

# Alternate Amino Terminal Processing of Surfactant Protein A Results in Cysteiny Isoforms Required for Multimer Formation<sup>†</sup>

Baher M. Elhalwagi, Mamatha Damodarasamy, and Francis X. McCormack\*

Division of Pulmonary/Critical Care Medicine, Department of Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

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**ABSTRACT:** The biological functions of rat surfactant protein A (SP-A), an oligomer composed of 18 polypeptide subunits derived from a single gene, are dependent on intact disulfide bonds. Reducible and collagenase-reversible covalent linkages of as many as six or more subunits in the molecule indicate the presence of at least two NH<sub>2</sub>-terminal interchain disulfide bonds. However, the reported primary structure of rat SP-A predicts that only Cys<sup>6</sup> in this region is available for interchain disulfide formation. Direct evidence for a second disulfide bridge was obtained by analyses of a set of three mutant SP-As with telescoping deletions from the reported NH<sub>2</sub>-terminus. Two of the truncated recombinant proteins formed reducible dimers despite deletion of the domain containing Cys<sup>6</sup>. Edman degradation revealed that each mutant protein was a mixture of two isoforms with and without an isoleucine-lysine-cysteine (IKC) extension at the NH<sub>2</sub>-terminus, which was derived from the COOH-terminal end of the reported signal peptide. Large variations in the abundance of the IKC isoforms between truncated SP-As suggested that the amino acid sequences located downstream from the signal peptide modulated alternate-site cleavage by signal peptidase. Elution of the newly identified cysteine in the position of DiPTH-Cys indicated participation in disulfide linkage, which was interchain based on the direct correlation between prevalence of the IKC variant and the extent of dimerization for each truncated protein. Sequencing of both native rat SP-A and human SP-A also revealed isoforms with disulfide-forming NH<sub>2</sub>-terminal extensions. The extended rat SP-A isoforms were enriched in the more fully glycosylated and multimeric SP-A species separated on SDS–PAGE gels. Thus, a novel post translational modification results in naturally occurring cysteiny isoforms of rat SP-A, which are essential for multimer formation.

Pulmonary surfactant is a complex mixture of phospholipids and proteins which lines the gas exchanging surface of the lung and prevents collapse of alveolar sacs during expiration (Pattle, 1955). Three proteins which are specific to the lung and intimately associated with the surfactant phospholipids have been described: surfactant proteins (SP-) A (King et al., 1974), B (Glasser et al., 1987; Hawgood et al., 1987; Jacobs et al., 1987), and C (Warr et al., 1987). SP-A<sup>1</sup> is a hydrophilic glycoprotein which is synthesized and secreted by alveolar type II cells and Clara cells of the pulmonary epithelium. There is *in vitro* and *in vivo* evidence that SP-A is important for the formation of surfactant aggregates such as tubular myelin (Korfhagen et al., 1996; Suzuki et al., 1989) and that it assists in the maintenance of the surface activity of surfactant in the presence of serum protein inhibitors (Bruni et al., 1996; Cockshutt et al., 1990). SP-A has also been shown to modulate the uptake and secretion of surfactant from isolated alveolar type II cells by a receptor-mediated mechanism (Dobbs et al., 1987; Rice et al., 1987; Wright et al., 1987), but the physiologic

relevance of these *in vitro* observations is challenged by the lack of an obvious homeostatic defect in the recently developed SP-A-knockout mouse (Korfhagen et al., 1996). Increasing attention has focused on the activities of SP-A to bind to a variety of microorganisms via carbohydrate recognition and to activate macrophages, suggesting a possible host defense function as an antibody-independent opsonin (Gaynor et al., 1994; Pikaar et al., 1995; Tino & Wright, 1996; Williams et al., 1996).

SP-A is a member the C-type lectin superfamily subgroup called the collectins, named for the shared structural features of a collagen-like region and a carbohydrate recognition domain (CRD) (Sastry & Ezekowitz, 1993). There is only one gene for SP-A in the rat and the deduced primary structure of the protein reveals (Sano et al., 1987; White et al., 1985) (1) a short NH<sub>2</sub>-terminal segment; (2) a collagen-like region of 24 proline and hydroxyproline-rich gly-x-y repeats, with a single interruption after the twelfth repeat at Gly<sup>44</sup>; (3) a hydrophobic “neck” region; and (4) a COOH-terminal CRD which bears sequence homology to mannose binding protein A (Drickamer et al., 1986). Intracellular processing of SP-A results in cleavage of the signal peptide (Phelps et al., 1986), glycosylation of asparagines at positions 1 and 187 (McCormack et al., 1994a), and hydroxylation of prolines within the collagen-like region (Weaver & Whitsett, 1991). Subunits of SP-A form trimers by the helical folding of their collagen-like regions. Although the tertiary structure of rat SP-A has not been fully characterized, rotary shadowing of human and dog SP-As predict that the fully assembled

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\* To whom correspondence should be addressed. Tel: 513-558-0480. Fax: 513-558-0835. E-mail: frank.mccormack@uc.edu.

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<sup>1</sup> Abbreviations: SP-A, surfactant protein A; CRD, carbohydrate recognition domain; IKC, isoleucine-lysine-cysteine; NH<sub>2</sub>, amino; COOH, carboxy; PVDF, polyvinylidene difluoride; ELISA, enzyme-linked immunosorbent assay; PTH, phenylthiohydantoin, PAGE, polyacrylamide gel electrophoresis.

molecule is a hexagonal array of trimers which are laterally associated at their NH<sub>2</sub>-terminal ends, resembling a bouquet of tulips (Voss et al., 1988).

The biological activities of rat SP-A are critically dependent on intact disulfide bonds (Kuroki et al., 1988b). The available data do not identify whether the essential disulfides are intrachain, interchain, or both, in part because the organized disulfide-bridged structure of the protein is not fully understood. There are five cysteine residues in the reported sequence for mature rat SP-A, at position +6 near the NH<sub>2</sub>-terminus and positions +135, +204, +218, and +226 of the CRD (Sano et al., 1987). Comparison with the solved disulfide structures of dog (Benson et al., 1985) and human SP-A (Haagsman et al., 1989) predicts that Cys<sup>6</sup> of rat SP-A forms an intermolecular disulfide and Cys<sup>135</sup>/Cys<sup>226</sup> and Cys<sup>204</sup>/Cys<sup>218</sup> form a pair of intrachain bonds. Because SP-A forms disulfide-dependent multimers larger than dimers, at least two cysteines must form interchain cross-links. Furthermore, all intermolecular bonds are restricted to the NH<sub>2</sub>-terminal domains of the protein, since cleavage of the collagen-like domain with collagenase dissociates multimers (Murata et al., 1993). These observations have been difficult to reconcile with the reported primary structure of rat SP-A since only one cysteine, Cys<sup>6</sup>, is present at the NH<sub>2</sub>-terminal end of the reported mature protein (Sano et al., 1987).

