Structure of a truncated human surfactant protein D is less effective in agglutinating bacteria than the native structure and fails to inhibit haemagglutination by influenza A virus

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Surfactant protein D (SP-D) is a lung-specific protein that is synthesized and secreted by lung epithelial cells and is believed to play an important role in lung host defence. This protein belongs to the C-type lectin family, which is characterized by an N-terminal cysteine-rich domain, a collagen-like domain, a neck domain and a carbohydrate recognition domain (CRD). To elucidate the biological actions of this animal lectin against such pathogens as micro-organisms, the biological activities of a recombinant partial SP-D lacking a collagen-like domain were examined. A recombinant human SP-D, consisting of a short collagen region (two repeats of Gly-Xaa-Yaa amino acid sequences), the neck domain and the CRD, was expressed in *Escherichia coli*. The recombinant SP-D was purified on a nickel column and then on a maltose-agarose column. This protein can form a trimeric structure owing to the neck domain and exhibits sugar-binding activity and specificity similar to those of native human SP-D. The recombinant SP-D caused dose-dependent and calcium-dependent agglutination of *E. coli* Y1088. The agglutination titre (the concentration required to achieve a 50% decrease in light transmission by agglutination) of recombinant SP-D was approx. 6-fold that of native SP-D. As for conglutination, the recombinant trimeric conglutinin required 8–16-fold higher concentrations than the native counterpart. In haemagglutination inhibition (HI) of influenza A virus, although native and recombinant conglutinin showed similar levels of HI activity, the recombinant SP-D was unable to inhibit haemagglutination, even at a concentration approx. 120-fold that of the native SP-D. The lectin precipitation and lectin blot assays showed that the truncated SP-D could bind to influenza A virus as well as native SP-D did. These results indicate that the agglutination activity of trimeric collectins can be largely retained, and furthermore that the oligomeric structure with several hands at opposite sites can enhance agglutination activity. The difference in HI activity against influenza A virus between native and recombinant SP-D suggests that SP-D uses a different mechanism from that of conglutinin to inhibit viral haemagglutination.

INTRODUCTION

Pulmonary surfactant is a complex mixture of lipids and proteins synthesized by alveolar type II cells and is secreted into alveolar spaces [1]. Surfactant lines the alveolar surfaces of these spaces to prevent alveoli from collapsing during expiration by decreasing surface tension [2]. There are four surfactant proteins of which two are hydrophobic, surfactant protein B (SP-B) and surfactant protein C (SP-C), and two are hydrophilic, proteins SP-A and SP-D [3,4]. SP-A and SP-D belong to a family of collectins characterized by a collagen-like domain and a carbohydrate recognition domain (CRD) [5]. Other collectins are serum proteins synthesized in the liver, and these are mannan-binding protein (MBP) [6], collectin 43 (CL-43) [7] and bovine conglutinin [8]. Several studies suggest that these lectins might play an important role in immunoglobulin-independent host defence. MBP can destroy bacteria via a complement-mediated mechanism [9,10] or by opsonization [11]. Conglutinin has been identified as a β-inhibitor of influenza A virus and exhibits haemagglutination inhibition (HI) activity and neutralizing activity towards influenza A viruses [12–14]. SP-A enhances the phagocytosis of bacteria [15] by alveolar macrophages and acts as an opsonin for herpes simplex virus [16]. SP-D acts as an opsonin for influenza A virus [17] and also causes agglutination of bacteria [18]. SP-D consists of tetramers of disulphide-bonded trimers, each containing 12 identical subunits. SP-D is similar in structure to the cruciform figure of conglutinin seen with electron microscopy. The binding surface in collectins is usually made up of three CRDs joined together to create the binding surface. Collectins conglutinin and SP-D with opposite binding sites seem to be particularly well suited to agglutinating micro-organisms. We previously succeeded in producing recombinant conglutinin with deletion of the N-terminal and collagenous domain in *Escherichia coli* [19]. This recombinant conglutinin can form a trimeric structure by association of the neck domain; it exhibits HI activity against influenza A virus as well as some capacity for conglutination. To elucidate the biological function of other C-type lectins active against pathogenic micro-organisms, we produced a recombinant truncated human SP-D, consisting of a short collagen region (two repeats of Gly-Xaa-Yaa amino acid

Abbreviations used: CRD, carbohydrate recognition domain; HA, haemagglutinin; HI, haemagglutination inhibition; MBP, mannan-binding protein; NA, neuraminidase; PV-maltose, poly[*N*-*p*-vinylbenzyl-*O*-α-D-glucopyranosyl-(1 $\rightarrow$ 4)-D-gluconamide]; SP-D, surfactant protein D (similarly SP-A, SP-B and SP-C).

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sequences), a neck domain and a CRD, in a bacterial expression system. Trimeric recombinant human SP-D was produced in *E. coli* and was characterized with respect to its agglutination activity and inhibition of haemagglutination by influenza A virus by comparing it with the recombinant conglutinin with the same trimer.

