion protein containing the *E. coli* maltose binding protein (MBP). After removal of the MBP, the recombinant structure, containing three CRDs of SP-D, was found to be comparable to native SP-D in terms of carbohydrate binding specificity, the binding to lipopolysaccharides (LPSs) of Gram-negative bacteria, and interaction with phospholipids. The CRD of SP-D, without the neck region peptide, was also expressed and shown to behave as a monomer that showed a very weak affinity for maltose-agarose, LPSs and phospholipids. The α-helical neck region on its own showed affinity for phospholipids and thus might contribute to the binding of SP-D to these structures. However, the possibility that hydrophobic patches, which are exposed only in the isolated neck region and not in the intact SP-D, plays a role in neck region–phospholipid interaction, cannot be excluded. The results confirm the importance of the neck region as a trimerizing agent in bringing together three CRDs and suggest that multivalency is important in the strong binding of SP-D to carbohydrate targets.

INTRODUCTION

Surfactant protein D (SP-D) is a collagenous, carbohydrate-binding glycoprotein that is synthesized and secreted into the air space of the lung by alveolar type II cells and also by non-ciliated bronchiolar epithelial cells [1]. SP-D belongs to the mammalian C-type lectin superfamily [2,3]. It has a characteristic primary structure that is organized into four regions, namely (1) an N-terminal region involved in interchain disulphide formation, (2) a collagenous region composed of Gly-Xaa-Yaa repeats, (3) an α-helical 'neck' peptide and (4) a carbohydrate recognition domain (CRD) [4]. SP-D has been shown to bind lipopolysaccharides (LPSs) of many Gram-negative bacteria and also to interact with alveolar macrophages, consistent with its having a role in pulmonary host defence [5]. Recent studies have also demonstrated interactions with phospholipids *in vitro* [6], suggesting that SP-D could play a role in the reorganization or turnover of pulmonary surfactant *in vivo*. SP-D shows structural similarities to pulmonary surfactant protein A (SP-A), serum mannose binding protein and bovine conglutinin [2]. Both SP-D and conglutinin have remarkably similar structures, being composed of four collagen-like rods joined to each other at a central point, and each rod having three globular, C-type, lectin domains (also called CRDs) at the other extremity [7]. It is likely that this type of multivalent structure is important in the recognition of carbohydrate targets by the CRDs, and also in clearance of the target via interaction of the SP-D–target complex with cell-surface receptors for SP-D. The binding of SP-D to cells is known to trigger events such as the enhancement of oxygen radical production by alveolar macrophages [8] and it has also been demonstrated that SP-D uses a receptor on the surfaces of alveolar macrophages that is distinct from the C1q-receptor [9].

We have expressed the CRDs of human SP-D in *Escherichia coli* as a trimeric structure. The recombinant fragment of SP-D was initially synthesized as a fusion protein consisting of *E. coli* maltose binding protein (MBP) linked by a peptide (containing a site susceptible for Factor Xa protease) to the neck plus CRD region of SP-D. After one-step affinity purification on an amylose column, the fusion protein was cleaved with Factor Xa to release the polypeptide containing the neck and CRD region of SP-D. This polypeptide behaved as a homotrimer in non-dissociating conditions. The three CRDs of SP-D [rSP-D(N/CRD)], held together by the 'neck' region, behaved similarly to native SP-D in terms of sugar specificities and also in the binding to LPSs of Gram-negative bacteria, and in its interaction with phospholipids. We have also expressed the CRD of SP-D without the neck peptide. This material, containing only a single CRD of SP-D [rSP-D(CRD)], behaved as a monomer in non-dissociating conditions, and showed very weak affinity for maltose-agarose and phospholipids. An unexpected finding was that the recombinant, α-helical, neck region [rSP-D(N)] on its own showed affinity for phosphatidylinositol (PI), which indicated that this region of SP-D might play a role in its binding to certain phospholipids. The production of the trimeric neck-CRD of SP-D

Abbreviations used: CRD, carbohydrate recognition domain; DPPC, dipalmitoyl phosphatidylcholine; IPTG, isopropyl β-D-thiogalactopyranoside; LPS, lipopolysaccharide; MBP, maltose binding protein; SP-A, surfactant protein A; SP-D, surfactant protein D; nSP-A, purified native human SP-A; nSP-D, purified native human SP-D; PI, phosphatidylinositol; PC, phosphatidylcholine; rSP-D(CRD), recombinant lectin domain of SP-D; rSP-D(N), recombinant neck region of SP-D; rSP-D(N/CRD), recombinant neck region and carbohydrate recognition domains of SP-D.

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highlights the significance of the neck region as a trimerizing agent and suggests that multivalency is important in the recognition of carbohydrate targets.