We produced a set of mutant SP-As with telescoping deletions from the end of the signal peptide through the collagen-like region to evaluate the role of the NH<sub>2</sub>-terminal domains in oligomeric assembly and function. In the process of characterizing the truncated proteins, we encountered isoforms of SP-A with tripeptide extensions at the NH<sub>2</sub>-terminus containing an additional cysteine, formerly attributed to the signal peptide. We report that these novel isoforms occur naturally in rat SP-A, probably via alternate site cleavage by signal peptidase, and are required for the complete disulfide-dependent oligomeric assembly of the protein.

## EXPERIMENTAL PROCEDURES

**DNA Constructs.** The 1.6 kB cDNA for rat SP-A was isolated (Sano et al., 1987) and ligated into the *Eco*R1 site of the PVL 1392 recombination vector (Luckow & Summers, 1988) as previously reported (McCormack et al., 1994a). We used mutagenic oligonucleotides and the polymerase chain reaction to generate telescoping deletions in the cDNA for SP-A by overlap extension (Horton et al., 1989). For the first mutant, nucleotides encoding Asn<sup>1</sup>–Ala<sup>7</sup> were deleted and the signal sequence was directly juxtaposed to downstream sequences encoding Gly<sup>8</sup>–Phe<sup>228</sup>. Additional mutant cDNAs were generated in a similar fashion, by deletion of the nucleotides encoding Asn<sup>1</sup>–Gly<sup>44</sup> and Asn<sup>1</sup>–Pro<sup>80</sup> and ligation of the native signal sequence to downstream nucleotide sequences encoding Gly<sup>45</sup>–Phe<sup>228</sup> and Ala<sup>81</sup>–Phe<sup>228</sup>, respectively. The mutant cDNAs were ligated into the *Eco*R1 site of PVL 1392, and orientation was confirmed with *Kpn*I. The coding region for all mutant cDNAs was sequenced by the dideoxy method of Sanger (1977) to confirm the intended deletions and exclude spurious mutations.

**Expression of the cDNAs for SP-A in Insect Cells.** The production of recombinant SP-A in the baculovirus system

was performed as previously described (McCormack et al., 1994a,b). Briefly, recombinant baculoviruses containing mutant cDNAs for SP-A were produced by homologous recombination in *Spodoptera frugiperda* (Sf-9) cells following cotransfection with linear viral DNA and the PVL 1392/SP-A constructs (Baculogold, Pharmingen). Fresh monolayers of 10<sup>7</sup> *Trichoplusia ni* (*T. ni*) cells were infected with plaque purified recombinant viruses and incubated with serum-free media (IPL-41) supplemented with 0.4 mM ascorbic acid and antibiotics for 72 h. Recombinant SP-A was purified from the culture media by adsorption to mannose-Sepharose 6B columns in the presence of 1 mM calcium and elution with 2 mM EDTA (Fornstedt & Porath, 1975). The purified recombinant SP-A was dialyzed against 5 mM Tris (pH 7.4) and stored at –20 °C.

**Purification of Native SP-A.** Surfactant was isolated by bronchoalveolar lavage of silica-pretreated Sprague Dawley rats (Dethloff et al., 1986), floated on NaBr gradients and sedimented by centrifugation, as described (Kuroki et al., 1988b). SP-A was isolated and purified from the surfactant pellet by delipidation, mannose-Sepharose affinity chromatography, and gel permeation chromatography with Biogel A-15m (Hawgood et al., 1985). The purification method for normal rats was the same except that the NaBr gradient purification and gel permeation steps were omitted.

**Protein Assays.** The SP-A content of tissue culture media containing recombinant SP-A was determined with a rabbit polyclonal IgG against rat SP-A using a sandwich ELISA (McCormack et al., 1990). The lower limit of sensitivity of the assay was 0.20 ng/mL, and the linear range extended from 0.16 to 10.0 ng/mL. Routine protein concentrations were determined with the bicinchoninic protein assay kit (BCA) (Pierce) using bovine serum albumin as a standard.

**Analysis of Recombinant SP-A.** Protein samples were separated by 8–16% SDS–PAGE and either stained with Coomassie blue or transferred to polyvinylidene difluoride (PVDF) membranes for sequencing. Protein species were quantitated by laser densitometry.

**Protein Microsequencing.** The NH<sub>2</sub>-terminal amino acid sequence of native and recombinant SP-As was determined using a modification (Hewick et al., 1981) of the technique of Edman degradation (Edman, 1956) on an automated gas phase microsequencer (Applied Biosystems 470A). The elution of phenylthiohydantoin (PTH) derivatized amino acids was monitored with a Model 120A PTH analyzer. Proteins were submitted for analysis in 5 mM Tris (10 µg/mL) or bound to PVDF membranes after transfer from SDS–PAGE gels. The lower limit of detection for this method is about 1 pmol of protein. The relative abundance of the SP-A isoforms was estimated by comparison of the yield of representative amino acids from each sequence. Residues in the second or third cycle were usually selected for quantitation, to avoid the higher background in the first cycle and the declining yields due to technical limitations in subsequent cycles.