MATERIALS AND METHODS

Buffers and media

The Tris-buffered saline (TBS) used contained 20 mM Tris/HCl (pH 7.4)/140 mM NaCl. TBS/C was TBS containing 5 mM $CaCl_2$. *E. coli* lysis buffer contained 6 M guanidine hydrochloride, 20 mM sodium phosphate and 500 mM NaCl, pH 7.8. The coating buffer contained 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.05 % NaN_3, pH 9.6. SOB medium contained 2 % (w/v) Bacto-tryptone, 0.5 % Bacto-yeast extract, 0.05 % NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 50 μg/ml ampicillin. Rich medium contained 1 % (w/v) Bacto-tryptone, 0.5 % Bacto-yeast extract, 0.5 % NaCl, 0.2 % glucose and 100 μg/ml ampicillin. *E. coli* lysis buffer for XL1-Blue contained 10 mM phosphate, pH 7.2, 30 mM NaCl, 0.25 % Tween-20, 10 mM 2-mercaptoethanol, 10 mM EDTA and 10 mM EGTA. Column buffer contained 10 mM phosphate, pH 7.2, 500 mM NaCl, 1 mM NaN_3, 10 mM 2-mercaptoethanol and 1 mM EGTA. Column buffer/T was column buffer containing 0.25 % Tween-20. Denaturing buffer was TBS/C containing 6 M urea. LB medium contained 1 % (w/v) Bacto-tryptone, 0.5 % Bacto-yeast extract and 1 % (w/v) NaCl.

Bacteria strains and virus

E. coli strains Y1088, JM109 and XL1-Blue were purchased from Clontec Labs and Invitrogen. A/Ibaraki/1/90 (H3N2), passaged less than 10 times and sensitive to animal sera, was used for the HI assay. The virus was grown in chorioallantoic membranes of embryonated hen eggs by a standard procedure and stored at −70 °C.

Purification of human SP-D from amniotic fluid

Human SP-D was purified from amniotic fluid as previously described [20]. Briefly, crude SP-D was isolated from a pool of human amniotic fluid by using two maltose-agarose affinity chromatography steps. The fractions containing crude SP-D were further purified on a HiTrap column (Pharmacia Biotech) conjugated with anti-(human immunoglobulin) antibody and then purified on a Protein G column (Pharmacia Biotech).

Construction of human SP-D expression plasmids

Two-step polymerase chain reactions were performed to obtain a cDNA clone for human SP-D. A 10 μl [(1–9) × 10^7 phages] portion of a human lung λgt11 cDNA library (Clontec Labs) was used as a first PCR template with λgt11 primers (forward, 5′-GGTGGCGACGACTCCTGGAGCCCG-3′; reverse, 5′-TTGACACCAGACCAACTGGTAATG-3′). This amplified DNA (2 μl) was used as a second PCR template. Two primers were designed and synthesized on the basis of the human SP-D cDNA sequence [10]: 5′-GGCTCGAGGGAGAAAGTGGGC-TTCCAGA-3′ and 5′-GGGAATTCTCAGAACTCGCAGAC-CACAA-3′, which contain *Xho*I and *Eco*RI sites respectively. Each PCR assay employed 1 × commercially supplied reaction buffer, 1 mM of each primer, 200 mM dNTP species, and 1 unit of Deep Vent DNA polymerase (New England Biolabs), and was performed in a Zymoreactor (Atto Co.) for 35 cycles consisting of denaturation at 92 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. PCR products were digested with *Eco*RI and *Xho*I, then inserted into the bacterial expression vector pRSET-A (Invitrogen Co.) and transfected into *E. coli* JM109 cells. To obtain the truncated SP-D without the histidine tag and two Gly-Xaa-Yaa repeats, two primers (5′-GGGGAA-TTCCATCGAAGGTAGGGGAGAAAGTGGGCTTCCAG-A-3′, which contain the sequence coding for the Factor Xa site, 5′-GGGAAGCTTTCAGAACTCGCAGACCACAA-3′), were designed, and PCR was performed as described above. PCR products were digested with *Eco*RI and *Hin*dIII and inserted into expression vector pMAL-c2 (New England Biolabs) and transformed into *E. coli* XL1-Blue cells.

Expression and purification of recombinant proteins

The recombinant proteins were expressed in an *E. coli* expression system as previously described [19]. *E. coli* JM109, which carries the proper insert, was grown to a D_{600} of approx. 0.3 in 200 ml of SOB medium. After the addition of isopropyl β-D-thiogalactoside to a final concentration of 1 mM, the culture was grown for an additional 1 h. The cells were infected with an M13 phage, containing the T7 RNA polymerase gene driven by the *E. coli* lac promoter, at a multiplicity of infection of 5 plaque-forming units per cell, incubated for a further 3 h and harvested. The cell pellets were suspended in 20 ml of lysis buffer and lysed by sonication (15 s, 70 % output, ten times). After centrifugation at 43000 ***g*** for 30 min, the supernatant was incubated with nickel-nitrilotriacetic acid-agarose (Qiagen) for 15 min, and the gel was loaded on a column. The column was washed with TBS and TBS/C, after which the fusion protein was eluted with TBS/C containing 0.5 M imidazole. The eluate was dialysed against three changes of 1000 vol. of TBS/C and applied to a maltose-agarose (Seikagaku Co.) column. After washing with TBS/C, bound protein was eluted with TBS/C containing 100 mM maltose.