MATERIALS AND METHODS

DNA constructs used in the expression of rSP-D(N), [rSP-D(N/CRD)] and [rSP-D(CRD)] of human SP-D

The Bluescript plasmid containing the cDNA coding for human SP-D [4] was digested with *Sma*I and *Eco*RI, as well as with *Msc*I and *Eco*RI, giving rise to DNA fragments of 532 and 364 bp respectively. Both fragments were subcloned into the pGex-2T vector, double-digested with *Sma*I and *Eco*RI. The DNA inserts coding for the SP-D fragments were excised from the pGex-2T vector by using *Bam*HI and *Eco*RI and the resulting fragments were ligated into a chloramphenicol-resistant gene carrying the Bluescript-plasmid, pBCSK, which had been digested with *Bam*HI and *Eco*RI. The plasmid containing the sequences encoding the neck region plus CRD region was designated pBCSK1 and the plasmid containing the sequences encoding the CRD alone was designated pBCSK2.

The plasmid pMAL-c (6148 bp) (New England Biolabs, Hitchin, MA, U.S.A.) which contains the *E. coli malE* gene under the inducible P_{tac} promoter, which codes for MBP fused in-frame to the *LacZ*α gene, was used as expression vector for the neck region plus the CRD [10]. Sites between *malE* and *LacZ*α are available for cloning, which interrupts the *malE–LacZ*α fusion (blue to white selection on 5-bromo-4-chloroindol-3-yl β-δ-galactopyranoside plates). An *Xba*I–*Hin*dIII cassette was lifted from pBCSK1 and subcloned into pMAL-c cleaved with the same restriction enzymes. The new recombinant construct, carrying the neck region plus CRD of human SP-D, designated pSPD(N/CRD), was used to transform competent *E. coli* TB1 cells.

To express the carbohydrate recognition domain [rSP-D(CRD)] alone, the open reading frame was lifted from pBCSK2 with *Xba*I and *Eco*RI and subcloned into the Studier expression vector [11], pET-21b, between the *Nhe*I and *Eco*RI restriction sites. On ligation, the vector supplied the T7ϕ10 promoter and the ribosome binding site. The resultant plasmid, pSPD(CRD), was introduced into an appropriate host cell, *E. coli* BL21(λDE3), that had an integrated copy of T7 RNA polymerase gene under the transcriptional control of the lacUV5 promoter. Addition of isopropyl β-D-thiogalactopyranoside (IPTG) induced the expression of T7 RNA polymerase and hence the CRD of human SP-D. Three hours after induction with 0.4 mM IPTG, the recombinant product accumulated to more than 15% of the total *E. coli* extract.

The neck region [rSP-D(N)] was expressed and purified as described by Hoppe et al. [12].

Expression and purification of the recombinant fragments of human SP-D

The *E. coli* TB1 containing *p*SP-D(N/CRD) was grown in Luria broth (containing 50 μg/ml ampicillin) at 37 °C with vigorous aeration to an absorbance of 0.8 at 600 nm. IPTG (NOVA Biochem) was added to a final concentration of 0.4 mM to induce the P_{tac} promoter in the plasmid. The cells were shaken under these conditions for 2 h and harvested. Cells from 1 litre of culture (3 g of cell pellet) were suspended in 50 ml of lysis buffer [20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 0.25% (v/v) Tween-20, 1 mM EGTA and 1 mM EDTA]. Lysozyme and PMSF (Sigma) were added to final concentrations of 100 μg/ml and 0.1 mM respectively. All subsequent steps of purification were performed at 4 °C. The cell suspension was incubated with lysis buffer over ice for 30 min and the lysate was then sonicated at 60 Hz for 30 s with an interval of 1 min (15 cycles) to shear the chromosomal DNA. After centrifugation at 16000 rev./min (6000 ***g***) at 4 °C for 30 min, the supernatant of sonicated lysates was collected and diluted 5-fold with column buffer [20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.25% (v/v) Tween-20 and 1 mM EDTA], and loaded on to an amylose resin column (50 ml bed volume) (New England Biolabs) equilibrated with the same column buffer. The column was washed successively with three bed volumes of column buffer, five bed volumes of column buffer without Tween-20, and five bed volumes of Factor Xa buffer [20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 2 mM $CaCl_2$ and 5% (v/v) glycerol]. The fusion protein was eluted with 100 ml of Factor Xa buffer containing 10 mM maltose. Peak fractions were pooled and Factor Xa (1 unit/μl) (New England Biolabs) was added (1 unit of Factor Xa per 100 μg of fusion protein) to release SP-D(N/CRD) polypeptide from the fusion partner MBP. Enzymic digestion was performed for 24 h at room temperature. The recombinant material was purified by ion-exchange chromatography on DEAE-Sepharose followed by affinity chromatography on maltose-agarose.

