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A 50-kDa variant form of human surfactant protein D

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A 50-kDa variant form of human surfactant protein D. R.J. Mason, L.D. Nielsen, Y. Kuroki, E. Matsuura, J.H. Freed, J.M. Shannon. ©ERS Journals Ltd 1998.

ABSTRACT: The dominant form of human surfactant protein D (SP-D) is a multimeric collagenous glycoprotein composed of monomeric subunits that have a molecular mass of 43 kDa under reducing conditions. However, in evaluating monoclonal antibodies to human SP-D, an additional monomeric subunit was identified with a reduced molecular mass of 50 kDa.

This 50-kDa variant was detected in approximately half of the samples evaluated and was found in lavage fluid from normal subjects, patients with alveolar proteinosis or idiopathic pulmonary fibrosis and in amniotic fluid. This 50-kDa variant had the same amino-terminal sequence, amino acid composition and apparent size of the carboxy-terminal collagenase-resistant fragment (20 kDa) as the 43-kDa subunit. The major difference was in the amino-terminal portion of the molecule and was due to altered glycosylation, as determined by carbohydrate staining, chemical deglycosylation, treatment with *N*-glycanase and neuraminidase and reduced signals for threonine at positions 5, 9 and 10 during amino-terminal sequencing.

After gel filtration chromatography, the 50-kDa form was not present in the high molecular weight fraction, which is commonly used in purification of SP-D, but was found only in the smaller molecular weight fraction of monomers and trimers of SP-D.

In conclusion, the 50 kDa-form of surfactant protein D is produced by post-translational glycosylation and does not form higher ordered oligomers, but its precise physiological function remains to be determined.
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Surfactant protein (SP) D is thought to be an important host defence molecule in the small airways and gas exchange units in the lung [1]. SP-D is a calcium-dependent lectin, and because of its collagen-like sequence, is referred to as a "collectin", as are SP-A, mannose-binding protein and bovine conglutinin [2]. Although SP-D is associated with surface active material, it can be easily separated from surfactant lipids, because it binds these lipids weakly. Human SP-D is thought to arise from a single gene [3, 4] that codes for a large collagenous glycoprotein composed of multiple identical units, usually 12, each of which has an apparent molecular mass of 43 kDa under reducing conditions [5–8]. Each subunit is comprised of an amino-terminal portion, a collagenous portion with 59 Gly-XY repeats, a neck region and a globular carbohydrate recognition domain. Mature multimeric SP-D has 12 carbohydrate recognition domains and is thought to function as a multivalent lectin [3]. The carbohydrate recognition domain of SP-D has specificity for maltose, mannose and glucose residues [9]. By means of this lectin property SP-D has been reported to bind a variety of viruses, bacteria, mycobacteria and fungi [10–14]. SP-D has been reported to increase the aggregation and phagocytosis of bacteria and viruses [15]. In addition, SP-D has moderate affinity for phosphatidylinositol and glucosylceramide [9, 16]. SP-D is synthesized by both type II and nonciliated bronchiolar cells in the rodent lung. In Clara or nonciliated bronchiolar cells of rodents, SP-D is found in the dense secretory granules [17–19]. SP-D is not found in the lamellar bodies of type II cells or in the tubular myelin form of surfactant and is, therefore, thought to be secreted independently of the lipids of surface active material and probably plays little role in the metabolism or processing of surfactant. Recently, SP-D has been found to be elevated in the serum of patients with interstitial lung disease and adult respiratory distress syndrome [20, 21].

In evaluating a series of monoclonal antibodies to SP-D by Western analysis, we identified a new 50-kDa variant of SP-D, in addition to the usual 43-kDa form. This report describes the isolation and characterization of this 50-kDa variant.

Materials and methods

Sources of material

Lavage samples were obtained from alveolar proteinosis patients as part of a therapeutic lavage, from normal volunteers and from patients with idiopathic pulmonary fibrosis attending a Specialized Center for Research in Occupational and Immunologic Lung Diseases. Amniotic fluid was obtained at the time of uncomplicated caesarean sections. The collection of these samples was approved by the Institutional Review Board of the National Jewish Medical and Research Center.

Purification of human surfactant protein D

SP-D was isolated from human bronchoalveolar lavage fluid (BALF) and near-term human amniotic fluid. Methods similar to those reported by CROUCH *et al.* [22] and LU *et al.* [23] were used to prepare native SP-D. However, as will be discussed below, gel filtration was not used for purification of SP-D; instead, preparative polyacrylamide gel electrophoresis (PAGE) was used to prepare large amounts of sodium dodecyl sulphate (SDS)-denatured SP-D. In brief, lavage and amniotic fluids were centrifuged at either 100,000×*g* for 45 min (small volumes) or 28,000×*g* for 16 h (large volumes) to remove the surfactant as a pellet. The supernatant, which contained most of the SP-D, was used for the subsequent studies. $CaCl_2$ was added to a final concentration of 10 mM and the solution was applied to a maltose sepharose column [24] that had been equilibrated with 5 mM tris-hydroxymethyl-amino methane (Tris), 0.15 M NaCl and 1 mM $CaCl_2$, pH 7.4 (Tris-buffered saline (TBS) + Ca) at 4°C. The column was washed with TBS + Ca buffer and then the SP-D was eluted with TBS + 10 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA). The eluted material was dialysed against 10 mM ammonium bicarbonate and lyophilized.

To obtain denatured SP-D, the freeze-dried material from maltose sepharose chromatography was dissolved in a small volume of reducing SDS-PAGE sample buffer [25] and partially purified by electrophoresis through a 9% polyacrylamide gel in a BioRad Model 491 Prep Cell (Hercules, CA, USA) according to the manufacturer's instructions. Two monomeric forms of SP-D (43 kDa and 50 kDa) were resolved and pooled separately. The two forms were finally purified to apparent homogeneity by reverse-phase high-performance liquid chromatography (HPLC) on a C_8 column (Vydac 208 TPS10, Vydac, Hesperia, CA, USA; 10 mm×25 cm). The sample was loaded in 0.1% trifluoroacetic acid (TFA) in H_2O and the column was washed with the loading buffer for 10 min at a flow rate of 4 mL·min^{-1}. The column was then eluted with a gradient of 0–60% 0.1% TFA in acetonitrile over a period of 60 min (1%·min^{-1}). Both forms of SP-D eluted at approximately 43% acetonitrile.