## RESULTS

**Synthesis and Characterization of Mutant Recombinant Proteins.** Recombinant SP-A proteins were synthesized in insect cells using baculovirus vectors. The wild-type recombinant SP-A produced in this expression system has levels of activity which are comparable to rat SP-A in *in vitro* assays of protein function, despite incomplete hydroxyl-

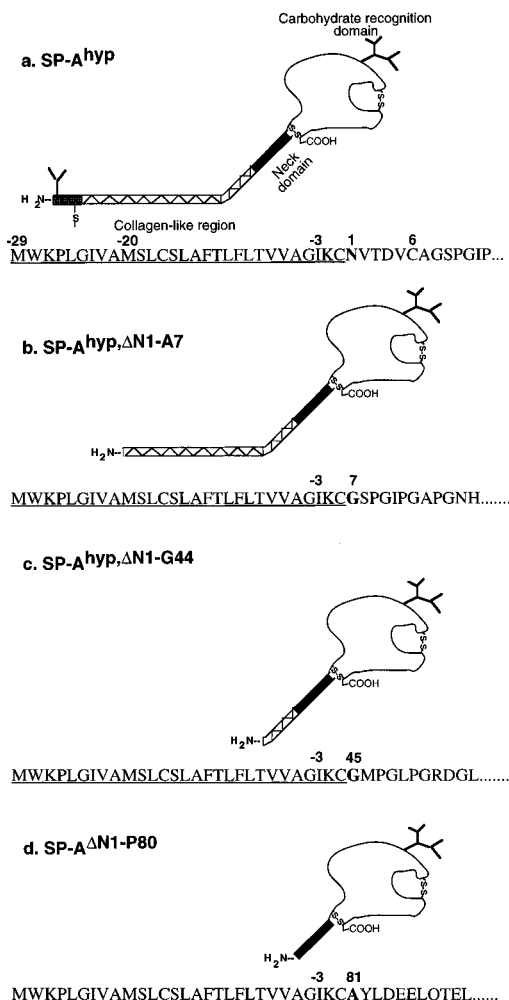


FIGURE 1: Structure of truncated mutant recombinant forms of SP-A. Mutant cDNAs for SP-A were generated by overlap extension PCR. Telescoping deletions from the NH<sub>2</sub>-terminus of SP-A (Asn<sup>1</sup>) were created by juxtaposition of nucleotide sequences encoding the signal peptide (underlined) to downstream sequences encoding NH<sub>2</sub>-termini of Gly<sup>8</sup>, Gly<sup>45</sup>, and Ala<sup>81</sup> (bold). The schematic structures and NH<sub>2</sub>-terminal boundary amino acid sequences of wild-type recombinant SP-A<sup>hyp</sup> (A), SP-A<sup>hyp,ΔN1-A7</sup> (B), SP-A<sup>hyp,ΔN1-G44</sup> (C), and SP-A<sup>hyp,ΔN1-P80</sup> (D) truncated mutant proteins are shown.

ation of prolines in the collagen-like region (denoted SP-A<sup>hyp</sup>) (McCormack et al., 1994a,b). In this study, we used oligonucleotide-based mutagenesis of the 1.6 kb cDNA for SP-A to generate three truncated proteins with telescoping deletions from the reported NH<sub>2</sub>-terminus at Asn<sup>1</sup>. The native signal sequence was retained in each mutant cDNA construct, to provide for secretion into the culture media, and was ligated to downstream sequences encoding the beginning (Gly<sup>8</sup>), middle (Gly<sup>45</sup>), and end (Ala<sup>81</sup>) of the collagen-like region. Nucleotide sequencing of the full coding region for each mutant cDNA confirmed the desired deletions and the absence of spurious mutations. The boundary amino acids at the re-engineered NH<sub>2</sub>-termini and a schematic representation of each protein is shown in Figure 1. The mutant proteins were denoted by (1) SP-A<sup>hyp,ΔN1-A7</sup> lacking the NH<sub>2</sub>-terminal segment, (2) SP-A<sup>hyp,ΔN1-G44</sup> lacking the NH<sub>2</sub>-terminal segment and the proximal portion of the collagen-like domain, and (3) SP-A<sup>hyp,ΔN1-P80</sup> lacking the NH<sub>2</sub>-terminal segment and the entire collagen-like domain. All proteins were purified from the culture media by Ca<sup>2+</sup>-dependent adsorption to mannose-Sepharose affinity col-

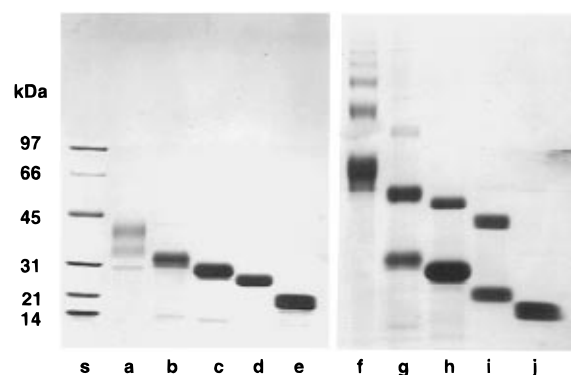


FIGURE 2: Electrophoretic analysis of recombinant forms of SP-A. Proteins were size-fractionated on 8–16% SDS–PAGE gels under reducing (a–e) and nonreducing (f–j) conditions. Rat SP-A (a, f), SP-A<sup>hyp</sup> (b, g), SP-A<sup>hyp,ΔN1-A7</sup> (c, h), SP-A<sup>hyp,ΔN1-G44</sup> (d, i), SP-A<sup>hyp,ΔN1-P80</sup> (e, j), and standards (s) are shown.

umns. The yield ranged 1–3 mg/100 mL/10<sup>8</sup> *T. ni* cells in culture. The results clearly indicate that the NH<sub>2</sub>-terminal domains of SP-A are not required for secretion from eucaryotic cells or for the binding to carbohydrate. Figure 2 shows the SDS–PAGE analysis of native SP-A, SP-A<sup>hyp</sup>, and the truncated SP-As. Under reducing and denaturing conditions, rat SP-A appeared as a triplet at 26, 32, and 38 kDa. The heterogeneity in molecular weight under these conditions is due to differential glycosylation of Asn<sup>1</sup> and Asn<sup>187</sup> (McCormack et al., 1994a; Whitsett et al., 1985b). Because the insect cells modify proteins with simple, sialic acid deficient oligosaccharide structures, the wild-type recombinant protein SP-A<sup>hyp</sup> migrates slightly more rapidly than rat SP-A as a broad band between 27–31 kDa, as described (McCormack et al., 1994a) (predicted nonglycosylated SP-A<sup>hyp</sup> = 24.2 kDa). The apparent molecular masses of the SP-A<sup>hyp,ΔN1-A7</sup>, SP-A<sup>hyp,ΔN1-G44</sup>, and SP-A<sup>hyp,ΔN1-P80</sup> were approximately 28, 24, and 21 kDa, respectively, consistent with sizes of the deleted peptide sequences and the loss of the oligosaccharide moiety attached to Asn<sup>1</sup>. Under nonreducing and denaturing conditions, rat SP-A formed disulfide-dependent multimers which migrated with apparent masses of 67 kDa, 131 kDa, and less abundant higher order species, as described (Kuroki et al., 1988b). The SP-A<sup>hyp</sup> migrated as a 29 kDa monomer and multimers with apparent masses of 49 and 97 kDa, as recently reported (McCormack et al., 1994a). The appearance of monomers under nonreducing conditions is never seen for rat SP-A and indicates that intermolecular disulfide bridge formation in SP-A<sup>hyp</sup> is not fully supported in the invertebrate expression system. However, higher order oligomer formation of SP-A<sup>hyp</sup> is preserved, since species with masses greater than 97 kDa which are poorly detected by Coomassie staining are apparent on immunoblots (McCormack et al., 1994a). The SP-A<sup>hyp,ΔN1-A7</sup> appeared as a monomeric band at 28 kDa and an unexpected disulfide-dependent dimer at 47 kDa, despite the Asn<sup>1</sup>–Ala<sup>7</sup> deletion which included the Cys<sup>6</sup> interchain crosslink. The SP-A<sup>hyp,ΔN1-G44</sup> was also both monomeric (24 kDa) and dimeric (41 kDa), but the SP-A<sup>hyp,ΔN1-P80</sup> was exclusively monomeric. The protein species identified by immunoblot analysis were identical to those seen on SDS–PAGE, and no oligomers greater than dimers appeared for the truncated proteins (not shown). The relative abundance of the oligomeric species and the monomeric species on the