A 1 litre batch of *E. coli* BL21(λDE3) containing the *p*SP-D(CRD) was grown and harvested as described above. After sonication and high-speed centrifugation, most of the expressed product was found to be insoluble and recovered in the pellet. Therefore denaturing and refolding procedures were applied to the cell pellet by using dialysis against decreasing concentrations (6 M to zero) of urea. The supernatant containing rSP-D(CRD) protein was freeze-dried, then redissolved in 2 ml of gel filtration buffer [20 mM Tris/HCl, 100 mM NaCl and 5% (v/v) glycerol, pH 8.0] and loaded on to a Superose 12 FPLC column (Pharmacia, Milton Keynes, U.K.). After monitoring at 280 nm, the fractions containing the major peak were found to elute at a position corresponding to approx. 16 kDa. These fractions were pooled, dialysed against a low-salt buffer (20 mM Tris/HCl, pH 7.4, and 50 mM NaCl), and subjected to Mono-Q ion-exchange chromatography. The purified rSP-D(CRD) was eluted at 250 mM NaCl (50–500 mM NaCl gradient).

Characterization of recombinant proteins

Gel-filtration chromatography of rSP-D(N/CRD) and rSP-D(CRD) was performed on an FPLC system (Pharmacia) with a Superose 12 HR 10/30 (Pharmacia Biotech, Stockholm, Sweden) column. Approx. 0.1–0.2 mg (in 0.2 ml) of recombinant protein was applied to the column. Proteins were eluted with 20 mM Tris/HCl, pH 7.4, 100 mM NaCl, containing 5 mM $CaCl_2$ or 10 mM EDTA at a flow rate of 0.5 ml/min. The carbohydrate binding characteristics were tested by a solid-phase carbohydrate assay as previously described [13,14].

Comparison of LPS binding between native and recombinant surfactant protein

Human SP-D was purified, as described previously [4,15], from a pool of bronchoalveolar lavage fluid obtained from patients with alveolar proteinosis. The purification procedure involves two steps of lectin-affinity chromatography followed by gel filtration on Superose 6. The polyclonal antibodies against native human SP-D(nSP-D) and rSP-D(N/CRD) were raised in rabbits by an initial intramuscular injection of antigen with Freund's complete adjuvant, followed by intravenous booster injection. Immunoblotting of recombinant proteins was performed by the method of Towbin et al. [16], with polyclonal rabbit anti-(human

SP-D) as a probe, as previously described [17]. The binding of native and recombinant SP-D to the LPSs from Gram-negative bacteria such as *E. coli* (L8274; Sigma), *Klebsiella pneumoniae* (L4268; Sigma), and *Pseudomonas aeruginosa* (L9143; Sigma) was examined by using sandwich ELISA methods [13]. Tris buffer (50 mM Tris/HCl, 100 mM NaCl, pH 7.4) containing 5 mM EDTA or 2 mM $CaCl_2$ with 100 mM maltose was used to determine whether the binding was calcium-dependent and carbohydrate-specific.

Competitive solid-phase binding of recombinant SP-D to phospholipids on microtitre wells

The phospholipids dipalmitoylphosphatidylcholine (DPPC; Calbiochem, La Jolla, CA, U.S.A.), PI (Calbiochem) and phosphatidylcholine (PC; Sigma) were dissolved in ethanol (10 μg/ml), applied separately to microtitre wells (Nunc polysorb, Intermed, Kamstrup, Denmark) and air dried. The wells were incubated with Tris buffer (300 μl) containing 2% (w/v) BSA (blocking buffer) for 1 h to block non-specific binding. The biotinylated proteins, unlabelled native proteins or recombinant fragments (0–20 μg/ml in blocking buffer) were added to the wells and incubated for 2 h at room temperature. After the microtitre plates had been washed five times with Tris buffer, extravidin–alkaline phosphatase conjugate (Sigma, Poole, Dorset, U.K.), diluted 1:5000 in blocking buffer, was added to each well. After the plate had been washed with Tris buffer, *p*-nitrophenyl phosphate (Sigma) was added as a substrate for the phosphatase reaction, and the plates were incubated for 30 min at 37 °C. The reaction was stopped by the addition of 1 M NaOH and the absorbance was read at 405 nm.

RESULTS

Expression of recombinant fragments of human SP-D

A fusion protein expressed in *E. coli* (at a level of more than 50% of the total *E. coli* extract) was purified on an amylose resin column and cleaved with Factor Xa protease to release the neck region plus CRD of SP-D (Figure 1). The fusion protein present in the total cell lysate, after induction with IPTG, appeared as a major band of 60 kDa on SDS/PAGE (lane 3). The purified fusion protein (lane 4), composed of MBP linked to the SP-D neck region and CRD, was cleaved by Factor Xa for 24 h at room temperature to yield the 42 kDa band of MBP, and a 20 kDa band (lane 5) that corresponds to rSP-D(N/CRD). The rSP-D(N/CRD) was further purified by DEAE-cellulose ion-exchange chromatography followed by affinity chromatography on maltose-agarose and eluted with EDTA (lane 6).