To obtain the truncated SP-D without the histidine tag and two Gly-Xaa-Yaa repeats, *E. coli* XL1-Blue was grown to D_{600} approx. 0.5 in 200 ml of rich medium. After the addition of isopropyl β-D-thiogalactoside to a final concentration of 0.3 mM, the culture was incubated for 3 h and then harvested. The cells were suspended in 10 ml of lysis buffer and incubated at 4 °C for 30 min. After sonication, the lysate were centrifuged at 9000 ***g*** for 30 min. The supernatant was diluted with 5 vol. of column buffer/T and applied to an amylose resin (New England Biolabs) column. The column was washed with column buffer/T and column buffer; the fusion protein was then eluted with column buffer containing 10 mM maltose. The eluant was dialysed against Factor Xa buffer. The fusion protein was cleaved with Factor Xa (New England Biolabs; 1 mg of Factor Xa per 100 mg of fusion protein) by incubation at 23 °C for 24 h. After cleavage with Factor Xa the protein was denatured by using dialysis against denaturing buffer (6 M urea). For refolding, stepwise dialysis was performed against 6-fold (1 M urea) and 2-fold (0.5 M urea) denaturing buffer and TBS/C. The pure fragment of SP-D was purified by affinity chromatography on a maltose-agarose column. Purification and identification of the recombinant human SP-Ds were confirmed by SDS/PAGE and Western blotting with rabbit anti-(human SP-D) serum.

Chemical cross-linking study

The recombinant protein was dissolved at a concentration of 25 μg/ml in PBS containing 10 mM $CaCl_2$. Samples were

incubated with bis-(sulphosuccinimidyl)suberate (Pierce Chemical Co.) at 37 °C for 30 min. Samples were boiled in SDS/PAGE sample buffer for 10 min. SDS/PAGE was performed on a 4–20 % (w/v) gradient gel. Peptide bands were silver-stained with a Silver Stain II Kit Wako (Wako Pure Chemical).

Gel-filtration chromatography

Gel-filtration chromatography was performed on a Superose 12 HR 10/30 column (Pharmacia Biotech) at a flow rate of 0.5 ml/min with TBS containing 2 mM EDTA at pH 8.0. Recombinant SP-D (20 μg), after treatment with TBS containing 10 mM dithiothreitol, was applied to the column. Proteins were monitored at 280 nm. Calibration of the column was performed with a Gel Filtration Standard (Bio-Rad) containing thyroglobulin, bovine γ-globulin, chicken ovalbumin, equine myoglobin and vitamin B_{12}.

Bacterial agglutination

Bacterial agglutination assays [18] were performed with *E. coli* Y1088. Y1088 was incubated in 5 ml of LB medium at 37 °C overnight. The cells were harvested and washed with TBS three times, and diluted to a D_{700} of 1.0 with TBS/C. Then 800 μl portions of these diluted cell suspensions were mixed with lectins (recombinant or native human SP-D) alone, or together with various inhibitors, and the volumes were adjusted to 1 ml with TBS/C. Absorbance was measured continuously at 700 nm in a spectrophotometer Ubest-30 (Japan Spectroscopic Co.). Bacterial agglutination was assessed by measuring the decrease in light transmission through the *E. coli* suspension after the addition of various concentrations of SP-D. The agglutination titre was defined as the concentration required to achieve a 50 % decrease in light transmission by agglutination.

Binding of recombinant truncated human SP-Ds to sugar

The binding of recombinant human SP-D to mannan or poly[*N*-*p*-vinylbenzyl-*O*-α-D-glucopyranosyl-(1 $\rightarrow$ 4)-D-gluconamide] (PV-maltose) (Seikagaku Co.) was measured by ELISA [19] with rabbit anti-(human SP-D) serum. The sugar inhibition assay was performed with mannan. PV-maltose or mannan-coated microtitre plates were incubated with the recombinant protein alone or together with an increasing concentration of sugars. I_{50} was defined as the concentration of sugar that resulted in 50 % inhibition of binding [20].

HI test

HI tests were performed with a standard microtitre assay method in 96-well microtitre plates with 0.5 % chicken erythrocytes [19]. For this test, ether-treated egg-derived virus antigens were prepared in our laboratory and the mixture was incubated in TBS/C only, or in TBS/C with 10 mM EDTA. The inhibition of virus-mediated agglutination of chicken erythrocytes by human SP-D was observed after incubation for 1 h at room temperature.