To obtain native SP-D, lyophilized material from maltose sepharose columns was dissolved in a small volume of TBS + 10 mM ethylenediaminetetraacetic acid (EDTA) and further purified by gel filtration on a column of 4% agarose (BioRad A 15m) according to the method of CROUCH *et al*. [26].

Amino acid composition and amino-terminal sequence analysis

Amino acid analyses of HPLC fractions were performed by means of standard procedures [27]. To perform amino-terminal sequence analysis, fractions isolated by HPLC were applied to a Pro-Spin membrane (Applied Biosystems/Perkin Elmer, Foster City, CA, USA) and sequenced by gas-phase Edman degradation on a Model 470A/120A protein microsequencer (Applied Biosystems/Perkin Elmer). Data analysis was performed using Dyna-max chromatography software (Rainen Instrument Co., Woburn, MA, USA).

Western blot analyses

To identify SP-D in different amniotic and lavage fluids, 10–20 mL of fluid was partially purified on maltose sepharose, eluted with EDTA and freeze-dried. The samples were dissolved in 100 µL of reducing SDS–PAGE sample buffer and heated briefly. Aliquots were then fractionated by electrophoresis through precast 8–16% polyacrylamide gradient slab gels in a Novex Xcell Mini-cell (San Diego, CA, USA) according to the manufacturer's instructions. Specific proteins (*e.g.* SP-D) were visualized by Western immunoblot analysis. In brief, the proteins were transferred to nitrocellulose using the Novex Western transfer apparatus. The nitrocellulose was blocked using phosphate-buffered saline containing 5% nonfat dry milk and probed using primary antibodies according to methods reported by HARLOW and LANE [28] for immunoblotting. Primary antibodies (see below) were detected using horseradish peroxidase-conjugated secondary antibodies and diaminobenzidine as the substrate. In some experiments, the proteins in PAGE gels were visualized directly by silver staining according to the method of MORRISSEY [29].

The antibodies used in this study were obtained from the following sources: monoclonal antihuman SP-D antibodies (1G11, 6B2, and 7C6) [30] were from E. Matsuura and polyclonal rabbit antihuman SP-D was a generous gift from E. Crouch (Washington University, St Louis, MO, USA); antihuman immunoglobulin (Ig) G (heavy and light chains) was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA); monoclonal anti-human SP-A was from T. Akino (Sapporo Medical University); polyclonal antihuman SP-A and polyclonal rabbit antirat SP-D were from D. Voelker (National Jewish Medical and Research Center, Denver, CO, USA). E. Aronsen (Dept of Medicine, National Jewish Medical and Research Center) prepared a rabbit antibody to the SP-D peptide S-P-R-S-A-A-E-N-A-A-L-Q-Q [31], which was coupled to keyhole limpet haemocyanin (KLH; Pierce, Rockford, IL, USA) according to the methods reported by HARLOW and LANE [28] and used for immunization and antibody production.

Carbohydrate staining

Sugars in SP-D glycoproteins were detected in samples which were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose using a First Choice glycan detection kit (Boehringer Mannheim Corp., Indianapolis, IN, USA). The procedure for glycoproteins immobilized on filters was carried out according to the method described by the supplier.

Treatments with neuraminidase, O-*glycanase and* N-*glycanase*

Sialic acid was removed from SP-D glycoproteins with neuraminidase as described by PERSSON *et al.* [31], with the following changes. It was necessary to reduce the Sig-ma type V neuraminidase to 0.05 per 25 µL reaction mix and the reaction was complete after 30 min at 37°C.

To remove *O*-linked sugars from SP-D, sialic acids were first removed with neuraminidase as described above. The pH of the reaction mix was then adjusted to 6–7 using 0.5 M Tris base. *O*-glycanase (2 mU; Genzyme Corp., Cambridge, MA, USA) was added to each 25 µL reaction mix,

which contained about 200 ng protein, and incubated at 37°C for the times indicated in the text.

N-linked sugars were removed from SP-D using recombinant *N*-glycanase (Genzyme Corp.) and reaction conditions recommended by the supplier. In brief, aliquots of HPLC-purified 43-kDa and 50-kDa SP-D were dried in a Speed Vac centrifuge (Savant, Hicksville, NY, USA) and redissolved in a small volume of 50 mM Tris (pH 8.4) containing 0.5% SDS, 50 mM 2-mercaptoethanol and 1 mM EDTA. After denaturing for 5 min by boiling, Nonidet P-40 (NP-40) and enzyme were added and the mixture was incubated for 18 h at 37°C. The digested glycoproteins were analysed by SDS-PAGE and Western blotting.

Chemical deglycosylation

Prep Cell fractions containing SP-D adjacent to the 43-kDa or 50-kDa peak fractions (the peak fractions were used for HPLC purification; see fig. 1) were dialysed exhaustively against 10 mM ammonium bicarbonate and freeze-dried. Some freeze-dried aliquots were dissolved in 1× Laemmli PAGE sample buffer for Western analysis. Other aliquots were chemically deglycosylated using anhydrous trifluoromethanesulphonic acid (TFMS), which has been shown to cleave both *N*- and *O*-linked glycans from glycoproteins [32]. The reagents and procedures used for TFMS-deglycosylation were obtained in the Glyco Free deglycosylation kit from Oxford Glyco Sciences (Wakefield, MA, USA). After chemically removing glycans from SP-D, the samples were dialysed against 10 mM ammonium bicarbonate, freeze-dried and dissolved in PAGE sample buffer for Western analysis.