Table 1: Disulfide-Dependent Oligomeric Species of SP-A Variants on Nonreducing SDS-PAGE

	rat SP-A		SP-A <sup>hyp</sup>		SP-A <sup>hyp,ΔN1-A7</sup>		SP-A <sup>hyp,ΔN1-G44</sup>		SP-A <sup>ΔN1-P80</sup>	
	% <sup>a</sup>	kDa	%	kDa	%	kDa	%	kDa	%	kDa
monomer	0		40	29	83	28	49	24	100	21
dimer	77	68	54	49	17	47	51	41	0	
trimer <sup>b</sup>	0		6	97	0		0			
tetramer <sup>b</sup>	16	131	0							
higher <sup>c</sup>	7									

<sup>a</sup> Data is the integrated area of each band, quantified by laser densitometry and expressed as percent of total. <sup>b</sup> The precise subunit composition cannot be assigned with certainty based on electrophoretic migration alone. <sup>c</sup> The densitometric quantification of the lesser abundant higher order oligomers is probably less precise than the lower molecular weight species.

Table 2: Amino Terminal Sequence of Recombinant SP-A

		amino acid no. <sup>a</sup>												% <sup>e</sup>
		-3	-2	-1	1	2	3	4	5	6	7	8		
SP-A <sup>hyp</sup>														
sequence 1 <sup>b,c</sup>					N <sub>10</sub>	V <sub>43</sub>	T	D	V	C	A			78
sequence 2 <sup>d</sup>		I	K <sub>12</sub>	—	—	V	T	D						22
		amino acid no.												% <sup>e</sup>
		-3	-2	-1	8	9	10	11	12	13	14	15		
SP-A <sup>hyp,ΔN1-A7</sup>														
sequence 1					G	S	P <sub>35</sub>	G	I	P	G	A		55
sequence 2		I	K <sub>30</sub>	C	G	S	P	G						45
		amino acid no.												% <sup>e</sup>
		-3	-2	-1	45	46	47	48	49	50	51			
SP-A <sup>hyp,ΔN1-G44</sup>														
sequence 1					G	M <sub>16</sub>	P	G	L	P	G			15
sequence 2		I	K <sub>89</sub>	C	G	M	P	G						85
		amino acid no.												% <sup>e</sup>
		-3	-2	-1	81	82	83	84	85	86	87	88		
SP-A <sup>ΔN1-P80</sup>														
sequence 1					A	Y <sub>90</sub>	L	D	E	E	L	A		97
sequence 2		I	K <sub>3</sub>	—										3

<sup>a</sup> Numbered according to position in the reported native SP-A sequence. <sup>b</sup> Subscripted numbers represent molecular yield (picomoles) of the associated amino acid. <sup>c</sup> Italicized cysteines were disulfide-linked based on elution in the position of DiPTH-Cys. <sup>d</sup> (—) = blank cycle. <sup>e</sup> Calculated using the molecular yield (picomoles) of representative amino acids from each sequence [e.g., % sequence 1 isoform = pmol sequence 1 / (pmol sequence 1 + pmol sequence 2)].

SDS-PAGE gel were determined by laser densitometry and compared in Table 1. The dimeric species represented 17% of the SP-A<sup>hyp,ΔN1-A7</sup>, 51% of the SP-A<sup>hyp,ΔN1-G44</sup>, and 0% of the SP-A<sup>ΔN1-P80</sup>.

**Amino-Terminal Variants of Recombinant SP-A.** Experiments were performed to investigate the mechanism of the disulfide-dependent dimerization of the truncated mutant SP-As. The NH<sub>2</sub>-terminal protein sequence of the purified proteins (approx. 10 μg) was analyzed by Edman degradation using a gas phase microsequencer, and the data are shown in Table 2. The results confirmed the intended, re-engineered NH<sub>2</sub>-terminal sequences beginning with Gly<sup>8</sup> for SP-A<sup>hyp,ΔN1-A7</sup>, Gly<sup>45</sup> for SP-A<sup>hyp,ΔN1-G44</sup>, and Ala<sup>81</sup> for SP-A<sup>ΔN1-P80</sup>. The analysis also revealed another isoform for each truncated protein, with sequences which were identical to the first except that they were preceded by Ile-Lys-Cys (IKC) in the first three cycles. Examination of the coding

sequence for mature rat SP-A revealed that the IKC extension was derived from the COOH-terminal end of the leader peptide (Sano et al., 1987). For clarity, the positions of these amino acids were labeled according to their relationship to the reported NH<sub>2</sub>-terminus of the wild type protein (Asn<sup>1</sup>), as Ile<sup>-3</sup>, Lys<sup>-2</sup>, and Cys<sup>-1</sup>. The IKC variants represented 45, 85, and <5% of the mutant proteins SP-A<sup>hyp,ΔN1-A7</sup>, SP-A<sup>hyp,ΔN1-G44</sup>, and SP-A<sup>ΔN1-P80</sup>, respectively, based on comparisons of the molar yield of representative PTH-derivatized amino acids from the paired isoforms. Thus, variations in the context of amino acid sequences downstream from the leader peptide resulted in markedly different levels of the IKC isoforms between the truncated proteins. The incorporation of the IKC extension into the sequence of the secreted SP-As was not an artifact of the mutations introduced, however, since the IKC variant comprised 22% of the wild-type recombinant protein, SP-A<sup>hyp</sup>. Collectively, these data provide evidence the cDNA for rat SP-A encodes two isoforms of the protein, which result from alternate NH<sub>2</sub>-terminal processing. The marked effect that downstream NH<sub>2</sub>-terminal sequence variations have on the abundance of the IKC isoforms suggests alternate cleavage points for a specific protease, probably signal peptidase.