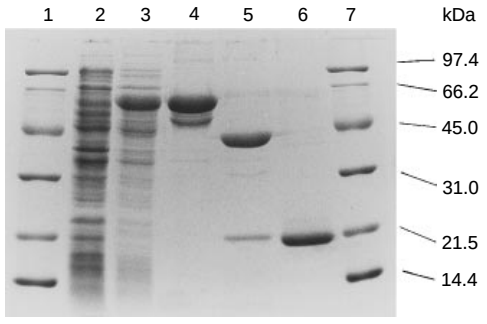

Figure 1 Purification of the recombinant protein composed of the neck region plus CRD of SP-D

Lanes 1 and 7 contain the molecular mass standards as shown. Lanes 2–6 show various steps in the purification: lane 2, total cell lysate without induction with IPTG; lane 3, total cell lysate after induction with IPTG; lane 4, fusion protein purified from the amylose resin column; lane 5, cleaved fragments derived from the fusion protein after incubation with protease Factor Xa for 24 h at room temperature; lane 6, purified rSP-D(N/CRD) eluted from DEAE chromatography. All the samples were run on SDS/PAGE [15% (w/v) gel] after reduction of disulphide bonds.

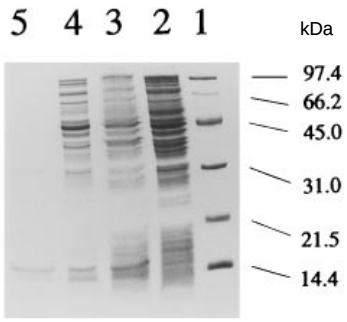

Figure 2 Purification of the recombinant protein composed of the CRD of SP-D

Lane 1 contains molecular mass standards as shown. Lanes 2–5 show various steps in the purification: lane 2, total cell lysate without induction with IPTG; lane 3, insoluble fraction of total cell lysate after induction with IPTG; lane 4, refolded soluble material recovered from the pellet after dialysis against decreasing concentrations of urea; lane 5, purified recombinant SP-D(CRD) after chromatography on Superose 12 and Mono-Q. All the samples were run on SDS/PAGE [15% (w/v) gel] after reduction of disulphide bonds.

rSP-D(CRD) was expressed as a monomer. After complete denaturation with 6 M urea and gradual refolding by dialysis against decreasing concentrations of urea, the soluble rSP-D(CRD) was purified by two steps, first by gel filtration on a Superose 12 column and then by Mono-Q ion-exchange chromatography. Figure 2 shows that a band of the expected molecular mass of approximately 16 kDa (lane 5) was obtained as the SP-D lectin domain. The identity of the recombinant material was confirmed by immunoblotting analysis with rabbit anti-rSP-D(N/CRD) as a probe (results not shown).

Characterization of recombinant SP-D proteins

The molecular sizes of non-denatured recombinant SP-D(N/CRD) and SP-D(CRD) were determined by gel filtration on Sepharose 12 chromatography at room temperature in the presence of 20 mM Tris/HCl buffer, pH 7.4, containing 100 mM NaCl. Either EDTA (10 mM) or $CaCl_2$ (5 mM) was added to the buffer to determine whether calcium had any influence on the state of oligomerization of the rSP-D(N/CRD) or rSP-D(CRD) preparations. It was found that rSP-D(N/CRD) eluted from the column, in the presence of 5 mM $CaCl_2$, as a main peak of apparent molecular mass 66 kDa plus a minor peak of 20 kDa (Figure 3A). This retention profile did not change when the eluting buffer contained 10 mM EDTA instead of calcium ions. The main peak fractions that were eluted from the column, run in the presence of either $CaCl_2$ or EDTA, were collected and examined by SDS/PAGE [15% (w/v) gel] with or without the reduction of disulphide bonds. The main peaks from the EDTA or the $CaCl_2$ elution showed the same behaviour on SDS/PAGE, giving a major, 20 kDa band with or without the reduction of disulphide bonds (Figure 4). The rSP-D(CRD) preparation had an apparent molecular mass of approx. 16 kDa, in the presence of either calcium or EDTA, and therefore behaved as a monomer on gel filtration under non-dissociating conditions (Figure 3B).