Collagenase digestion

For digestions using collagenase, 150-μL aliquots (~450 ng protein) of Prep Cell fractions containing either the 43-kDa or 50-kDa form of SP-D were first mixed with 3 μL of 10% NP-40. This allowed the formation of micelles containing NP-40 and the 0.1% SDS from the Prep Cell fractions, thus preventing interference with the subsequent enzyme reactions. $CaCl_2$ was added to a final concentration of 20 mM, and the solution then aliquoted into three equal 52-μL portions. Purified form of type III collagenase (0.8 U; Advance Biofactures, Lynbrook, NY, USA) was added to one of the 52 μL aliquots. The collagenase reaction mixtures were incubated at 37°C for 16 h along with untreated (*i.e.* no collagenase) control aliquots. The third control aliquot was not incubated. After incubation, 18 μL 4×Laemmli PAGE sample buffer was added to each mixture. Samples (20 μL; ~50 ng protein) of each were analysed by SDS-PAGE and silver staining.

In vitro *translation*

Human lung samples were homogenized in 4 M guanidinium isothiocyanate, 0.5% *N*-lauryl sarcosine and 0.1 M 2-mercaptoethanol in 25 mM sodium citrate buffer, pH 7.0. Total ribonucleic acid (RNA) was purified by centrifuging the homogenate through a cushion of 5.7 M CsCl at 150,000×*g* for 18 h at 22°C. The RNA was precipitated with ethanol, washed with 70% ethanol, and then dissolved in ribonuclease (RNase)-free water. Polyadenylic acid (Poly A^+) was isolated using a Poly (A) Quik Kit (Stratagene, La Jolla, CA, USA) according to the supplied instructions. Cell-free protein translation was performed using a rabbit reticulocyte cell-free system according to the manufacturer's instructions (Novagen Red Nova Lysate protocol; Novagen, Madison, WI, USA). Immunoprecipitation of proteins after cell-free translation of messenger RNA (mRNA) was conducted according to the methods of Anderson and Blobel [33], using polyclonal anti-hu-man SP-D (E. Crouch).

Results

During the analysis of human SP-D from the lavage fluids of patients with alveolar proteinosis, a protein band with an apparent molecular weight of 50 kDa was observed in all of the samples from different patients when the crude supernatant or pellet was analysed by SDS-PAGE (fig. 2).

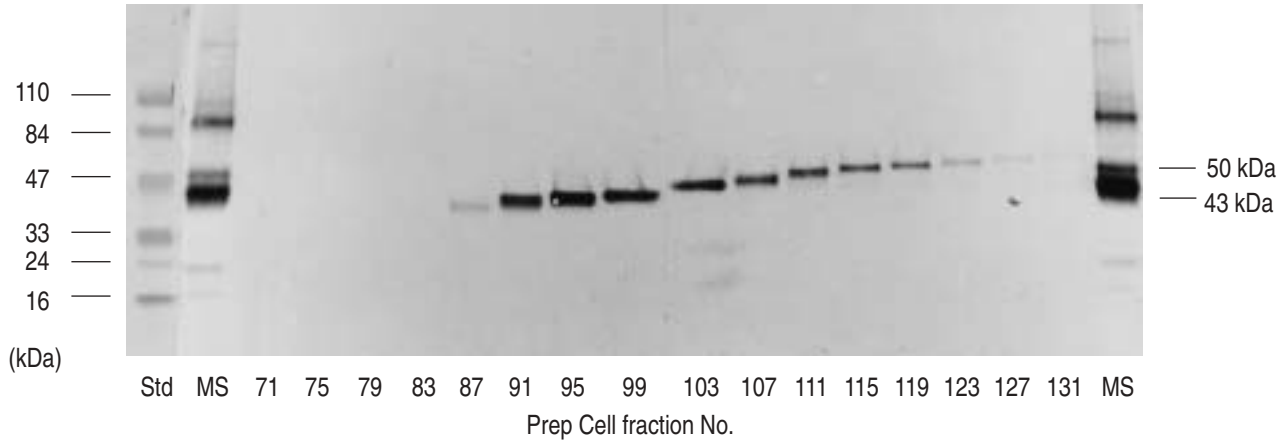

Fig. 1. – Western-blot analysis of fractions isolated by preparative electrophoresis. Surfactant protein D (SP-D) was partially purified by chromatography on maltose sepharose (MS) and then further purified by preparative electrophoresis under reducing and denaturing conditions (numbered fractions). Aliquots were then analysed by polyacrylamide gel electrophoresis and SP-D was identified by transfer and Western blotting with the 6B2 monoclonal antibody. Fractions 91–95 were pooled to isolate the 43 kDa form of SP-D and fractions 107–111 were pooled to isolate the 50 kDa form. Std: prestained molecular weight standards.

By Western blot analysis, the observed 50-kDa variant was in addition to the expected 43-kDa band, which was also seen in all samples and has been reported by many investigators to be the dominant reduced and denatured monomer of SP-D [22, 31]. Higher molecular weight dimers and trimers of SP-D were also observed as bands with apparent molecular weights of 100 and 150 kDa, respectively. To determine whether this unusual 50 kDa form of SP-D was unique to alveolar proteinosis, BALF was analysed from normal and volunteer patients with idiopathic pulmonary fibrosis and from amniotic fluid. These fluids all contained the expected 43-kDa monomer, but approximately half also contained detectable amounts of the 50-kDa monomer. The 50-kDa band was detected in four out of four samples from alveolar proteinosis patients, five out of eight normal controls, six out of 10 patients with interstitial lung disease and one out of two amniotic fluids of normal term infants. No correlation was seen between any clinical diagnosis or source and the presence of this 50 kDa SP-D monomer. It is important to note that a similar larger molecular weight form of SP-D was not detected in rat lung or lavage fluid [34].

