Structural Analysis and Lipid-Binding Properties of Recombinant Human Surfactant Protein A Derived from One or Both Genes†

I. García-Verdugo,‡,§ G. Wang,‡,|| J. Floros,||,⊥ and C. Casals*,§

Department of Biochemistry and Molecular Biology I, Complutense University of Madrid, 28040-Madrid, Spain, and Departments of Cellular and Molecular Physiology and Pediatrics, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

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ABSTRACT: Surfactant protein A (SP-A) constitutes an important part of the innate immune defense in the lung. In humans there are two functional genes (*SP-A1* and *SP-A2*). The functional importance of having two distinct chain types in human SP-A is undefined. Amino acid substitutions in the primary structure of the protein may have effects on structural stability or on activity. To address this issue, SP-A1, SP-A2, and coexpressed SP-A1/SP-A2 variants were in vitro expressed in insect cells, purified, and used for study. We found the following: (1) Human SP-A variants expressed in insect cells, derived from one gene (*SP-A1* or *SP-A2*) or both genes, differ in the relative extent and heterogeneity of oligomerization. SP-A1 and SP-A2 exist in small oligomeric forms, whereas coexpressed SP-A1/SP-A2 products favor the formation of larger oligomers. (2) Circular dichroic and fluorescence spectroscopic studies identified structural differences between SP-A variants in the collagen domain, with SP-A2 being more stable than SP-A1 but not in the calcium binding region. Recombinant human SP-A variants expressed in insect cells exhibit a lower melting temperature compared to native human SP-A. Oligomerization does not increase the thermal stability of the collagen domain of coexpressed SP-A1/SP-A2. (3) The ability of SP-A to undergo self-aggregation and induce phospholipid and bacterial lipopolysaccharide aggregation is greater for SP-A2 than for coexpressed SP-A1/SP-A2, which in turn is greater than that observed for SP-A1. The presence of SP-A1 polypeptide chains in coexpressed products modulates functional capabilities of SP-A, which depend on both the collagen and globular domains.

Surfactant protein A (SP-A)[1] belongs to the collectin class of C-type lectins along with surfactant protein D, mannose binding protein, collectin 43, and conglutinin (*1*). Together with C1q, these proteins are also called defense collagens, and they play important roles in innate immunity. Substantial evidence indicates that SP-A is involved in innate host defense and inflammatory processes of the lung, as well as in several aspects of pulmonary surfactant biology (*1*−*4*). In humans, the *SP-A* locus consists of two functional genes (*SP-A1* and *SP-A2*) and one pseudogene (*5*). Of the SP-A alleles that have been identified and characterized, four SP-A1 alleles (6A, $6A^2$, $6A^3$, and $6A^4$) and six of the SP-A2 alleles (1A, $1A^0$, $1A^1$, $1A^2$, $1A^3$, and $1A^5$) are found frequently in the general population (*6*). The SP-A variants have documented differences in the coding and noncoding regions (*5*−*9*). Such heterogeneity may point to functional, structural, and regulatory differences among SP-A variants.

The sequence of mature SP-A consists of four structural domains: (1) an N-terminal domain involved in intermolecular disulfide bond formation; (2) a collagen domain important for the stability and oligomerization of SP-A; (3) a neck region consisting of amino acid residues with high α-helical propensity, primarily involved in protein trimerization; and (4) a globular carbohydrate recognition domain involved in lipid binding and also in Ca^{2+}-dependent binding of carbohydrates (10). Allelic differences in the sequence of SP-A are observed as amino acid substitutions at 10 positions (*5*, *11*). Of those, two are in the signal sequence (amino acids 9 and 19), six are in the collagen domain (amino acids 50, 66, 73, 81, 85, and 91), and two are in the carbohydrate/calcium binding domain (amino acids 219 and 223).

Voss et al. (*12*) reported that native SP-A from bronchoalveolar lavage fluid is an octadecamer consisting of six trimers. They also postulated that the human SP-A trimer is a heterotrimer composed of two SP-A1 molecules and one SP-A2 molecule (*13*). Whether the two gene products are

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* To whom correspondence should be addressed at the Department of Biochemistry and Molecular Biology I, Faculty of Biology, Complutense University of Madrid, 28040 Madrid, Spain. Phone 34-91-3944261; fax 34-91-3944672; e-mail casals@solea.quim.ucm.es.

‡ I.G.-V. and G.W. contributed equally to this study.