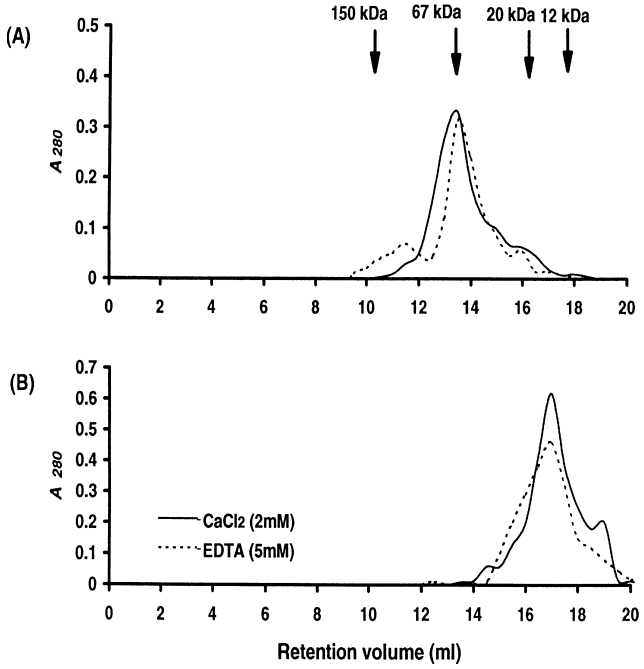

Figure 3 Elution profiles of gel filtration chromatography of the recombinant fragments of SP-D on Superose 12 FPLC

Elution of (**A**) rSP-D(N/CRD) and (**B**) rSP-D(CRD) from an FPLC Superose 12 column (HR 10/30). The absorbance at 280 nm was measured and 0.5 ml fractions were collected and examined by SDS/PAGE. Calibration of the column was performed with the following marker proteins: alcohol dehydrogenase (150 kDa), BSA (67 kDa), soybean trypsin inhibitor (20.3 kDa), cyctochrome *c* (12.4 kDa); their approximate elution positions are shown.

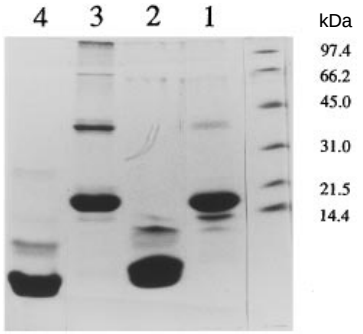

Figure 4 SDS/PAGE [15% (w/v) gel] analysis of the recombinant fragments of rSP-D(N/CRD) (lanes 1 and 3) and rSP-D(N) (lanes 2 and 4)

The samples in lanes 1 and 2 were run after reduction of disulphide bonds, and the samples in lanes 3 and 4 were run without reduction of disulphide bonds. Comparison of the reduced and non-reduced samples of rSP-D(N/CRD) (lanes 1 and 3) shows that the trimerization of recombinant SP-D(N/CRD) is due to the self-association of the α-helical portion of the neck region, rather than CRDs being cross-linked via aberrant disulphide bonding.

Sugar binding specificities of the recombinant proteins

In view of the large difference in molecular size and number of carbohydrate-binding sites seen between native SP-D and rSP-D(N/CRD), it is difficult to compare accurately the responses seen in the immunochemical analysis of their binding to the maltosyl-BSA (Figure 5). This is because the native SP-D is a tetrameric structure that may bind to maltosyl-BSA by any one

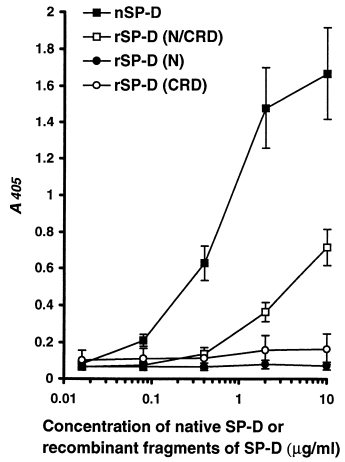

Figure 5 Binding of native SP-D or the recombinant SP-D fragments to immobilized maltosyl-BSA

Table 1 Comparison of the sugar specificities of the recombinant neck region plus CRDs of SP-D [(SP-D(N/CRD)] and native human SP-D

The values given are IC_{50} (mM), i.e. the concentration required for 50% inhibition of binding of biotinylated lectin to mannan. The values in parentheses denote the IC_{50} concentration relative to that of mannose. Conditions for the assay are described in the Materials and methods section. Abbreviation: n.d., not determined.

Sugar inhibitor	rSP-D(N/CRD) (the present study)	nSP-D [4]
Maltose	7.3 (0.50)	5.1 (0.68)
Glucose	12.5 (0.86)	9.6 (1.28)
ManNAc	23.7 (1.64)	n.d.
ManN	> 50	n.d.
GluN	n.d.	18.6 (2.48)
D-Fucose	n.d.	n.d
GalN	> 50	n.d.
Mannose	14.5 (1.0)	7.5 (1.0)
Galactose	38 (2.62)	22 (2.93)
L-Fucose	18.5 (1.28)	6.5 (0.86)
GluNAc	42 (2.90)	28 (3.73)
GalNAc	> 50	> 50
Lactose	n.d.	23 (3.1)