To ensure that this observation was not limited to a single monoclonal antibody, several monoclonal (1G11, 6B2 and 7C6) [30] and polyclonal antibodies were evaluated for detection of SP-D by Western blot analysis. All anti-SP-D antibodies (monoclonal and polyclonal) that we have tested recognize both the 43-kDa and 50-kDa forms of SP-D. One of the polyclonal antibodies was prepared to a synthetic peptide as described previously [33] and, hence, could not recognize carbohydrate determinants on SP-D. The detection of the 50-kDa protein with several different monoclonal and peptide polyclonal antibodies suggested strongly that this was a variant of SP-D, as opposed to a protein which shared an antigenic epitope with SP-D.

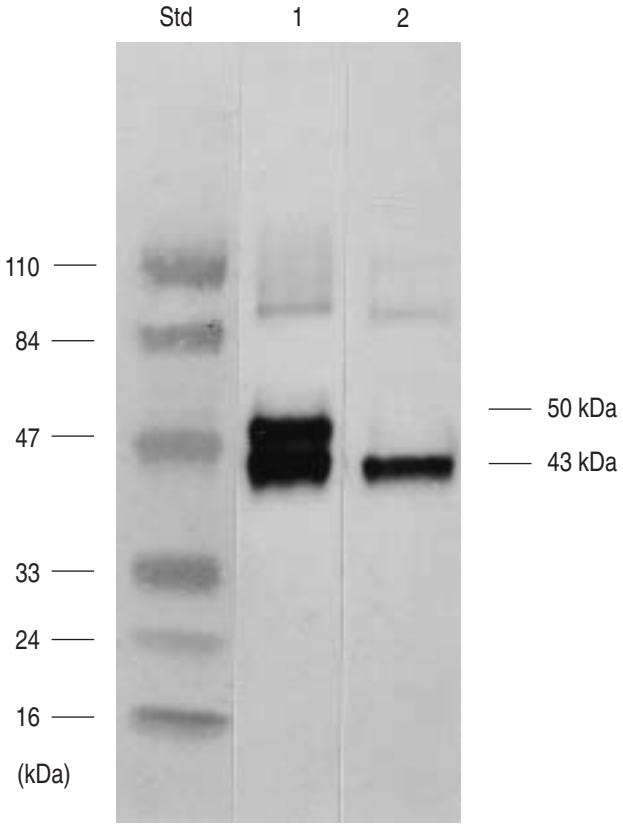

Fig. 2. – Western-blot demonstrating the 43-kDa and 50-kDa forms of surfactant protein D (SP-D). SP-D was partially purified by maltose sepharose chromatography and then separated by polyacrylamide electrophoresis, transferred to a nitrocellulose membrane and identified by Western analysis. Lane 1 is lavage from a patient with idiopathic pulmonary fibrosis; and lane 2 is from lavage from a normal subject. Std: prestained protein molecular weight standards.

Since SP-A and IgG are the proteins most likely to contaminate SP-D preparations, antibodies to these proteins were also used to probe Western blots. After partial purification by maltose sepharose affinity chromatography, the SP-D preparations contained traces of SP-A and IgG. SP-D and SP-A were effectively separated from each other by electrophoresis under reducing and denaturing conditions in the Prep Cell. However, the 50-kDa form of SP-D still contained IgG heavy chains, by Western blot analysis, after electrophoresis. These two 50-kDa proteins were com-pletely separated from each other by subsequent reverse-phase HPLC. Both the 43-kDa and 50-kDa forms of SP-D eluted from the HPLC column at a concentration of 43% acetonitrile.

The most effective means by which to separate the 50-kDa variant from the 43-kDa form of SP-D was by preparative electrophoresis as shown in figure 1. SP-D was partially purified from the supernatant fraction of alveolar proteinosis fluid by maltose sepharose affinity chromatography. The material was concentrated by freeze-drying and further purified by preparative electrophoresis. There was an apparent continuum of molecular weights, which suggested that there was not a single alteration in the primary structure of SP-D. Fractions 91–95 were pooled to isolate the 43-kDa form of SP-D and fractions 107–111 to isolate the 50-kDa variant.

Table 1. – Amino acid composition of the 43 kDa and 50 kDa forms of surfactant protein D (SP-D)

Amino acid	Predicted from cDNA [7]	Measured [7]	Measured [23]	Current study 43 kDa	Current study 50 kDa
Cys	1.2	-	2.1	-	-
Thr	3.0	3.1	2.4	0.8	0.8
Ser	4.8	4.6	5.9	5.8	8.2
Gly	21.8	20.6	23.0	21.3	20.4
Ala	10.3	10.4	9.4	12.3	10.1
Val	5.4	5.8	4.7	7.4	6.9
Met	1.2	-	1.6	1.9	1.6
Ile	1.2	1.2	1.5	0.8	1.7
Leu	5.4	5.8	4.3	6.6	7.4
Tyr	0.9	0.9	1.2	0.6	2.5
Phe	3.0	2.7	2.0	4.5	3.7
His	0.6	0.5	0.7	0.6	0.5
Arg	3.9	3.9	4.1	5.3	1.6
Pro	-	5.5	6.2	7.6	7.0
HyPro	-	6.6	3.1	3.7	3.8
Lys	-	4.2	3.9	2.0	2.3
HyLys	-	1.2	1.2	1.0	1.1
Asx	6.7	7.0	7.0	7.5	9.5
Glx	12.4	13.0	13.0	15.2	15.7
Trp	0.7	-	-	-	-
Pro+HyPro	10.9	12.1	9.3	11.3	10.8
Lys+HyLys	6.7	5.3	5.1	3.0	3.4

cDNA: complementary deoxyribonucleic acid.