§ Complutense University of Madrid.

|| Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine.

⊥ Department of Pediatrics, The Pennsylvania State University College of Medicine.

[1] Abbreviations: SP-A, surfactant protein A; $SP\text{-}A^{hyp}$, hydroxyproline-deficient recombinant surfactant protein A; CRD, carbohydrate recognition domain; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; Re-LPS, rough lipopolysaccharide; CD, circular dichroism; T_m, midpoint transition melting temperature; PAGE, polyacrylamide gel electrophoresis.

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expressed in a 2:1 (SP-A1:SP-A2) ratio and actually form heterotrimeric structures remains to be defined. However, Karinch et al. (*8*) have shown that the SP-A1/SP-A2 mRNA ratio varies considerably in adult lung among unrelated individuals. Karinch et al. (*14*) reported that the SP-A1 mRNA content was higher than the SP-A2 in explant cultures, whereas McCormick and Mendelson (*15*) found more SP-A2 than SP-A1 mRNA in lung tissues from four adults. Interestingly, while both genes are expressed in lung alveolar type II cells, the SP-A2 gene is expressed primarily (if not exclusively) in tracheal and bronchial submucosal gland cells (*16*−*18*). SP-A isolated from bronchoalveolar lavage of one patient with alveolar proteinosis consisted primarily of the SP-A2 gene product (*19*).

It is known that neither SP-A from bronchoalveolar lavage (*20*) nor recombinant SP-A (*13*, *21*) exists only as fully assembled complexes of octadecamers, but SP-A also exists in other oligomeric forms (e.g., dodecameric, nonameric, or hexameric). Patients with birch pollen allergy have lower levels of high oligomeric size forms compared to healthy subjects (*20*). Voss et al. (*13*) reported that recombinant human SP-A1 and SP-A2 produced in mammalian cells differ in their pattern of disulfide-linked polypeptide chains and in the macromolecular structure revealed by electron microscopy. Wang et al. recently reported that SP-A2 variants produced in either the baculovirus (*22*) or the mammalian (*23*) cell system stimulate significantly more TNF-α and IL-8 production by THP-1 cells than SP-A1 variants. Differences between SP-A1 and SP-A2 variants produced in mammalian cells in their susceptibility to ozone-induced oxidation have also been reported (*23*).

Amino acid substitutions in the primary structure of the protein may have effects on structural stability or on activity. Since the signal peptide sequence is cleaved during the N-terminal processing of SP-A, the remaining major differences among alleles are in the collagen-like domain and in the carbohydrate/calcium binding domains. It is reasonable to suggest that the amino acid substitutions in the collagen domain could have effects on the structural stability of the SP-A molecule. Consequently, the first objective of this study was to investigate whether single-gene products have similar thermal stability of the collagen domain and whether there are structural differences between single-gene products and SP-A variants containing coexpressed products from both genes. The second objective was to assess differences among SP-A variants derived from one or both SP-A genes by studying the ability of human SP-A (a) to self-aggregate, which depends on a structurally intact collagen domain (*24*), and (b) to induce phospholipid vesicle aggregation and bacterial lipopolysaccharide aggregation. SP-A selectively interacts with several insoluble amphipathic lipids that are present in surfactant or microbial membranes (*25*). This ability of SP-A to bind lipids may be of physiological and immunological relevance in the lung.

SP-A has been implicated in the susceptibility to and pathogenesis of many pulmonary diseases of environmental, infectious, and idiopathic origin (*26*). In addition, SP-A alleles have been associated with increased or decreased risk for certain pulmonary diseases (*27*−*31*). Because recombinant human SP-A has the potential to be used as a drug itself, either as a part of surfactant therapy or alone, it could be relevant to explore biochemical differences or similarities between recombinant human SP-A variants and to compare SP-A from one or both genes with human SP-A isolated from bronchoalveolar lavage.

Table 1: Amino Acid Differences among SP-A Alleles[a]

		amino acid							
gene	allele	50	66	73	81	85	91	219	223
SP-A1	$6A^2$	Val	Met	Asp	Ile	Cys	Pro	Arg	Gln
SP-A1	$6A^4$		Met	Asp	Ile	Cys	Pro	Trp	Gln
SP-A2	$1A^1$	Val	Thr	Asn	Val	Arg	Ala	Arg	Lys
SP-A2	$1A^0 = 1A^2$	Val	Thr	Asn	Val	Arg	Ala	Arg	Gln

[a] Amino acid differences at residues 66, 73, 81, and 85 constitute the "core" differences that distinguish one SP-A gene and its corresponding alleles from the other SP-A gene and its corresponding alleles (*7*). These differences are located in the collagen domain of SP-A. Residues 219 and 223 are located in the globular domain. Alleles $1A^0$ and $1A^2$ have identical amino acid sequence following removal of the signal peptide.