Lectin blot and lectin precipitation assay

The lectin blot assay was performed as below. A 10 μl sample containing 10^3 haemagglutination units of A/Ibaraki/1/90 (H3N2) was resolved by SDS sample buffer, separated by SDS/PAGE and transferred to BioBlot-NC membrane (Coastor Co.). After the membrane had been blocked with TBS/C containing 0.05 % Tween-20 and 5 % (w/v) BSA, the membranes were incubated with SP-Ds (truncated or native human SP-D). This was followed by incubation with rabbit anti-(human SP-D) serum, anti-(rabbit IgG)-conjugated biotin (Vector), alkaline phosphatase-conjugated streptavidin (Gibco BRL) and the substrate for alkaline phosphatase. The binding of SP-Ds to influenza A virus was measured by precipitation under low-speed centrifugation. A 10 μl sample containing 10^3 haemagglutination units of the same virus was mixed with lectins at a concentration of 1 μg/ml in TBS/C or TBS containing 10 mM EDTA. After incubation at 37 °C for 60 min, the mixture was centrifuged at 600 ***g*** for 10 min. The supernatant and the pellet were analysed by Western blotting, with rabbit anti-(human SP-D) serum.

RESULTS

Expression of recombinant human SP-Ds in *E. coli*

The 496 bp PCR-product was amplified from a human lung λgt11 cDNA library and cloned into the bacterial expression vector pRSET-A. Sequencing of this fragment revealed that this product encoded residues 197–355 of human SP-D protein, including the two Gly-Xaa-Yaa repeats of the collagen region, the neck domain, and the CRD. A high level of expression of recombinant protein was obtained after 3 h of M13/T7 phage infection. The purity of the recombinant human SP-D is shown in Figure 1(A). After treatment with guanidine hydrochloride, truncated protein was able to bind to a nickel column via a histidine tag. After washing the column, bound recombinant protein was eluted with imidazole buffer and was then dialysed with TBS/C to allow it to refold to a native form. After dialysis for 5 days the recombinant protein was able to bind to the maltose-agarose column and was eluted with 100 mM maltose. This indicates that the recombinant protein has an affinity for

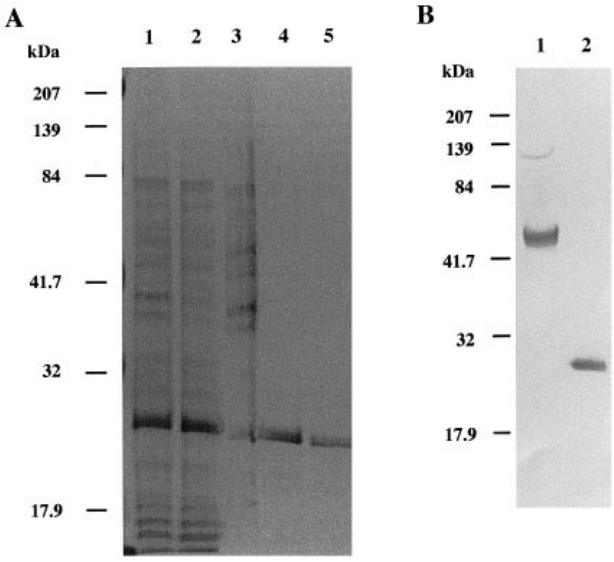

Figure 1 SDS/PAGE analysis and immunoblotting of recombinant SP-D

Aliquots of recombinant proteins at each purification step were analysed by SDS/PAGE [4–20 % (w/v) gradient gel] under reducing conditions, stained with Coomassie Blue (**A**), transblotted on a membrane and used for immunostaining with rabbit anti-(human SP-D) serum (**B**). (**A**) Lane 1, total cell lysate; lane 2, soluble fraction with guanidine hydrochloride; lane 3, insoluble fraction with guanidine hydrochloride; lane 4, recombinant protein eluted from the nickel column; lane 5, recombinant protein eluted from the maltose-agarose column. (**B**) Lane 1, purified native human SP-D; lane 2, purified recombinant fusion protein [same sample as in (**A**), lane 5]. The positions of molecular mass markes are shown at the left.

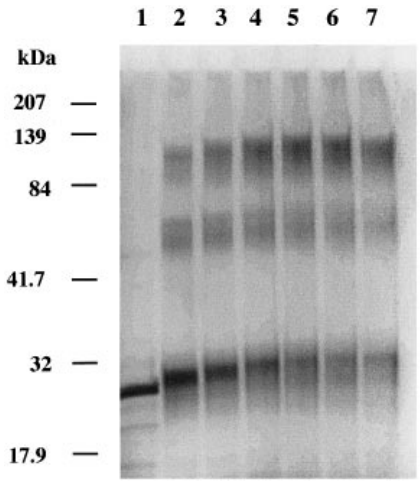

Figure 2 Chemical cross-linking analysis of recombinant SP-D

Aliquots were treated with 0 mM (lane 1), 0.25 mM (lane 2), 0.5 mM (lane 3), 1.0 mM (lane 4), 2 mM (lane 5), 5 mM (lane 6) or 10 mM (lane 7) bis-(sulphosuccinimidyl)suberate at 37 °C for 30 min. After SDS/PAGE [4–20% (w/v) gradient gel under reducing conditions], cross-linked complexes were revealed by silver staining. The positions of molecular mass markes are shown at the left.