PTH-derivatized cysteine is unstable and often produces blank cycles if not protected by disulfide-linkage, deliberate biochemical modification, or artifactual acylation during polyacrylamide gel electrophoresis. The PTH-derivatized Cys<sup>-1</sup> of SP-A<sup>hyp,ΔN1-A7</sup> and SP-A<sup>hyp,ΔN1-G44</sup> eluted from the microsequencing column in a position consistent with DiPTH-Cys, in front of the PTH-Tyr peak, indicating participation in disulfide-bond formation (Haniu et al., 1994). In both cases, it is apparent that the Cys<sup>-1</sup> formed an interchain rather than an intrachain disulfide, since the densitometric abundance of disulfide-dependent dimers on nonreducing SDS-PAGE analysis (Table 1) correlated with the percentage of the IKC isoform in each sample (Table 2). Specifically, the SP-A<sup>hyp,ΔN1-G44</sup> was 85% IKC isoform and more than 50% dimeric, SP-A<sup>hyp,ΔN1-A7</sup> was 45% IKC isoform and 17% dimeric, and the SP-A<sup>ΔN1-P80</sup> had less than 5% IKC isoform and did not form dimers. The observation that deletion of Cys<sup>6</sup> in SP-A<sup>hyp,ΔN1-A7</sup> and SP-A<sup>hyp,ΔN1-G44</sup> limits thiol-dependent assembly to dimerization demonstrates that there is only one other interchain disulfide-forming cysteine in the molecule. The additional interchain linkage was mapped to Cys<sup>-1</sup> by analysis of SP-A<sup>ΔN1-P80</sup>, which revealed that the combined deficiency of Cys<sup>-1</sup> and Cys<sup>6</sup> blocks dimerization.

**Amino-Terminal Variants of rat SP-A.** We next examined the NH<sub>2</sub>-terminal protein sequence of SP-A isolated from the lungs of rats to determine if the cysteinyl isoforms are physiologically relevant (Table 3). SP-A was isolated and purified from the alveolar lavage of normal rats and rats which were pretreated with intratracheal silica (Dethloff et al., 1986). Determination of the structure of silica-derived SP-A is important because silica instillation is commonly used to enhance the yield of SP-A from rats and most studies of rat SP-A function use this reagent (Kuroki et al., 1988b). The NH<sub>2</sub>-terminal protein sequences of rat and silica-derived SP-A each revealed the reported primary peptide sequence (beginning with Asn<sup>1</sup>), and the IKC-containing sequence representing 16% of the normal rat SP-A and 22% of the silica-treated rat SP-A, respectively. The results clearly indicate that the IKC isoforms of SP-A occur naturally in

Table 3: Amino terminal sequence of rat SP-A

		amino acid no <sup>a</sup>										% <sup>e</sup>
		-3	-2	-1	1	2	3	4	5	6	7	
N1 rat SP-A												
sequence 1 <sup>b,c</sup>					N <sub>4</sub>	V <sub>16</sub>	T	D	V	C	A	84
sequence 2 <sup>d</sup>		I	K <sub>3</sub>	C	—	V	T	D				16
silica rat SP-A												
sequence 1					N <sub>4</sub>	V <sub>15</sub>	T	D	V	C	A	79
sequence 2		I	K <sub>4</sub>	—	—	V	T	D				21

<sup>a</sup> Numbered according to position in the reported native SP-A sequence. <sup>b</sup> Subscripted numbers represent molecular yield (picomoles) of the associated amino acid. <sup>c</sup> Italicized cysteines were disulfide-linked based on elution in the position of DiPTH-Cys. <sup>d</sup> (—) = blank cycle. <sup>e</sup> Calculated using the molecular yield (picomoles) of representative amino acids from each sequence [e.g., % sequence 1 isoform = pmol sequence 1 / (pmol sequence 1 + pmol sequence 2)].

Table 4: Amino Terminal Sequence of Rat SP-A Separated on SDS-PAGE Gels

		amino acid no. <sup>a</sup>										% <sup>e</sup>
		-3	-2	-1	1	2	3	4	5	6	7	
26 kDa-reducing sequence 1 <sup>b</sup>					N <sub>2</sub>	V <sub>6</sub>	T	D	V	C	A	100
32 kDa-reducing sequence 1					N <sub>9</sub>	V <sub>15</sub>	T	D	V	C	A	83
sequence 2 <sup>d,c</sup>		I	K <sub>3</sub>	C	—	V	T	D				17
38 kDa-reducing sequence 1					N <sub>2</sub>	V <sub>29</sub>	T	D	V	C	A	76
sequence 2		I	K <sub>9</sub>	C	—	V	T	D				24
68 kDa-nonreducing sequence 1					N <sub>7</sub>	V <sub>19</sub>	T	D	V	C	A	83
sequence 2		I	K <sub>4</sub>	C	—							17
131 kDa-nonreducing sequence 1					N <sub>3</sub>	V <sub>1</sub>	T	D	V	—	A	50
sequence 2		I	K <sub>1</sub>	—								50

<sup>a</sup> Numbered according to position in the reported native SP-A sequence. <sup>b</sup> Subscripted numbers represent molecular yield (picomoles) of the associated amino acid. <sup>c</sup> Italicized cysteines were disulfide-linked based on elution in the position of DiPTH-Cys. <sup>d</sup> (—) = blank cycle. <sup>e</sup> Calculated using the molecular yield (picomoles) of representative amino acids from each sequence [e.g., % sequence 1 isoform = pmol sequence 1 / (pmol sequence 1 + pmol sequence 2)].

rats and that the abundance of the isoforms is not markedly altered by silica pretreatment. Further, the similar abundance of the IKC variants from the invertebrate expression system and from whole animals demonstrate that tissue-specific processing is not required for expression of the variant.