EXPERIMENTAL PROCEDURES

Experiments presented in this study were performed with two different preparations of native human SP-A from normal subjects, six different preparations of individual $1A^2$ and $6A^2$ alleles and the coexpressed SP-A1/SP-A2 variant $1A^2/6A^2$, two different preparations of the $6A^4$ allele, and one preparation of the variants $1A^0$ and $1A^1$ and coexpressed variant $1A^1/6A^4$. Experiments were repeated at least twice with each protein preparation. Coexpressed variants are said to be when the two SP-A alleles (one from each SP-A gene) are expressed together. The amino acid differences (after signal peptide removal) among SP-A alleles (*5*) used in this study are shown in Table 1. Please note that alleles $1A^0$ and $1A^2$ are identical in amino acid sequence following removal of signal peptide.

Insect Cell Culture Conditions. Insect cell line Sf9 from *Spodoptera frugiperda* was purchased from Invitrogen (Carlsbad, CA). The insect cell culture and transfection were carried out according to our previously described protocol (*22*). In brief, for cell growth and protein expression, insect cells were cultured in Sf-900 II SFM medium (Invitrogen, Carlsbad, CA) in an incubator at 27−28 °C with shaking about 100 rpm. For transfection, a monolayer culture was used and transfected with recombinant bacmid DNA (baculovirus shuttle vector containing SP-A cDNA).

Preparation of Plasmids and Bacmids. The preparation of plasmids and bacmids was described previously (*22*). The cDNA fragments of human SP-A1 alleles ($6A^2$ and $6A^4$) and SP-A2 alleles ($1A^0$, $1A^1$, and $1A^2$) were isolated from the recombinant plasmids by digestion with *Xho*I and *Sph*I. The cDNA fragments were then cloned into donor plasmid pFastBac DUAL (Invitrogen, Carlsbad, CA), and the sequence was confirmed by DNA sequencing. The SP-A gene was driven by the p10 promoter. Recombinant plasmid pFastBac DUAL-SP-A was transformed into *Escherichia coli* DH10Bac. The promoter−SP-A region of pFastBac DUAL-SP-A plasmid was transferred into baculovirus genome (bacmid) by site-specific transposition processing.

Transfection, SP-A Expression, and Purification from Baculovirus-Mediated Insect Cell System. SP-A variants were expressed and purified as described (*22*). Briefly, insect cells Sf9 were cultured in Sf-900 SFM medium. When the cell density of insect cells reached 1×10^6 cells/mL, the 50 mL

culture was inoculated with 1 mL of a virus solution containing about 1×10^9 titer/mL of virus particles. The medium containing SP-A was harvested 84 h after inoculation. Insect cells and fragments of cells were removed from the medium by centrifugation at 500*g* for 10 min at 4 °C. Then, SP-A was purified by the mannose-binding affinity chromatographic method (*22*). The purified SP-A was then dialyzed against 5 mM Tris-HCl (pH 7.5) that was changed at least three times at 4 °C. Lipopolysaccharide (LPS) contamination was removed with polymyxin B−agarose (1 mL/mg of SP-A) according to the manufacturer's instructions (Sigma, St. Louis, MO). SP-A concentration was determined by the micro-BCA method (Pierce, Rockford, IL) with RNase A as a standard. SP-A was stored at −70 °C until use.

Human Lung Tissue Procurement and Isolation of Native SP-A. Human lung tissue was obtained from male multiple organ donors. Lungs from donors with either a recent history of tobacco smoking or who had been on mechanical ventilation for more than 72 h, or samples that had any radiological evidence of pulmonary infiltrate, were excluded. Lungs were lavaged twice with 4 °C saline and pulmonary surfactant obtained as previously described (*32*). SP-A was purified from isolated surfactant by sequential butanol and octyl glucoside extractions (*33*). The purity of SP-A was checked by one-dimensional SDS−PAGE in 12% polyacrylamide gel under reducing conditions (50 mM dithiothreitol). Quantification of SP-A was carried out by amino acid analysis in a Beckman System 6300 high-performance analyzer. The protein hydrolysis was performed with 0.2 mL of 6 M HCl, containing 0.1% (w/v) phenol, in evacuated and sealed tubes at 108 °C for 24 h. Norleucine was added to each sample as the internal standard (*34*).