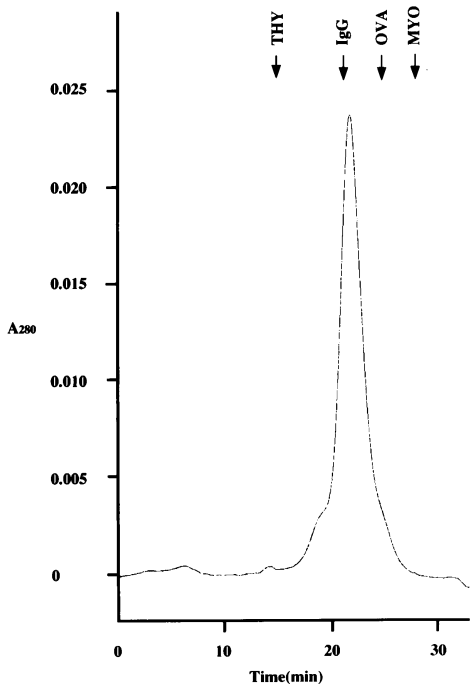

Figure 3 Gel-filtration chromatography of recombinant truncated SP-D on Superose 12 column

The recombinant truncated SP-D was subjected to chromatography on a Superose 12 column. The absorbance at 280 nm was measured. Arrows show the peak positions of the eluted standards: THY, thyroglobulin (670 kDa); IgG, bovine γ-globulin (158 kDa); OVA, chicken ovalbumin (44 kDa); MYO, equine myoglobin (17 kDa).

maltose similar to that of the native human SP-D. The molecular mass of recombinant human SP-D was estimated to be approx. 24 kDa by SDS/PAGE, although its actual molecular mass is 21.6 kDa from the deduced amino acid sequence. Recombinant conglutinin in *E. coli* [19], lacking the collagen domain, showed similar differences between its molecular mass and that of native conglutinin. However, the N-terminal amino acid sequence of recombinant conglutinin after enterokinase cleavage showed an amino acid sequence identical with that of mature conglutinin. In this case we also confirmed that the N-terminal amino acid sequence of recombinant SP-D was identical with that of mature human SP-D (results not shown). The recombinant protein was identified by Western blotting analysis, with rabbit anti-(human SP-D) serum (Figure 1B). The final yield of the recombinant truncated protein was 0.3 mg from 1 litre of *E. coli* culture.

Structural characterization of recombinant human SP-D

As revealed by a cross-linking study, this recombinant protein contained monomers (26–28 kDa), dimers (44–56 kDa) and trimers (87–118 kDa) (Figure 2). An increase in the concentration of the chemical cross-linker seemed to make the trimer band clearer rather than decreasing the intensity of the monomer band. Furthermore gel-filtration analysis confirmed that this recombinant protein mainly showed a trimer peak of 100 kDa (Figure 3). These results indicate that this protein was able to assemble into trimers without the collagen domain. This is not inconsistent with the fact that recombinant human MBP [21], human SP-A [22], bovine SP-D [23] and bovine conglutinin [19], without the collagen domain, can form a trimeric structure by association of the neck domains.

Carbohydrate-binding activities of truncated human SP-Ds

To examine the carbohydrate-binding specificity of truncated human SP-D, a sugar inhibition assay was performed by ELISA in the presence or absence of various sugars (Table 1). The results of this assay indicated that the specificity of truncated human SP-D was similar to that of native human SP-D. Figure 4 shows that the binding activity of truncated SP-D was Ca^{2+}-dependent and dose-dependent, as is that of native human SP-D [20]. The binding activity of the truncated SP-D to PV-maltose was as strong as that of native SP-D, although the more truncated SP-D without two Gly-Xaa-Yaa repeats and the histidine tag showed less binding activity to PV-maltose (Figure 4).

Table 1 Sugar specificities of trimeric recombinant human SP-D and native SP-D

Each I_{50} value shows the concentration required for 50% inhibition of binding. The values for native SP-D are taken from [20]. The symbol ∞ indicates no inhibition.