Rat (and mouse) SP-A has the unusual feature of a carbohydrate attachment site at the NH<sub>2</sub>-terminus of the protein. Analyses were performed to determine if there is a relationship between the occupancy of the NH<sub>2</sub>-terminal glycosylation site and the prevalence of the IKC extension. Rat SP-A migrates heterogeneously on reducing SDS-PAGE gels, appearing as bands at 26, 32, and 38 kDa, due to differential N-linked glycosylation of the protein at both Asn<sup>1</sup> and Asn<sup>187</sup> (McCormack et al., 1994a; Whitsett et al., 1985a). The NH<sub>2</sub>-terminal sequence of the three molecular species was determined after transfer to PVDF membranes and the results are shown in Table 4. Reduction in the yield of Asn<sup>1</sup> relative to Val<sup>2</sup> during microsequencing provides indirect evidence that the site is modified by carbohydrate. The yield of Asn<sup>1</sup> and Val<sup>2</sup> was identical in SP-A synthesized in the presence of the inhibitor of N-linked glycosylation, tunicamycin (McCormack, F. X., unpublished observation). In the non-IKC sequences, the yield of Asn<sup>1</sup> suggested an oligosac-

charide occupancy rate of approximately 25% for the 26 kDa species, 50% for the 32 kDa species, and 95% for the 38 kDa species. These data, while qualitative, are consistent with the expected ascending gradient in the extent of glycosylation at the NH<sub>2</sub>-terminus from the smaller to the larger mass species separated by SDS-PAGE. The distribution of the IKC isoform demonstrated enrichment in the more fully glycosylated species, representing 0% of the 26 kDa band (*n* = 2), 17% of the 32 kDa partially glycosylated species, and 24% of the more fully glycosylated 38 kDa species. Comparison of the IKC and non-IKC isoforms revealed that the molecular yield of PTH-derivatized asparagine was zero in the cycle corresponding to the Asn<sup>1</sup> position of all IKC variant sequences and always greater than zero in non-IKC isoforms, suggesting quantitative glycosylation of the site in the extended isoform. The concordance between the extent of glycosylation and the abundance of the IKC isoform, and the indirect sequence evidence for the quantitative glycosylation of the Asn<sup>1</sup> site in the IKC variants suggest that glycosylation may favor the expression of the extended isoform by protecting the Cys<sup>-1</sup>–Asn<sup>1</sup> peptide bond from hydrolysis by signal peptidase. Alternatively, “pre-cleaved” transcripts which contain the IKC extension may be preferred substrates for glycosylation.

To determine if the IKC isoform of rat SP-A participates in disulfide-dependent oligomeric assembly, we performed NH<sub>2</sub>-terminal sequencing of the 68 and 131 kDa multimeric species cut from PVDF membranes after transfer from nonreducing SDS-PAGE gels (Table 4). We found that the IKC isoform represented 17% of the sequence of the 68 kDa band and 50% of the 131 kDa band. The Cys<sup>-1</sup> and Cys<sup>6</sup> of the 68 kDa band eluted in the position of DiPTH-Cys, consistent with disulfide bond formation (Haniu et al., 1994). The yield of the cysteines in the 131 kDa band was too low to determine if they were disulfide linked. The enrichment of the IKC variants in the more extensive disulfide-bridged 131 kDa band suggests that the IKC isoform participates in oligomeric assembly and identifies the second cysteine of rat SP-A which is essential for the formation of disulfide-linked multimers larger than dimers.

**Amino-Terminal Variants of Human SP-A.** The nucleotide sequence of the cDNA for human SP-A predicts a cysteine residue adjacent to the reported NH<sub>2</sub>-terminus (White et al., 1985). To determine if cysteinyl NH<sub>2</sub>-terminal variant forms of SP-A occur naturally in humans, we performed NH<sub>2</sub>-terminal sequencing of SP-A isolated from the lavage of a healthy volunteer (Table 5). Like the rat and recombinant SP-A, human SP-A was composed of isoforms which contained amino acid extensions derived from the reported signal peptide. Polypeptide subunits with the reported NH<sub>2</sub>-terminus (EVK...) represented 34% percent of the protein, while isoforms with an additional Cys<sup>-1</sup> (C-isoform) and Val<sup>-2</sup>–Cys<sup>-1</sup> (VC isoform) comprised 45% and 21% of the sequence, respectively. The sequence of amino acids from positions +1 to +7 in all three isoforms was identical to the reported NH<sub>2</sub>-terminal sequence for human SP-A. The Cys<sup>-1</sup> and Cys<sup>6</sup> both eluted in the position of diPTH-Cys, indicating participation in disulfide bonds. We also examined the isoform structure of SP-A isolated from patients with Alveolar Proteinosis (AP), a disease characterized by accumulation of surfactant lipids and proteins in the alveolar space, including an abnormal nonreducible dimeric

Table 5: Amino terminal sequence of human SP-A

		amino acid no. <sup>a</sup>										% <sup>e</sup>
		-3	-2	-1	1	2	3	4	5	6	7	
Human SP-A												
soluble												
sequence 1 <sup>b,c</sup>					E	V <sub>10</sub>	K	D	V	C		34
sequence 2				C	E <sub>13</sub>	V	K	D	V	C		45
sequence 3			V	C	E <sub>6</sub>	V	K	D	V	C		21
AP-SP-A												
soluble												
sequence 1 <sup>b,c</sup>					E	V <sub>4</sub>	K	D	V	C		31
sequence 2				C	E <sub>7</sub>	V	K	D	V	C		54
sequence 3			V <sub>2</sub>	C	E	V	K	D	V	C		15
35 kDa band from reducing SDS-PAGE												
sequence 1					E	V <sub>11</sub>	K	D	V	C		35
sequence 2				C	E	V <sub>12</sub>	K	D	V	C		39
sequence 3			V <sub>8</sub>	C	E	V	K	D	V	C		26
68 kDa band from reducing SDS-PAGE												
sequence 1 <sup>d,f</sup>				—	E <sub>0.3</sub>	V	K	D	V	C		100