Gel Electrophoresis under Native Conditions and Silver Staining. Gel electrophoretic analysis was done following the procedure described by Laemmli (*35*). In vitro expressed SP-A variants were subjected to electrophoresis under native conditions. SP-A protein samples were prepared with a 4× loading buffer containing 0.2 M Tris-HCl (pH 7.5) and 40% glycerol and were not denatured by chemicals and heating. Electrophoresis was performed at 4 °C with a 4−20% acrylamide gradient gel at 50 V for 1 h and 110 V for 13 h. The gel was stained by the silver staining method as reported by Rabilloud (*36*).

N-Terminal Sequencing of SP-A Variants. The N-terminal amino acid sequence of SP-A variants was determined by a modified Edman degradation method (Biotechnology Laboratory, University of British Columbia, Vancouver, Canada). Pure SP-A protein, about 3 μg, was absorbed onto poly(vinylidene difluoride) (PVDF) membranes by incubating SP-A with PVDF membranes (about 4 mm^2) at 4 °C overnight. After being washed with dH_2O, the PVDF membranes absorbing SP-A were directly used for microsequencing. The relative percentage of each SP-A isoform was estimated by comparing the molecular yield of a representative amino acid, such as valine, at the N-terminus of each sequence.

CD Measurements. CD spectra were obtained on a Jasco J-715 spectropolarimeter fitted with a 150 W xenon lamp (*24*, *37*). Quartz cells with a 1-mm path length were used and the spectra were recorded in the far-UV region (190−260 nm) with a scanning speed of 50 nm/min and at the indicated temperature. Four scans were accumulated and averaged for each spectrum. The acquired spectra were corrected by subtracting the appropriate blanks, subjected to noise-reduction analysis, and presented as molar ellipticities (degrees·centimeter^2·decimole^{-1}), assuming 110 Da as the average molecular mass per amino acid residue. At least two independent preparations of SP-A were measured. All measurements were performed in 5 mM Tris-HCl buffer, pH 7.2, and protein concentrations in all cases were 80 μg/mL. The concentration of each SP-A sample was always determined by amino acid analysis.

For the analysis of thermal stability, the samples (80 μg/mL) were allowed to equilibrate for 10 min at each temperature (from 20 to 65 °C) and were then scanned from 260 to 190 nm. The results were plotted as the molar ellipticity at 207 nm as a function of temperature. For natural human SP-A, melting curves were also monitored at 207 nm while the sample temperature was raised from 20 to 65 °C, with an average heating rate of 12 °C/h. SP-A concentrations were 120 or 200 μg/mL, and quartz cells with a 1- or 0.5-mm path length were used. Because the amount of SP-A variants from insect cells was limited, the analysis of thermal stability of these samples was done at 80 μg/mL in quartz cells with a 1-mm path length, recording spectra at different temperatures just as described above. The fraction folded was calculated from the equation $F = ([\theta] - [\theta]_u)/([\theta]_n - [\theta]_u)$, where $[\theta]$ is the observed mean residue molar ellipticity at 207 nm, and $[\theta]_n$ and $[\theta]_u$ are the mean values for native and unfolded SP-A, respectively. These values were obtained from the plateau before and after the transition. The temperature where the protein was 50% unfolded ($F = 0.5$) was taken as the melting temperature.

Fluorescence Measurements. Fluorescence experiments were carried out on a SLM-Aminco AB-2 spectrofluorometer. Cells of 10 × 10 mm were used. The slit widths were 4 nm for the excitation and emission beams. The sample was stirred continuously in a sample compartment thermostated with a compact refrigerated circulator, Julabo F30-C. Fluorescence spectra of SP-A were measured at 25 °C in 1.6 mL of 5 mM Tris-HCl buffer (pH 7.2). The final protein concentration of SP-A was 10 μg/mL. The blanks and protein samples were excited at 275 nm for measuring the total protein fluorescence spectrum or at 295 nm to preferentially excite tryptophan residues. Emission spectra were recorded from 300 to 400 nm.