of the clusters of three CRDs found at the C-terminal ends of each of its four subunits, whereas the rSP-D(N/CRD), which is equivalent to the C-terminal half of a single subunit, binds directly to the BSA-maltose via its only cluster of three CRDs. Rabbit anti-(human SP-D), plus an anti-(rabbit IgG)–alkaline phosphatase indicator antibody, was used to detect the bound nSP-D or rSP-D(N/CRD). It is therefore likely that the signal generated would be expected to be 2–3-fold greater for the immunoassay of nSP-D. With this in mind it does seem, however, that recombinant human SP-D(N/CRD) bound as strongly as natural SP-D to microtitre plates coated with maltosyl-BSA, whereas rSP-D(CRD) showed very little binding to maltosyl-BSA coated plates (Figure 5) or to the maltose-agarose column (results not shown). The use of a variety of monosaccharides or disaccharides as inhibitors of the binding of rSP-D(N/CRD) to maltosyl-BSA indicated that recombinant material had a similar carbohydrate-binding specificity to that of natural SP-D (Table 1).

Table 2 Binding of native SP-D and recombinant fragments of SP-D to LPSs from *E. coli*

The binding of fixed quantities (10 μg/ml) of nSP-D, rSP-D(N/CRD), rSP-D(N) and SP-D(CRD) to immobilized LPSs from *E. coli* were tested in the presence of 5 mM $CaCl_2$, 100 mM maltose with 5 mM $CaCl_2$ or 10 mM EDTA as described in the Materials and methods section. Binding is shown as colour development (A_{405}) in the ELISA. Results are means $\pm$ S.D. for three determinations. Similar values were obtained for the binding of nSP-D and recombinant fragments of SP-D to LPS from *Ps aeruginosa* and *K. pneumoniae*.

Binding conditions	nSP-D	rSP-D(N/CRD)	rSP-D(CRD)	rSP-D(N)
$CaCl_2$	0.652 $\pm$ 0.103	0.543 $\pm$ 0.165	0.151 $\pm$ 0.065	0.322 $\pm$ 0.089
Maltose/$CaCl_2$	0.139 $\pm$ 0.066	0.075 $\pm$ 0.026	0.101 $\pm$ 0.032	0.332 $\pm$ 0.073
EDTA	0.073 $\pm$ 0.012	0.072 $\pm$ 0.013	0.067 $\pm$ 0.005	0.131 $\pm$ 0.034

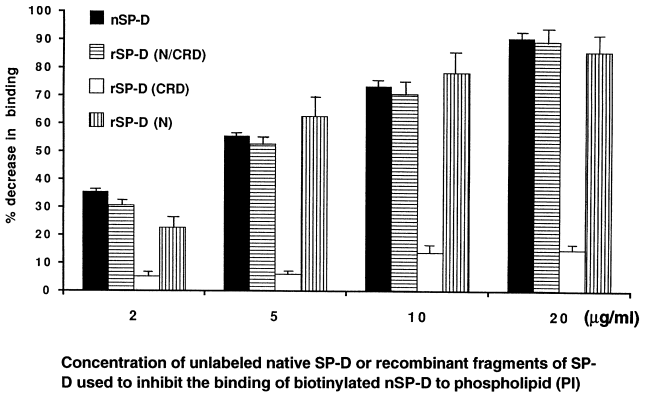

Figure 7 Study of the inhibition mediated by unlabelled native SP-D, rSP-D(N/CRD), rSP-D(CRD) and rSP-D(N) on the binding of biotinylated native SP-D to PI

LPS binding assays

The abilities of the recombinant fragments of SP-D or native human SP-D to bind LPSs from Gram-negative bacteria were tested by ELISA. LPSs from *E. coli*, *K. pneumoniae* and *Ps. aeruginosa* were tested (Table 2). The binding of nSP-D and SP-D(N/CRD) was completely inhibited by co-incubation with buffer containing 100 mM maltose or 5 mM EDTA, indicating that the interaction was mediated through CRD in a calcium-dependent manner. The recombinant SP-D(N/CRD) was able to bind to the LPS from *K. pneumoniae*, *Ps. aeruginosa* and *E. coli*, in a similar manner to native SP-D, whereas the rSP-D(CRD) showed very little binding to LPS from all three species. This indicates that the trimerization of the CRD markedly increases the affinity for the surfaces of the pathogens.

Phospholipid binding assays and inhibition studies

The phospholipid binding studies suggest a very weak interaction of the rSP-D(CRD) monomer with PI, DPPC and PC, whereas nSP-D and rSP-D(N/CRD) both showed the same pattern of interaction with PI and DPPC, and relatively weak binding to PC (Figure 6). The interaction of the PI and DPPC with nSP-D and rSP-D(N/CRD) was inhibited by EDTA. A competitive inhibition study was performed by testing the effectiveness of unlabelled nSP-D, rSP-D(N/CRD), rSP-D(CRD) or rSP-D(N) to inhibit the binding of biotinylated nSP-D to PI. The percentages of decreased binding seen on competition with unlabelled nSP-D, rSP-D(N/CRD) and rSP-D(N) were 85%, 72% and 79% respectively, whereas unlabelled rSP-D(CRD) could only inhibit up to 15% of the binding of biotinylated nSP-D to PI (Figure 7).