Compositional analysis

The amino acid composition of human SP-D has been reported by several laboratories [23]. SP-D is an unusual protein because it contains a large collagen-like region, and therefore, a high percentage of glycine, hydroxyproline and hydroxylysine. Table 1 shows the results of a compositional analysis of the purified 43-kDa and 50-kDa forms of human SP-D. It can be seen that although there were small differences, the amino acid compositions of the two forms of SP-D were similar to each other, to values in the literature [7, 23], and to those deduced from human SP-D complementary deoxyribonucleic acid (cDNA) [7]. Importantly, the 50-kDa protein contained significant amounts of hydroxyproline and hydroxylysine. For comparison, figure 3 includes the composition of 43-kDa human SP-D as reported by Rust *et al.* [7] and Lu *et al.* [23].

Sequence analysis

Amino-terminal sequencing was performed on both the 43-kDa and 50-kDa forms and the results compared with the published human SP-D cDNA sequence [23]. Both sequences were identical and matched the sequence predicted from the cDNA [23]. However, there was one significant difference. The signals for threonine at positions 5, 10 and 11 of the 50-kDa variant were markedly decreased compared with similar analysis of the 43-kDa form, which is compatible with the presence of *O*-linked sugars at these positions.

Carbohydrate staining and enzymatic digestions

Carbohydrate staining. Amino-terminal sequencing suggested that the difference in molecular weight was probably due to differential glycosylation. When the 43-kDa and 50-kDa fractions were stained for protein and carbohydrate, it was noted that a given amount of the 50-kDa protein stained much more heavily for carbohydrate (fig. 4).

Sequence deduced from cDNA [23]:

A E M K T Y S H R T T P S A C T L V M C S S V E S G L P G R

43-kDa SP-D:

A E M K T Y S H R T T P S A ? T L V M ? S S V E ? G L ? G ?

50-kDa SP-D:

A E M K T*Y S H R T*T*P S A ? T L V M ? S S V E ? G L ? ? R

Fig. 3. – Amino-terminal sequence of 50-kDa and 43-kDa human surfactant protein D (SP-D). The 50-kDa and 43-kDa forms of SP-D were isolated by preparative electrophoresis and further purified by high-performance liquid chromatography. These proteins were then sequenced as described in Materials and methods. The amino acid sequence corresponding to the complementary deoxyribonucleic acid (cDNA) for human SP-D [23] was compared with the results obtained for the 43-kDa and 50-kDa forms of SP-D. The amino acid sequences are identical and correspond exactly with the sequence deduced from the cDNA. The only significant differences were that the signals for threonines at position 5, and, especially, at positions 10 and 11 were very low for the 50-kDa form (*), which strongly suggests *O*-linked glycosylation at these sites. ?: the amino acid at this location could not be identified with certainty.

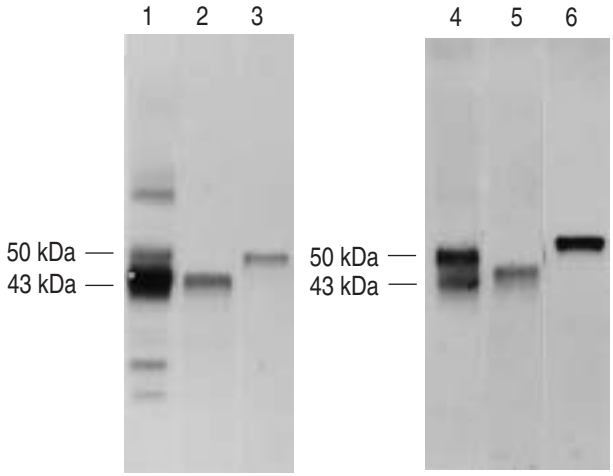

Fig. 4. – Detection of protein and carbohydrate. Surfactant protein D (SP-D) was isolated by maltose sepharose chromatography to give a product containing both the 43-kDa and the 50-kDa forms (lanes 1 and 4). The 43-kDa (lanes 2 and 5) and 50-kDa forms (lanes 3 and 6) were isolated separately, by preparative electrophoresis. The fractions (approximately 50 ng protein per lane) were subjected to standard polyacrylamide gel electrophoresis under reducing and denaturing conditions, transferred to nitrocellulose and stained for SP-D (lanes 1–3) by Western analysis with the 6B2 monoclonal antibody and for carbohydrate (lanes 4–6) using the glycan detection kit. The 50-kDa form stained much more intensely for carbohydrate than an equivalent amount of the 43-kDa form.

N-*Glycanase digestion.* Since it has been shown that SP-D is glycosylated by a single *N*-linked carbohydrate on an asparagine residue (Asn 70) in the collagen region of the protein [3, 5, 7], the two forms of SP-D were examined after deglycosylation with *N*-glycanase. Both forms shift to lower apparent molecular weights after deglycosylation (fig. 5). However, deglycosylated 50-kDa SP-D appears to split into two lower molecular weight peptides, both of which are still larger than deglycosylated 43-kDa SP-D. Therefore, the size difference observed does not appear to be due to a simple change in *N*-linked glycosylation. These digestions would not be expected to remove *O*-linked sugars.

Neuraminidase and O-*glycanase digestion*. Several attempts were made to remove the *O*-linked sugars with *O*-glycanase. In general, *O*-glycanase digestions are thought to proceed more towards completion if the terminal sialic acids are removed with neuraminidase before treatment with *O*-glycanase. Neuraminidase decreased the apparent molecular weight of the 50-kDa form much more than that of the 43-kDa form (fig. 6). Unfortunately, under the conditions used, *O*-glycanase did not reduce the molecular weights any further. Several concentrations of enzymes and several different conditions were used, without improvement.

Chemical deglycosylation. Chemical deglycosylation with anhydrous TFMS was used as another means of removing O-linked and *N*-linked sugars. This method removes approximately 90% of the carbohydrate on SP-D, as judged by carbohydrate staining (data not shown). Chemical deglycosylation of both the 43-kDa and the 50-kDa forms of SP-D produces a single protein with an apparent molecular mass of approximately 40 kDa, which is expected from the predicted primary structure of SP-D (fig. 7).