	I_{50} (mM)	
Sugars	Recombinant SP-D	Native SP-D
N-Acetyl-D-mannosamine	2.05	
Glucose	3.25	9.6
Maltose	3.75	5.1
D-Mannose	6.6	
L-Fucose	9.6	
Galactose	13.05	22.0
Lactose	25.6	23.5
D-Fucose	84	
N-Acetyl-D-galactosamine	> 200	
N-Acetyl-D-glucosamine	∞	28.5

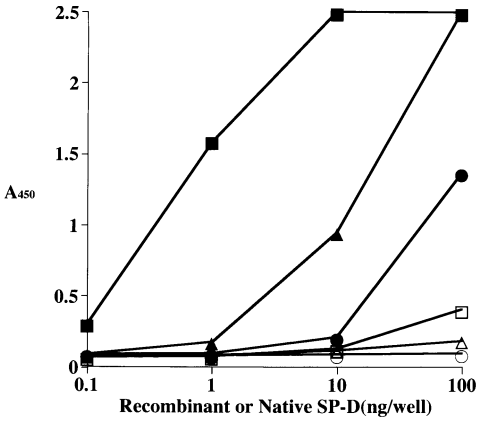

Figure 4 Binding of recombinant truncated SP-Ds and native SP-D to PV-maltose

PV-maltose (10 μg/ml) was coated on the plates. After blocking, samples of native or recombinant proteins at various concentrations (0, 1, 10, 100 and 1000 ng/ml) were added in TBS/NTC or TBS/NTC containing 10 mM EDTA. Anti-(native SP-D) rabbit serum and anti-(rabbit IgG)-conjugated horseradish peroxidase were diluted 1000-fold or 2000-fold respectively in TBS/NTC. The absorbance was read at 450 nm. Symbols: ▲, recombinant truncated SP-D with Gly-Xaa-Yaa repeats and histidine tag in TBS/NTC; △, recombinant truncated SP-D with Gly-Xaa-Yaa repeats and histidine tag in TBS/NTC with 10 mM EDTA; ●, recombinant truncated SP-D without Gly-Xaa-Yaa repeats and histidine tag in TBS/NTC; ○, recombinant truncated SP-D without Gly-Xaa-Yaa repeats and histidine tag in TBS/NTC with 10 mM EDTA; ■, native SP-D in TBS/NTC; □, native SP-D in TBS/NTC with 10 mM EDTA.

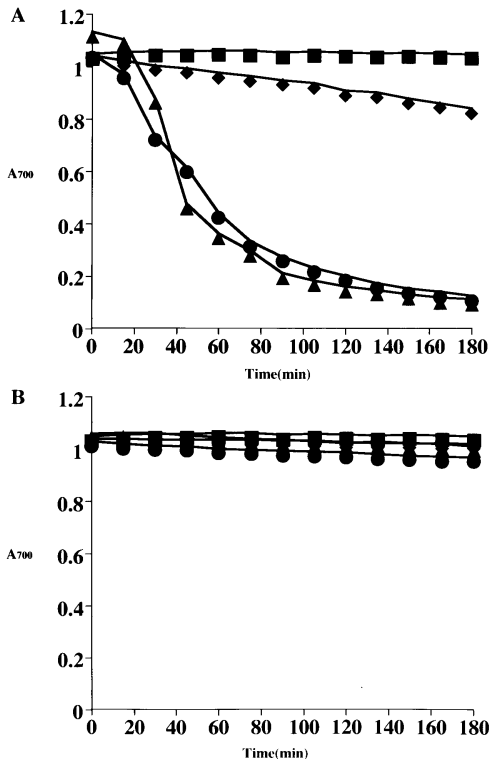

Figure 5 Bacterial agglutination by native or truncated SP-Ds

The effects of equivalent weights of truncated and native SP-Ds on the agglutination of *E. coli* Y1088 were examined. The decrease in D_{700} that accompanied the sedimentation of agglutinated bacteria was monitored continuously on a spectrophotometer. (**A**) Decreases in attenuance caused by 1 μg/ml of recombinant truncated SP-D with Gly-Xaa-Yaa repeats and histidine tag (▲), 1 μg/ml of native SP-D (●), 2 μg/ml of recombinant truncated SP-D without Gly-Xaa-Yaa repeats and histidine tag (◆) and in their absence (■). (**B**) Bacterial agglutinations were inhibited by the addition of 10 mM EDTA in same experiments.

Bacterial agglutination

We examined the ability of SP-D to cause agglutination of *E. coli* Y1088 cells, as measured by the decrease in light transmission through the bacterial suspension. The truncated SP-D with Gly-Xaa-Yaa repeats and the histidine tag caused the agglutination of *E. coli* in the same pattern as that of native SP-D (Figure 5A). The more truncated SP-D, without Gly-Xaa-Yaa repeats and the histidine tag, caused less agglutination than either the less truncated SP-D or the native molecule. Figure 5(B) shows that agglutination was inhibited by the presence of 10 mM EDTA, suggesting that agglutination by these human SP-Ds was due to Ca^{2+}-dependent lectin activity. Agglutination of *E. coli* by native or recombinant human SP-D was dose-dependent (results not shown). The agglutination titre was 0.3 μg/ml for truncated SP-D with Gly-Xaa-Yaa repeats and the histidine tag, and 0.05 μg/ml for native SP-D (Table 2). This indicates that an approx. 6-fold greater concentration of recombinant truncated SP-D is required to achieve an effect similar to that obtained with the native SP-D.