<sup>a</sup> Numbered according to position in the reported native SP-A sequence. <sup>b</sup> Subscripted numbers represent molecular yield (picomoles) of the associated amino acid. <sup>c</sup> Italicized cysteines were disulfide-linked based on elution in the position of DiPTH-Cys. <sup>d</sup> (—) = blank cycle. <sup>e</sup> Calculated using the molecular yield (picomoles) of representative amino acids from each sequence [e.g., % sequence 1 isoform = pmol sequence 1 / (pmol sequence 1 + pmol sequence 2)]. <sup>f</sup> Partially blocked.

form of SP-A (Voss et al., 1992). The NH<sub>2</sub>-terminal peptide sequence of soluble AP-SP-A contained the three NH<sub>2</sub>-terminal variants in roughly the same proportions as the SP-A from the healthy volunteer (Table 5). Following size fractionation of AP-SP-A by reducing SDS-PAGE, the monomeric 35 kDa band and nonreducible dimeric 68 kDa band were transferred to PVDF membranes and separately sequenced. The distribution of isoforms in the 35 kDa band were similar to the soluble form of the protein. The nonreducible dimeric species was predominantly blocked in two separate preparations of AP-SP-A. Less than 10% of the sample sequenced based on the molecular yield of amino acids before (0.3 pmol) and after (3–4 pmol) digestion with cyanogen bromide (not shown). Nevertheless, in both preparations the obtainable sequence was identical to the reported NH<sub>2</sub>-terminus for human SP-A except for the presence of a blank cycle in the first round of Edman degradation. The blank cycle indicates the presence of an additional amino acid at the NH<sub>2</sub>-terminus, which by inference is cysteine. The failure to directly detect cysteine may have been due to the low molecular yield of the residue or to a modification of the amino acid. We conclude that SP-A isolated from healthy volunteers and from patients with AP have cysteine-containing sequence variations at the NH<sub>2</sub>-terminus, in similar proportions.

## DISCUSSION

This data presented demonstrates that rat SP-A is a heterooligomer of two closely related polypeptide chains which differ by the presence or absence of a tripeptide extension at the NH<sub>2</sub>-terminus. The extended isoform was recognized by analyses of mutant recombinant forms of SP-A that were truncated at the NH<sub>2</sub>-terminus and then confirmed to occur in nature by protein sequencing of SP-A isolated from the alveolar lavage of rats and humans. Further analyses indicated that the IKC extension was critical for the structure of the rat protein, since the second disulfide

bond required for the multimeric assembly of rat SP-A forms at the newly identified cysteine at the (–1) position of the protein.

Protein isoforms may arise from a single gene by utilization of alternate start or termination sites, by alternate splicing of RNA or by alternate proteolytic processing. The hypothesis that the alternate use of the two potential in-frame translation start sites of SP-A (Sano et al., 1987) [at codons corresponding to Met<sup>–20</sup> and Met<sup>–29</sup> (see Figure 1)] results in distinct NH<sub>2</sub>-termini was excluded because deletion of the nucleotide sequences upstream from Met<sup>–20</sup> did not result in the loss of either isoform (data not shown). Alternate splicing of RNA was also excluded because the 1.6 kb cDNA for rat SP-A encodes isoforms both with and without IKC extension. We propose that the variation in the NH<sub>2</sub>-terminal sequence of SP-A is due to alternate-site cleavage by signal peptidase. The marked variation in the prevalence of the IKC isoform among the three truncated proteins suggests processing by a protease which is sensitive to the context of sequences downstream from the leader peptide. Signal peptidase has been shown to exhibit this property (Jain et al., 1994). Furthermore, the amino acids at the –1 and –3 positions with respect to both the primary (Cys<sup>–1</sup>–Asn<sup>1</sup>) and alternate cleavage point (Gly<sup>–4</sup>–Ile<sup>–3</sup>) for rat SP-A fulfill the small side chain requirement for signal peptidase cleavage (Izard & Kendall, 1994). Another possibility is that uniform signal peptidase cleavage at Ile<sup>–3</sup> is followed by exopeptidase processing. Although difficult to completely exclude, it is unlikely that a requirement for post signal peptidase proteolytic processing would be supported to the same extent in both the lung and in insect cells to produce the ~20% prevalence of the IKC isoform in both cases. To our knowledge, there is no reported precedent for alternate signal peptidase cleavage leading to expression of distinct and structurally important isoforms of a protein.

It is clear that Cys<sup>–1</sup> of rat SP-A forms disulfide bonds, since it has been shown that there are no free sulfhydryls in

the protein (Kuroki et al., 1988a). The elution profile of Cys<sup>-1</sup> in this study was also consistent with participation in a disulfide linkage, since the derivatized residue appeared in the position of DiPTH-Cys (Haniu et al., 1994). Evidence in favor of an interchain rather than an intrachain linkage at Cys<sup>-1</sup> was provided by the truncated proteins, which exhibited a close correlation between the abundance of the IKC isoform and the extent of dimerization on nonreducing SDS-PAGE gels. The lack of dimerization in Cys<sup>-1</sup> and Cys<sup>6</sup> deficient protein SP-A<sup>ΔN1-P80</sup> further indicates that Cys<sup>-1</sup> and Cys<sup>6</sup> account for all of the intermolecular disulfide bonds in SP-A. Since the rate of overexpression or the underhydroxylation of the collagen-like domains of SP-A variants may adversely affect the covalent assembly in the insect cell derived protein, analyses were also performed on the native rat protein. The IKC isoform was markedly enriched in the higher oligomeric species of rat SP-A separated on nonreducing SDS-PAGE, representing 17% of the SP-A species in the 68 kDa band and 50% of SP-A species in the 131 kDa band. The chromatographic profile of Cys<sup>-1</sup> of the dimeric rat SP-A species also indicated disulfide-linkage. The appearance of diPTH cystine in the third cycle of the dimeric SP-A 68 kDa species (Table 2) indicated that Cys<sup>-1</sup> is predominantly paired with Cys<sup>-1</sup> of adjacent chains, since mixed disulfides between Cys<sup>-1</sup> and Cys<sup>6</sup> would have delayed release until the sixth sequencing cycle. We cannot exclude low levels of mixed disulfides by these methods however. A model for interchain disulfide linkage at the NH<sub>2</sub>-terminus based on "in-register" alignment of polypeptide chains is proposed in Figure 3. The observed ratio of 4 extended chains per octadecamer is consistent with the observed predominance of disulfide-linked multimers through hexamers, and much lesser abundant higher species, on nonreducing SDS-PAGE.