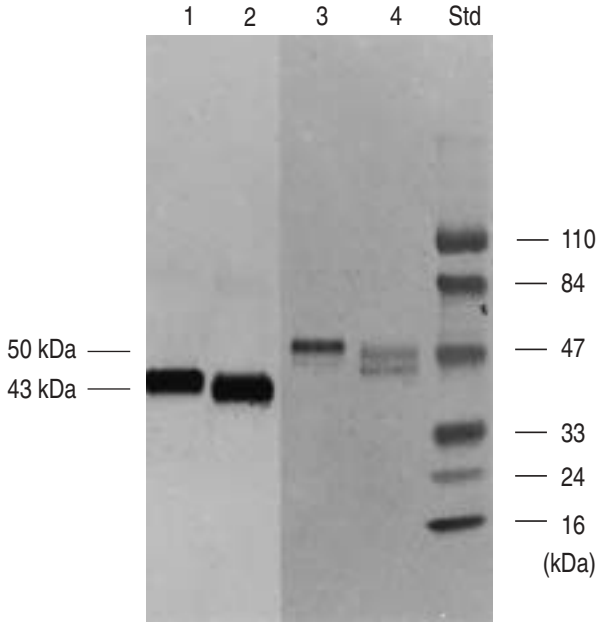

Fig. 5. – Digestion of the 43-kDa and 50-kDa forms of surfactant protein D (SP-D) with *N*-glycanase. The 43-kDa (lanes 1 and 2) and 50-kDa (lanes 3 and 4) forms of SP-D were purified by preparative electrophoresis and then treated with (lanes 2 and 4) or without (lanes 1 and 3) *N*-glycanase. The proteins were detected by Western blotting. Std: prestained standards.

Collagenase digestion. Samples of the separated 43-kDa and 50-kDa SP-D monomers were digested with highly purified bacterial collagenase. Both forms of SP-D produce a collagen-resistant carboxy-terminal fragment with the same apparent molecular weight of 20 kDa on SDS-PAGE (fig. 8). The very small amino-terminal collagen-resistant fragment was not identified or characterized. Therefore, it can be deduced that the difference in size observed is due to differences in the collagen region or the amino-terminal portion of the protein.

In vitro *translation*

To determine whether there were two primary translation products, SP-D produced by *in vitro* translation of mRNA from normal lungs, which could not be transplanted for technical reasons, was analysed. mRNA was extracted from biopsy specimens from three different adult lungs. After translation of the mRNA into proteins in the presence of ^{35}S-cysteine, the labelled SP-D translation products were selectively immunoprecipitated using a polyclonal antihuman SP-D. In these experiments, only one labelled protein band was obtained when the SP-D translation product was examined by SDS–PAGE and autoradiography (data not shown). This result indicates that there is only one primary translation product and that the two forms must result from secondary modification of the protein, *i.e*. post-translational modifications.

Isolation by gel filtration chromatography

To determine whether the discovery of the 50-kDa variant form of SP-D was due to the method of isolation of SP-D, SP-D was also purified by gel filtration. Several

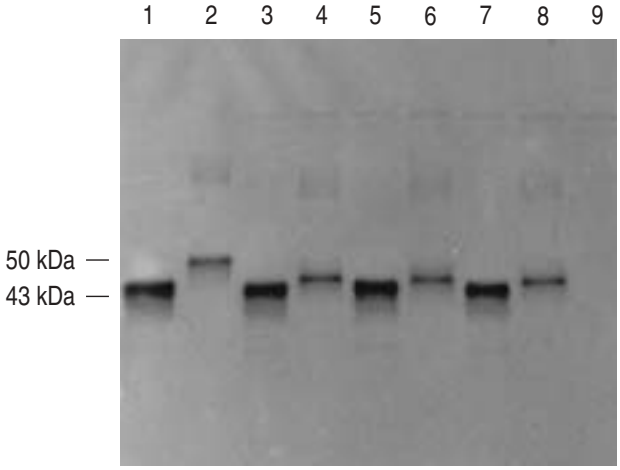

Fig. 6. – Digestion of surfactant protein D (SP-D) with neuraminidase and *O*-glycanase. The 43-kDa (lanes 1, 3, 5 and 7) and 50-kDa (lanes 2, 4, 6 and 8) forms of SP-D were isolated by preparative electrophoresis and then sequentially treated with neuraminidase and *O*-glycanase. Lanes 1 and 2: SP-D in the neuraminidase buffer; lane 3 and 4: after incubation of SP-D with neuraminidase for 18 h; lanes 5 and 6: after digestion with neuraminidase for 3 h; lanes 7 and 8: after digestion with neuraminidase (3 h) and *O*-glycanase (15 h); lane 9: neuraminidase alone. The protein bands were detected by Western blotting.

methods for isolating SP-D use gel filtration as part of the purification scheme [7]. SP-D from a patient with alveolar proteinosis was partially purified by maltose affinity chromatography and then fractionated by gel filtration through 4% agarose using the conditions reported by CROUCH *et al*. [26]. The elution profile based on the ultraviolet absorbance of the fractions is shown in figure 9a and the results of Western blot analysis of SP-D in different fractions in figure 9b. By comparing these results with those of CROUCH *et al*. [26], the peak at fraction 30–35 should contain the dodecameric form of SP-D, whereas the fractions in the region of fraction 55 should contain only the trimeric form. It can be seen in figure 9a that the gel filtration fractions containing the dodecameric form of SP-D contain the 43-kDa form of SP-D (and its apparent dimers and trimers). Fractions in the lower molecular weight range, however, contain both the 43-kDa and 50-kDa forms of SP-D (and their apparent dimers and trimers). Thus, it appears that the 50-kDa SP-D does not assemble into higher molecular weight multimers as has been shown for the 43-kDa SP-D [26].