HI activity of recombinant human SP-D

The HI activity of SP-Ds was determined with a microtitre plate assay system (Figure 6). Native SP-D had HI activity against influenza A virus. This HI activity was lectin dose-dependent and Ca^{2+}-dependent. The lowest concentration of native human SP-D capable of causing the HI phenomenon was 78–156 ng/ml. However, recombinant SP-D showed no HI activity at a concentration of 20 μg/ml (Table 2). In contrast, the HI activities of native and recombinant conglutinin showed similar values of 39–78 ng/ml (Table 2).

Interactions between SP-Ds and influenza A virus

The recombinant truncated SP-D with/without Gly-Xaa-Yaa repeats and the histidine tag failed to give viral aggregation in a light-transmission study (results not shown). However, its sedimentation by low-speed centrifugation showed that truncated SP-D (24 kDa) as well as native SP-D (45 kDa) could bind to its virion by lectin activity (Figure 7B). The lectin blot indicates that both SP-Ds can react on two envelope proteins of 85, 210 and 300 kDa of haemagglutinin (HA) and 55 kDa of neuraminidase (NA) although bovine conglutinin can bind to HA only (Figure 7A). Its binding to NA was weaker than to HA because the NA protein had less volume and had fewer high-mannose chains.

DISCUSSION

We previously reported that recombinant conglutinin with deletion of the N-terminal and collagenous domain is capable of conglutination activity by binding to sheep erythrocytes bearing iC3b on their surfaces (EAiC3b) and that it also displays HI activity towards influenza A virus [19]. To elucidate the biological function of other C-type lectins towards pathogenic microorganisms, we produced a recombinant human SP-D composed of synthetic peptides with a histidine tag, two Gly-Xaa-Yaa repeats of the collagen domain, a neck domain, and a CRD. This recombinant SP-D was capable of forming trimers without the collagen domain and showed similar binding activity and specificity to those of native SP-D. SP-D mediates the agglutination of Gram-negative bacteria [18] and basidiomycetous

Table 2 Comparison of biological characteristics of human SP-D and bovine conglutinin

The HI value shows the lowest protein concentration of human SP-D and bovine conglutinin causing HI. Ag_{50} shows the concentration of protein for a 50% decrease in light transmission by agglutination of bacteria. The conglutination value is the lowest protein concentration showing agglutination of sensitized SRBCs. The values for bovine conglutinin are taken from [19].

	Human SP-D (μg/ml)		Bovine conglutinin (μg/ml)	
	Native (oligomer)	Truncated (trimer)	Native (oligomer)	Truncated (trimer)
HI	0.078–0.156	> 20	0.039–0.078	0.039–0.078
Ag_{50}	0.05	0.3		
Conglutination			0.15	1.25–2.5

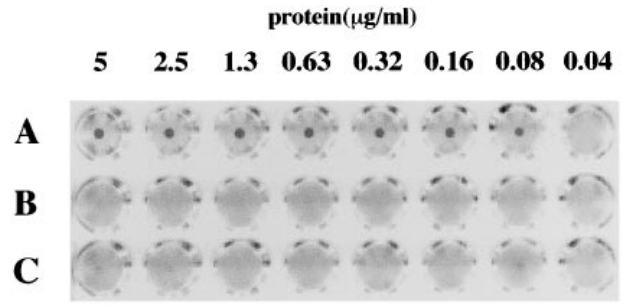

Figure 6 Inhibition of haemagglutination of influenza A virus by incubation with native SP-D or recombinant SP-D in the presence of Ca^{2+} or EDTA

The HA inhibition test was performed with a 2-fold dilution of native SP-D (**A**, **B**) or recombinant SP-D (**C**) in 96-well V plates to which 16 HA units (final concentration) of the influenza A virus (A/Ibaraki/1/90-H3N2) were then added. The mixture of recombinant or native SP-D was incubated with 0.5% chicken RBC in TBS, pH 7.5, with no other addition (**A**, **C**), or with 10 mM EDTA (**B**) at room temperature for 1 h.