DISCUSSION

Crouch et al. [18,19] have demonstrated that multimers of recombinant rat SP-D are secreted from Chinese hamster ovary

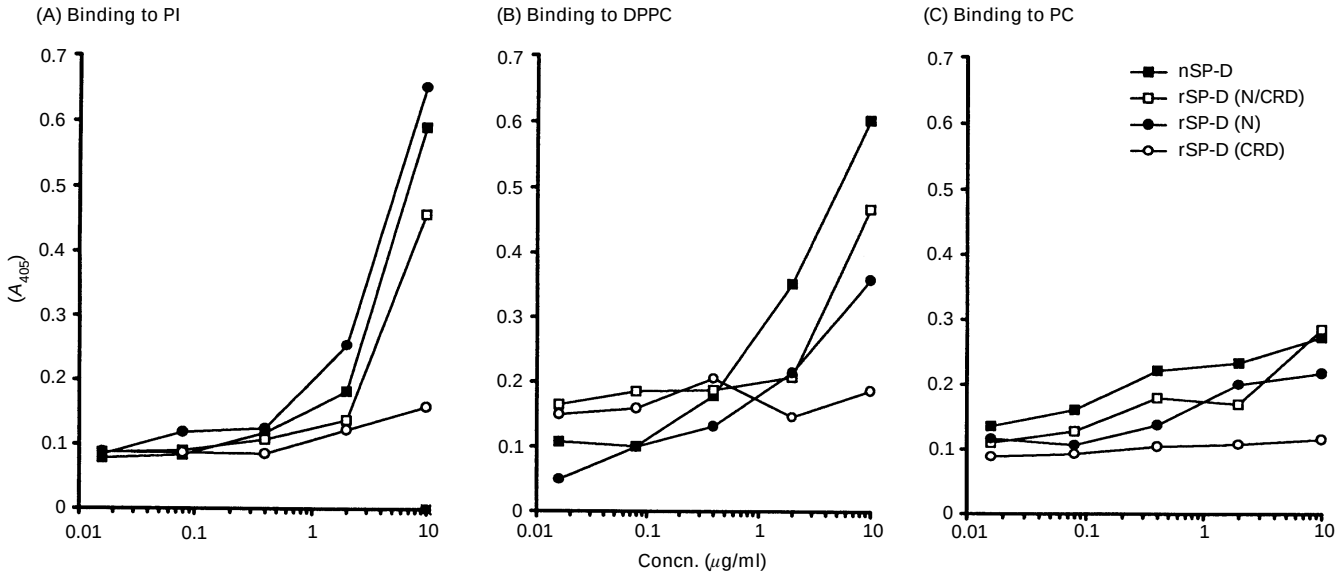

Figure 6 Dose-dependent binding of native SP-D and the recombinant SP-D fragments to PI (A), DPPC (B) or PC (C)

Phospholipids were applied to microtitre plates and then biotinylated nSP-D or recombinant fragments were tested for their ability to bind to the phospholipids, as outlined in the Materials and methods section.

K1 cells that had been transfected with rat SP-D cDNA. This recombinant rat SP-D also formed a triple-helical domain comparable in size and thermal stability to natural SP-D. They also demonstrated that the multimers of rat SP-D are assembled as homopolymers of four identical trimeric subunits that seem to be stabilized by interchain disulphide bonds between the N-terminal domains of the trimer. The expression of recombinant polypeptides corresponding to distinct regions of human SP-D has enabled us to study the important structural and functional features, shown by the C-type lectin domains and α-helical neck region. In this study we have expressed trimeric recombinant fragments of neck region plus the CRD as a trimer, and CRD alone as a monomer, of human SP-D in *E. coli*. The trimeric rSP-D(N/CRD) protein possesses the same properties as native human SP-D as demonstrated by a variety of structural and functional characteristics. It is recognized by rabbit anti-(human SP-D) polyclonal antibody and shows the same saccharide inhibition profile, LPS binding properties and phospholipid interactions *in vitro* as native human SP-D. Although it can be recognized by polyclonal antibody against human SP-D, the recombinant SP-D carbohydrate recognition domain [rSP-D(CRD)], which behaves as a monomer, was found to have a much lower affinity for carbohydrates, LPSs and phospholipids than do the native SP-D or rSP-D(N/CRD).