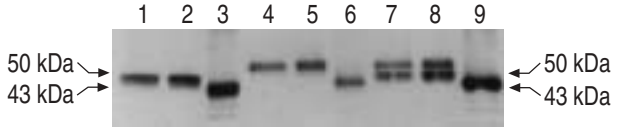

Fig. 7. – Chemical deglycosylation of surfactant protein D (SP-D). Samples containing approximately 50 ng protein after partial purification by preparative electrophoresis were deglycosylated or not. They were then subjected to standard polyacrylamide gel electrophoresis under reducing and denaturing conditions, transferred to nitrocellulose and stained for SP-D by Western analysis with the B62 monoclonal antibody. Lane 1: low molecular weight SP-D (fraction adjacent to peak 43-kDa fraction); lane 2: same as lane 1 after dialysis and freeze-drying; lane 3: same as lane 1 after chemical deglycosylation; lane 4: high molecular weight SP-D (fraction adjacent to peak 50-kDa fraction); lane 5: same as lane 4 after dialysis and freeze-drying; lane 6: same as lane 4 after chemical deglycosylation; lane 7: mixture of samples used in lanes 1 and 4; lane 8: mixture of samples used in lanes 2 and 5; lane 9: mixture of samples used in lanes 3 and 6.

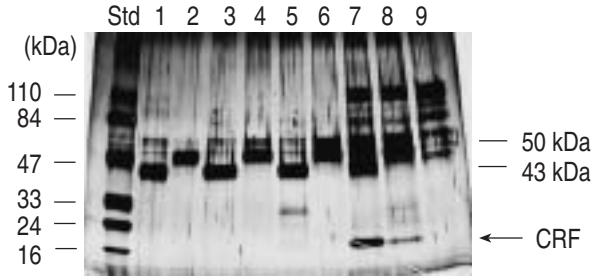

Fig. 8. – Collagenase digestion of the 43-kDa and 50-kDa forms of surfactant protein D (SP-D). The 43-kDa (lanes 1, 3, 5 and 7) and 50-kDa (lanes 2, 4, 6 and 8) forms of SP-D were isolated by preparative electrophoresis. Lanes 1 and 2: purified SP-D; lanes 3 and 4: SP-D in buffer for collagen digestion; lanes 5 and 6: SP-D incubated in buffer for collagen digestion for 18 h; lanes 7 and 8: SP-D incubated with collagenase for 18 h; lane 9: collagenase alone. The protein bands were identified by silver satin. CRF: collagenase-resistant fragment; Std: prestained protein.

Discussion

In evaluating monoclonal antibodies to human SP-D, a 50-kDa variant of SP-D was identified in addition to the well-described 43-kDa form. This report summarizes the characterization of this 50-kDa protein and the evidence to support the conclusion that it is a variant of human SP-D. In comparison to the 43-kDa form of SP-D, this 50-kDa protein had an identical amino-terminal amino acid sequence and amino acid composition and a carboxy-terminal collagenase-resistant fragment of identical size. The major difference in the two forms was in glycosylation, as evidenced by the intensity of the staining for carbohydrate, enzymatic digestions with *N*-glycanase and neuraminidase, chemical deglycosylation and the reduced signal in the 50-kDa form for threonines 5, 10 and 11, noted during amino-terminal sequencing, and presumably due to *O*-linked glycosylation. The reason that this form of human SP-D has not been described previously is due to the present method of isolation. Gel-filtration chromatography, a commonly used purification step in the isolation of SP-D [22, 23], was not used in our preparations. The 50-kDa form apparently does not form higher ordered oligomers and as such has a much smaller molecular mass than the usual multimeric structure composed of 43-kDa subunits. Hence, the 50-kDa form is separated from the multimeric form of SP-D composed of 43-kDa subunits by gel filtration.

It was concluded that the increase in the mass of the 50-kDa form is due to *O*-linked glycosylation. *N*-glycanase treatment shifted the molecular weight of the two forms approximately equally and resulted in two different-sized proteins. However, chemical deglycosylation of both the *N*- and *O*-linked sugars resulted in an apparent single-size protein close to the predicted size based on the primary structure deduced from cDNA. To the authors' knowledge, this is the first report of this post-translational modification of SP-D.

The function of the 50-kDa variant is not known. Because it was found in normal individuals and at a similar frequency to patients with idiopathic pulmonary fibrosis in this small sample, we do not believe it to be clinically detrimental. However, this hypothesis has not been tested directly. All patients had the 43-kDa form and some had both forms, but none had only the 50-kDa form. However, because the 50-kDa form of SP-D does not appear to form higher ordered multimers from the trimers, it is probably less effective as a multivalent lectin, and, therefore, less effective as a host defence molecule. This prediction of impaired function is based on functional studies reported with different forms of recombinant SP-D. In an analysis of different tertiary configurations of recombinant human SP-D, HARTSHORN and coworkers [35, 36] indicated that the large multimers (astral bodies) were more effective than the smaller cruciate form of SP-D in terms of aggregating influenza virus and preventing haemagglutination and much more effective than single arms or truncated SP-D (amino acids 197–349). The monomers and trimers failed to agglutinate influenza virus, enhance binding of the virus to neutrophils or prevent the deactivation of neutrophils by virus. Recombinant SP-D with a tandem substitution, $Cys^{15} \rightarrow Ser^{15}$, $Cys^{20} \rightarrow Ser^{20}$ produces a molecule which forms trimers but not dodecamers and has thermal lability and protease susceptibility. This recombinant protein binds to influenza A but does not aggregate the virus, enhance binding of the virus to neutrophils, augment the neutrophil respiratory burst or protect neutrophils from deactivation [37]. This tandem substitution may be functionally similar to the naturally occurring 50-kDa variant of human SP-D. The 50-kDa human variant cannot currently be isolated free of the 43-kDa form in the native state. The present method of isolation requires denaturation and reduction. Although the 50-kDa form was isolated by affinity chromatography on maltose sepharose and probably binds maltose, this is not absolutely certain because the lower molecular weight protein also contains the 43-kDa form, which could account for binding of the oligomer. Thus, the function of this 50-kDa form cannot be easily studied *in vitro* until a new method is devised for isolation which does not require denaturation.