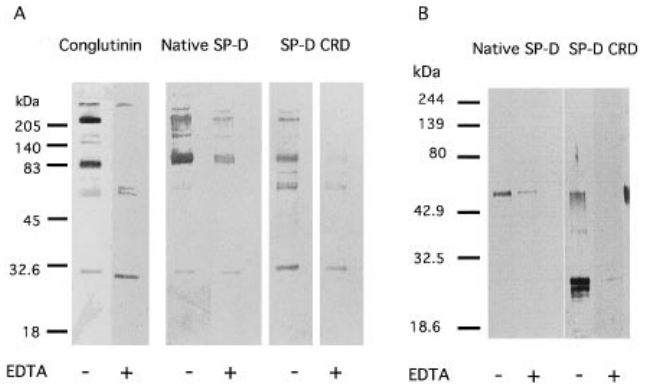

Figure 7 Interactions between SP-Ds and influenza A virus

(**A**) Lectin blot with bovine conglutinin, native SP-D and truncated SP-D (SP-D CRD). The influenza virion of A/Ibaraki/1/90 (H3N2) was resolved by SDS sample buffer, separated by SDS/PAGE [4–10% (w/v) gradient gel] and transferred to membrane. The membranes were incubated with conglutinin or SP-Ds [with EDTA (+) or in its absence (−)] and rabbit anti-human SP-D serum, and stained using a streptavidin–biotin alkaline phosphatase staining system with anti-(rabbit IgG)-conjugated biotin and alkaline phosphatase-conjugated streptavidin. (**B**) Binding of SP-Ds to the same influenza virus. The virus was incubated with native SP-D or truncated SP-D (SP-D CRD) in TBS/C (−) or TBS containing 10 mM EDTA (+). After centrifugation of the mixture, the pellets were analysed by Western blotting with rabbit anti-human SP-D serum. The positions of molecular mass markes are shown at the left.

yeast [24]. We investigated whether recombinant truncated SP-D could bind to bacteria and induce their agglutination and found that it was able to agglutinate Y1088 in a dose-dependent manner. Bacterial agglutination by truncated SP-Ds was inhibited in the presence of EDTA and 200 mM maltose, consistent with interaction with the bacterial cells through the CRDs of the recombinant protein. Under these assay conditions, an approx. 6-fold higher concentration of recombinant truncated SP-D was required to achieve an effect similar to those obtained with native SP-D. Truncated conglutinin has less conglutination activity than native conglutinin, as we previously reported [19]. The conglutination titre of truncated conglutinin was 1.25–2.5 μg/ml, whereas that of native conglutinin was 0.15 μg/ml. This indicates that truncated conglutinin requires an 8–16-fold greater concentration for conglutination than does the native one. Conglutinin and SP-D have a characteristic cruciform structure, which is assembled as tetramers of three identical polypeptides [25]. Their agglutination activity is considered to depend on oligomerization and their ability to bridge large distances between organisms. Therefore it is considered that recombinant truncated conglutinin with deletion of the collagen domain was unable to create the oligomeric form and was less effective in agglutinating EAiC3b [19]. The recombinant truncated rat SP-D without a collagen-like domain in CHO cells [26] and truncated human SP-D in *E. coli* [27] could form a trimer owing to the neck domain, and bind to phospholipids. As described in this paper, truncated SP-D, which consists largely of the trimeric form, also might have less ability than whole native SP-D to agglutinate bacterial cells. These results suggest that the trimeric structure of truncated SP-D as well as that of truncated conglutinin is basically sufficient for agglutinating bacteria or EAiC3b.

Recently, recombinant whole human SP-D was shown to protect neutrophils from influenza A virus-induced deactivation [28]. This protective ability of SP-D was associated with the extent of multimerization, which was sufficient to cause aggregation of influenza A viruses [29]. C-type lectins inhibit the haemagglutination activity of influenza A virus by binding to the high-mannose oligosaccharide near the sialic acid-binding site of viral HA [12]. This inhibition has been observed with MBP [30], conglutinin [13] and SP-D [17], which bind to the high-mannose oligosaccharide with its lectin domain. Although native SP-D exhibited HA inhibition activity, truncated SP-D was unable to inhibit haemagglutination, even when employed at higher concentrations (more than 20 μg/ml). The reason for this difference in HI is not clear. The saccharide specificity of human SP-D is different from that of conglutinin [20] and this SP-D CRD might possess a low affinity for the oligosaccharide attachment site of HA, compared with conglutinin CRD. Hence the truncated SP-D, consisting only of the trimeric form, does not inhibit haemagglutination. Another possibility, suggested in Figure 7, is that SP-D can bind to NA, which is another viral envelope protein of influenza virus, as well as to HA because of a difference in sugar specificity. The lectin precipitation study confirms that truncated

SP-D as well as native SP-D is able to bind to the influenza virion (Figure 7B). Lectin blotting data showed that human SP-D could bind weakly to NA as well as to HA, although bovine conglutinin could bind only to HA (Figure 7A). In a preliminary study, human MBP could also react with NA and HA and the truncated form failed to induce the viral HI phenomenon. In a different influenza A virus only NA protein was recognized by human MBP [30].

Finally, native and recombinant truncated conglutinin can bind to a special site on HA, which is critical for the haemagglutination reaction, so that an oligomeric structure has no advantage in the HI reaction. In contrast, native SP-D might bind to another HA site that is not as critical, so that the change to a trimeric structure might result in the loss of HI activity because its oligomeric structure and its multiple binding to both HA and NA could cause a high HI reaction owing to its steric interference. These results suggest that there is a difference in the mechanisms of inhibition of haemagglutination between conglutinin and SP-D.

In this study we produced a recombinant SP-D lacking the collagen domain and characterized several of its biological activities. This recombinant SP-D should prove useful in future studies on the structure and function of these types of protein.

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