The change in fluorescence emission intensity of SP-A upon addition of millimolar concentrations of Ca^{2+} was determined by adding a concentrated solution of $CaCl_2$ to the protein solution in the cuvette (2 mM final concentration). After equilibration for 15 min, the emission spectrum of SP-A was recorded on excitation at 275 nm. Next, the fluorescence spectrum of SP-A was recorded upon addition of 4 mM EDTA. The fluorescence intensity readings were corrected for the dilution caused by aliquot addition. The absorbance of the protein was measured after addition of calcium and EDTA by use of a Beckman DU-640 spectrophotometer.

SP-A Self-Aggregation Assays. Self-aggregation assays of native human SP-A were performed as previously described (*24*, *38*) by measuring the change in protein absorbance at 360 nm in a Beckman DU-640 spectrophotometer. The measurement of Ca^{2+}-dependent self-association of recombinant SP-A variants expressed in insect cells required higher

protein concentration (50 μg/mL) than native SP-A (20 μg/mL). The experiments were performed at 20 and 37 °C.

Lipid Aggregation Assays. Synthetic phospholipids DPPC and DPPG were purchased from Avanti Polar Lipids (Birmingham, AL), and their homogeneity was routinely tested on thin-layer chromatography. Rough LPS from *Salmonella minnesota* (serotype 595) was purchased from Sigma Chemical Co. (St. Louis, MO). The organic solvents (methanol and chloroform) used to dissolve lipids were HPLC-grade (Scharlau, Barcelona).

DPPC/DPPG (7:3 w/w) vesicles were prepared at a phospholipid concentration of 1 mg/mL by hydrating dry lipid films in a buffer containing 150 mM NaCl, 0.1 mM EDTA, 25 mM Tris-HCl (pH 7.2) and allowing them to swell for 1 h at 50 °C. Sonication, phosphorus determination, and vesicle-size analysis were performed as described previously (*37*). Re-LPS was hydrated for 1 h in 20 mM Tris-HCl buffer (pH 7.2) containing 150 mM NaCl and 0.1 mM EDTA.

LPS aggregation or DPPC/DPPG vesicle aggregation induced by recombinant SP-A were studied at either 25 or 37 °C by measurement of the change in absorbance at 400 nm in a Beckman DU-640 spectrophotometer.

SP-A-induced LPS aggregation assays were performed as follows: both sample and reference cuvettes were first filled with Re-LPS (40 μg/mL, final concentration) in 25 mM Tris-HCl buffer (pH 7.2) with 150 mM NaCl and 0.2 mM EDTA. After a 10-min equilibration at 37 °C, recombinant SP-A (20 μg/mL) was added to the sample cuvette, and the change in optical density at 400 nm was monitored. Next, Ca^{2+} (2.5 mM) was added to both the sample and reference cuvettes, and the change in absorbance was monitored again. Ca^{2+}-dependent LPS aggregation was reversed by adding EDTA (5 mM, final concentration). The extent of Ca^{2+}-dependent aggregation of Re-LPS in the absence of SP-A was also evaluated. In another set of experiments, sample and reference cuvettes were first filled with Re-LPS and 2.5 mM Ca^{2+}. After a 10-min equilibration at the indicated temperature, SP-A was added to the sample cuvette and the change in optical density at 400 nm was monitored.

SP-A-induced phospholipid vesicle aggregation assays were performed as described above for Re-LPS aggregation and elsewhere (*37*, *38*). The final concentrations of recombinant SP-A, phospholipids, Ca^{2+}, and EDTA were 20 μg/mL, 100 μg/mL, 2.5 mM, and 5 mM, respectively.

The contribution of self-aggregation of SP-A (at a concentration of 20 μg/mL) to the change of absorbance at 400 nm was routinely checked under the experimental conditions in which Re-LPS aggregation or phospholipid vesicle aggregation assays were done.