The cellular source of the 50-kDa protein is not known. Since the antibodies identify both the 50-kDa and the 43-kDa forms, any differences between alveolar type II cells and nonciliated bronchiolar cells cannot be evaluated. One possibility might be that glycosylation in type II cells and nonciliated bronchiolar cells might be different. SP-D may be processed slightly differently in bronchiolar cells than in type II cells. In nonciliated bronchiolar cells, SP-D is found in the dense secretory granules, which implies packaging and post-Golgi sorting mechanisms. However, in type II cells, SP-D is not found in lamellar bodies and may be processed differently.

The fact that the 50-kDa form was found in approximately half of the subjects, irrespective of source or disease suggests a genetic variant. This was not pursued further in terms of other genetic markers, and would have to be determined in future studies. In addition, in the subjects where the 50-kDa form was not detected, this form may have been either absent or simply below the limit of detection.

In summary, this study identified and characterized a 50-kDa variant of surfactant protein D that was found in the process of evaluating the specificity of monoclonal antibodies to surfactant protein D. The function and clinical significance of this variant remain to be determined. Investigators studying human surfactant protein D may want to assay for this variant in their preparations, with the caveat that it will be discarded if the preparation of

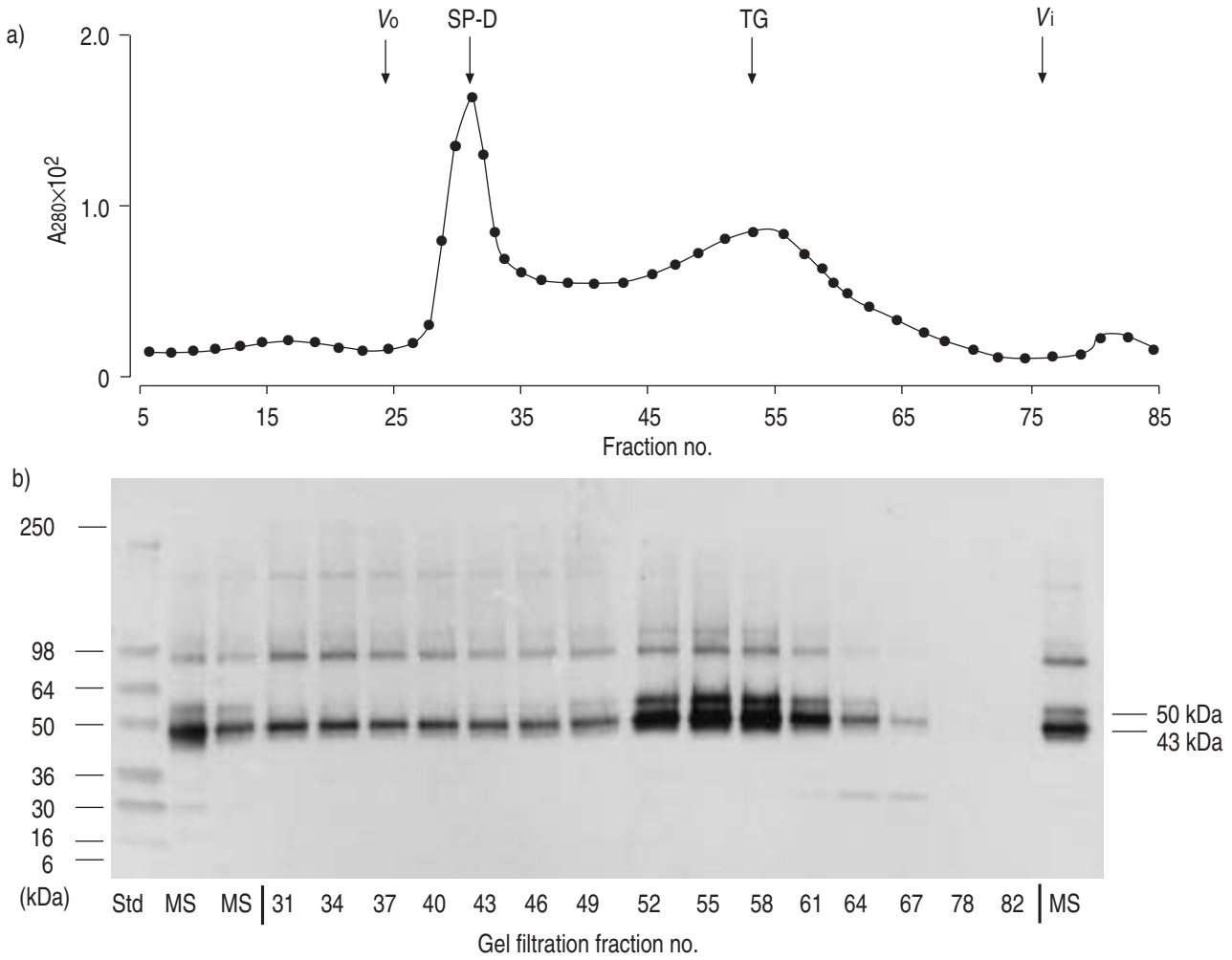

Fig. 9. – Purification of surfactant protein D (SP-D) by gel filtration. SP-D was partially purified by maltose sepharose (MS) chromatography from the supernatant of alveolar proteinosis lavage fluid, which was then further purified by gel filtration on 4% agarose. a) Absorbance of the different fractions at 280 nm (A_{280}). The void volume (V_o), the major peak for native SP-D (dodecameric form), thyroglobulin (TG), with a molecular mass of 670 kDa, and the inclusion volume (V_i) are shown. b) Western analysis of the fractions from (a). The 50-kDa form of SP-D was only found in the lower molecular weight fractions and was absent from the higher molecular weight fractions.

surfactant protein D is purified by gel filtration. The 50-kDa form has not been detected in numerous preparations of surfactant protein D from the rat, which is the only other species with which the authors have had extensive experience.

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