The factors which regulate differential NH<sub>2</sub>-terminal processing of rat SP-A are unclear, but the presence of an interchain disulfide bridge and a carbohydrate attachment site flanking the primary signal peptidase cleavage point suggests two possible mechanisms. There was a correlation between the extent of glycosylation of the NH<sub>2</sub>-terminus of SP-A and the abundance of the extended isoform. Specifically, the IKC variant was present in the 32 and 38 kDa glycosylated species of SP-A separated by SDS-PAGE and was absent from the lesser glycosylated 26 kDa species. In addition, the molecular yield of Asn<sup>1</sup> suggested that the extended isoform of SP-A was quantitatively glycosylated at the NH<sub>2</sub>-terminus but only partially modified in the shorter isoform. One potential explanation for these findings is that polypeptide chains are first cleaved by signal peptidase and then differentially modified with carbohydrate, with high efficiency in the presence of the IKC extension and low efficiency without the IKC extension. Alternatively, the cotranslational glycosylation of the nascent peptide chains may promote the expression of the extended isoform by protecting the Cys<sup>-1</sup>-Asn<sup>1</sup> cleavage site from hydrolysis by signal peptidase. Intermolecular disulfide bond formation at Cys<sup>-1</sup> might also affect Cys<sup>-1</sup>-Asn<sup>1</sup> cleavage, although studies of biosynthesis of the related collectin, SP-D, suggest that NH<sub>2</sub>-terminal crosslinks form very late in processing (Brown-Augsburger, 1996). Experiments are in progress to determine if glycosylation of Asn<sup>1</sup> or Cys<sup>-1</sup> disulfide bridge formation are important determinants of signal peptidase specificity for rat SP-A.

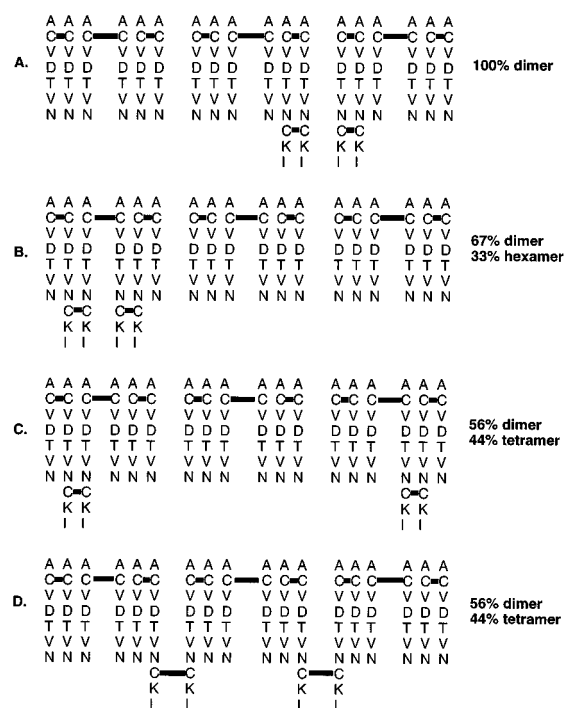


FIGURE 3: Proposed amino terminal disulfide linkages in SP-A. The four possible patterns of inter- and intratrimeric NH<sub>2</sub>-terminal disulfide linkage in the fully assembled octadecameric SP-A molecule are shown in panels A–D. Assignment of disulfides is based on the absence of free thiols (Kuroki, 1988) and the data presented supporting (1) a 4:14 ratio of isoforms with and without the IKC extension, respectively, (2) Cys<sup>-1</sup> and Cys<sup>6</sup> are the only intermolecular bonds in the molecule, (3) no mixed disulfides of Cys<sup>-1</sup> and Cys<sup>6</sup>. The relative proportions of disulfide-linked dimer, tetramers, and hexamers for each are shown for each pattern of disulfide pairing.

We also found heterogeneity at the NH<sub>2</sub>-terminus of human SP-A. Three isoforms were identified in SP-A isolated from a healthy subject, including cysteinyl variants with single and double amino acid extensions, and the sequence corresponding to the reported NH<sub>2</sub>-terminus of human SP-A (White et al., 1985). As with rat SP-A, the cysteines at positions -1 and +6 were demonstrated to form disulfide bonds. However, since the two known allelic forms of human SP-A contain two (α1 chain) and one (α2 chain) additional cysteine residues within the collagen domain that may also participate in interchain disulfide formation, the contribution of the newly identified Cys<sup>-1</sup> to the oligomeric assembly of the protein will be more difficult to assess (Floros et al., 1986). There is no N-linked oligosaccharide attachment site at the NH<sub>2</sub>-terminus of human SP-A. We also examined the NH<sub>2</sub>-terminal sequence of AP-SP-A isolated from patients with alveolar proteinosis, which has been shown to have structural differences from normal human SP-A (Voss et al., 1992). The most striking abnormality is a nonreducible dimeric form of the protein which is apparent on SDS-PAGE analysis of the protein. The NH<sub>2</sub>-terminal sequence and relative abundance of the isoforms of soluble AP-SP-A were very similar to the normal SP-A. However, the finding that the nonreducible 68 kDa species but not the monomeric 35 kDa species separated by SDS-PAGE was largely blocked invites speculation that a modification at the extreme NH<sub>2</sub>-terminus may play a role in the formation of the abnormal cross-link. The partial sequence which was obtained from the nonreducible dimeric form of SP-A was exclusively the C-isoform, but the blocked

isoforms which are present in the band may be the most relevant species.

The cDNAs for SP-A isolated from mouse (Korfthagen et al., 1992) and rabbit (Boggaram et al., 1988) but not dog (Benson et al., 1985) also predict cysteines at or near the COOH-terminus of the signal peptide, so the occurrence of disulfide-forming NH<sub>2</sub>-terminal variants may prove to be a common, if not universal, phenomenon.

In summary, we report cysteine-containing sequences at the NH<sub>2</sub>-terminus of rat SP-A which arise by alternate proteolytic processing. The cDNA for rat SP-A encodes for both isoforms, and mutations in the amino acid sequence upstream from the signal peptide influence their relative expression, indicating that the variants likely arise via utilization of two natural cleavage points for signal peptidase. The IKC isoforms are preferentially glycosylated and are required for the disulfide-dependent oligomeric assembly of rat SP-A. Human SP-A also has cysteinyl extensions at the NH<sub>2</sub>-terminus, but conclusions regarding their role in the disulfide-bridged structure of the protein will require further studies.

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