The study of the recombinant fragments of human SP-D confirms the view that the neck region is essential for the trimerization of the CRD region of human SP-D [20]. A recombinant peptide, composed of the amino acid sequence of the neck region of human SP-D, was shown by Hoppe et al. [12], by size-exclusion chromatography and cross-linking experiments, to form a trimer in solution. This trimeric peptide is held together by strong hydrophobic forces, mediated by hydrophobic residues showing a heptad repeat pattern resulting in a parallel, non-staggered, orientation of the α-helices in a three-stranded coiled coil. It is therefore reasonable to propose that the α-helical neck region acts as a trimerizing agent holding three CRDs together in the rSP-D(N/CRD) preparations whereas rSP-D(CRD), which lacks the neck region peptide, behaves as a monomer. Recently we produced a recombinant polypeptide composed of the α-helical neck region and CRD of bovine conglutinin (BK) in *E. coli* [21]. The purified product behaved as a homotrimer in non-dissociating conditions, with three CRDs held together by the α-helical neck regions. The recombinant trimeric material, although lacking the N-terminal and collagen regions of the native conglutinin, showed the same carbohydrate binding specificities as the native molecule for the complement fragment C3b and for LPSs derived from Gram-negative bacteria. Thus the neck region is likely to be an important feature in creating the type of multivalent bindings shown by all the collectins [3] for ordered arrays of carbohydrates found on the surfaces of bacteria and viruses.

There is good evidence that SP-A plays a critical role in lipid metabolism in the surfactant system [22]; however, the role of SP-D in lipid metabolism within the alveoli has not been clearly defined. The neck region of SP-A contains amino acid residues consistent with there being both an amphipathic α-helix followed by a strong non-polar region [23]. In contrast, SP-D lacks such a clearly identifiable non-polar region, which might account for the difference between SP-D and SP-A with respect to their interactions with surfactant lipids [24]. However, recent reports have demonstrated that SP-D can bind to PI [25,26] and also to glucosylceramide [27]. Furthermore it has been reported that SP-D-associated lipids were responsible for the observation that native SP-D can counteract the inhibitory effect of SP-A on phospholipid secretion by alveolar type II cells [28]. Using recombinant proteins of chimaeras of SP-A and SP-D produced in the baculovirus expression system, Ogasawara et al. [6] reported that the neck plus CRD regions of SP-D play a role in lipid binding and that the CRD is essential for PI binding.

It can be seen from Figure 6 that, in the present study, native SP-D appears to bind as well to DPPC as to PI. This is contrary to the findings of Ogasawara et al. [6,25], who found that SP-D bound specifically to PI and showed no binding to DPPC, as judged by direct binding of ^{125}I-labelled native SP-D with a protocol involving multilamellar liposomes. Because in the present study the examination of SP-D binding to phospholipids was performed by applying biotinylated SP-D to solid-phase DPPC, PI and PC spread out on the wells of microtitre plates, the difference in binding seen between this study and the previous one seems to lie in the different techniques used.

In this report we have shown that monomeric rSP-D(CRD) was not able to bind the phospholipids *in vitro* unless neck region was added to the N-terminal end of the CRD domain to form trimers of the expressed protein. Some of the phospholipid binding results (Figures 6 and 7) indicate that the neck region of SP-D might contribute to the overall phospholipid binding properties of native SP-D. The neck-region homotrimer, rSP-D(N), shows good binding to PI and to DPPC (Figure 6) and it also is as efficient as nSP-D in decreasing the binding of biotinylated native SP-D to PI (Figure 7). However, it is possible that hydrophobic residues at the N- and C-extremities of the rSP-D(N) sequence, which are normally buried within the intact native SP-D molecule, become exposed in the short recombinant peptide. Such exposed residues may then readily bind to phospholipids, especially as the physical state of phospholipids in microtitre plate wells is likely to differ from that seen in a lipid bilayer. Thus some caution is required before concluding that the neck region of SP-D plays an important role in the binding to phospholipids because the interaction observed might be between partly unfolded coiled-coil neck peptides and exposed lipid acyl chains, which is different from what would be expected to occur when native SP-D interacts with phospholipid liposomes. The finding that recombinant material, composed of the neck plus CRD of bovine conglutinin, forms a carbohydrate-binding homotrimer [21] that shows no binding to PI, DPPC or PC (J.-Y. Wang, unpublished work) supports the view that there is some specificity in the binding of PI by the neck plus CRD, and the neck region alone, of SP-D. However, further phospholipid-binding studies are required, involving the expression of the neck region alone of conglutinin, and of chimaeric structures composed of the neck and CRD regions of SP-D and conglutinin, before any firm conclusions can be drawn concerning the possible role of the neck region of SP-D in the binding of SP-D to PI and DPPC.

In conclusion, the results of these studies suggest that: (1) the neck peptide, which is known to form a triple-stranded α-helical bundle, is important in the trimerization of the CRDs of human SP-D; (2) the CRDs of human SP-D seem to fold correctly in *E. coli*, as judged by their carbohydrate binding properties; (3) the neck region of SP-D may also play a role in the binding of phospholipids in addition to being a trimerizing agent.

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