RESULTS

Oligomerization of Recombinant Human SP-A Expressed in Insect Cells. The relative extent of oligomerization of recombinant human SP-A produced by one or two genes was examined by electrophoresis under native conditions (Figure 1). SP-A molecules maintain their native conformation because they were not treated with SDS, reducing agents, or heating. Migration through a 4−20% gradient gel is based on the native molecular mass and structural molecular conformation. Figure 1 shows that the patterns of oligomerization among SP-A1, SP-A2, and coexpressed SP-A1/SP-A2 differed. SP-A1 ($6A^2$) consisted primarily of trimers (3X) (the basic trimer unit), and hexamers (6X). Higher oligomeric forms (dodecamers, 12X, and pentadecamers, 15X) were found in small amounts. SP-A2 ($1A^2$) consisted mainly of dimers (2X), tetramers (4X), and nonamers (9X). Smaller amounts of dodecamers and pentadecamers were also present. Similar results were found with $6A^4$ (SP-A1) and $1A^1$ (SP-A2) variants (data not shown). Coexpressed $6A^2/1A^2$ existed in higher oligomeric forms, with mainly octadecamers (18X). The pattern of oligomerization of coexpressed $1A^1/6A^4$ was similar to that of $1A^2/6A^2$ variant (data not shown), indicating that coexpressed SP-A variants have higher oligomeric forms than single alleles. Figure 1 also shows the oligomeric assembly of native human SP-A, which consists of octadecamers and higher oligomeric forms.

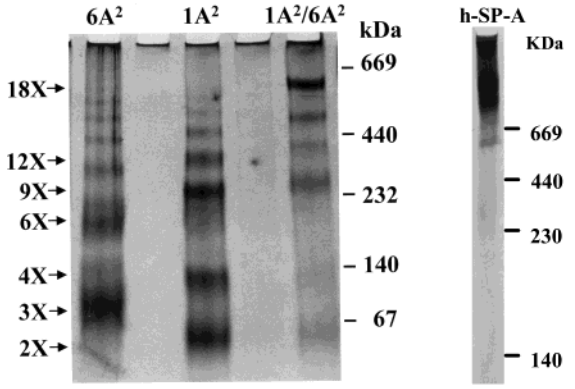

FIGURE 1: Patterns of oligomerization of native and recombinant human SP-A under native conditions. Native human SP-A was isolated from the bronchoalveolar lavage of previously healthy donors. SP-A1 ($6A^2$), SP-A2 ($1A^2$), and coexpressed SP-A ($1A^2/6A^2$) were produced in insect Sf9 cells by baculovirus-mediated expression system. These samples were subjected to 4−20% PAGE under native conditions followed by silver staining. Numbers on right denote molecular mass. Marks on the left indicate oligomers. The polypeptide chain [X] of SP-A from insect cells is about 28−30 kDa.

The amino-terminal sequences of SP-A variants from baculovirus-mediated insect cells were further analyzed by protein microsequencing. The results in Table 2 indicated that all SP-A variants have two isoforms: the first residues of the two isoforms are cysteine and glutamate (residues 20 and 21 of the SP-A precursor), respectively. The majority of SP-A1 ($6A^2$) and coexpressed SP-A1/SP-A2 ($1A^0/6A^2$) variants are found in the Cys^{20} isoform (60% and 80%, respectively), and the SP-A2 allele ($1A^0 = 1A^2$) is found almost exclusively in the Cys^{20} isoform.

Circular Dichroic Spectra of Recombinant Human SP-A Expressed in Insect Cells. The circular dichroic spectra of recombinant human SP-As and native human SP-A, in 5 mM Tris-HCl, pH 7.4, at different temperatures, are shown in Figure 2. The spectrum of native human SP-A isolated by butanol extraction is characterized by a strong negative extreme at 207 nm and a shoulder at 223 nm as previously reported for canine, porcine, or alveolar proteinosis-human SP-A (*24*, *34*, *37*, *39*, *40*). Recombinant human SP-As expressed in insect cells displayed a shape similar to that of native human SP-A at low temperatures. However, recombinant SP-A1 ($6A^2$ and $6A^4$) showed a decrease in CD signal. The molar ellipticity at the negative extreme (207 nm) of the $6A^2$ variant had values between −4000 and −6000

Table 2: Amino-Terminal Sequence of in Vitro Expressed SP-A Variants[a]

	amino acid[b]								single gene products		coexpression product
	20	21	22	23	24	25	26	27	SP-A2 ($1A^0$)	SP-A1 ($6A^2$)	SP-A2/SP-A1 ($1A^0/6A^2$)
sequence 1		Glu	Val	Lys	Asp	Val	Cys	Val	2%	42%	21%
sequence 2	Cys	Glu	Val	Lys	Asp	Val	Cys	Val	98%	58%	79%

[a] The percentage of each isoform was calculated by the molecular yield (picomoles) of representative amino acid from each sequence divided by the molecular yield (picomoles) of total isoforms {e.g., % sequence 1 isoform = (picomoles of sequence 1)/(picomoles of total sequences)}. [b] Numbered according to the amino acid number of SP-A precursor.

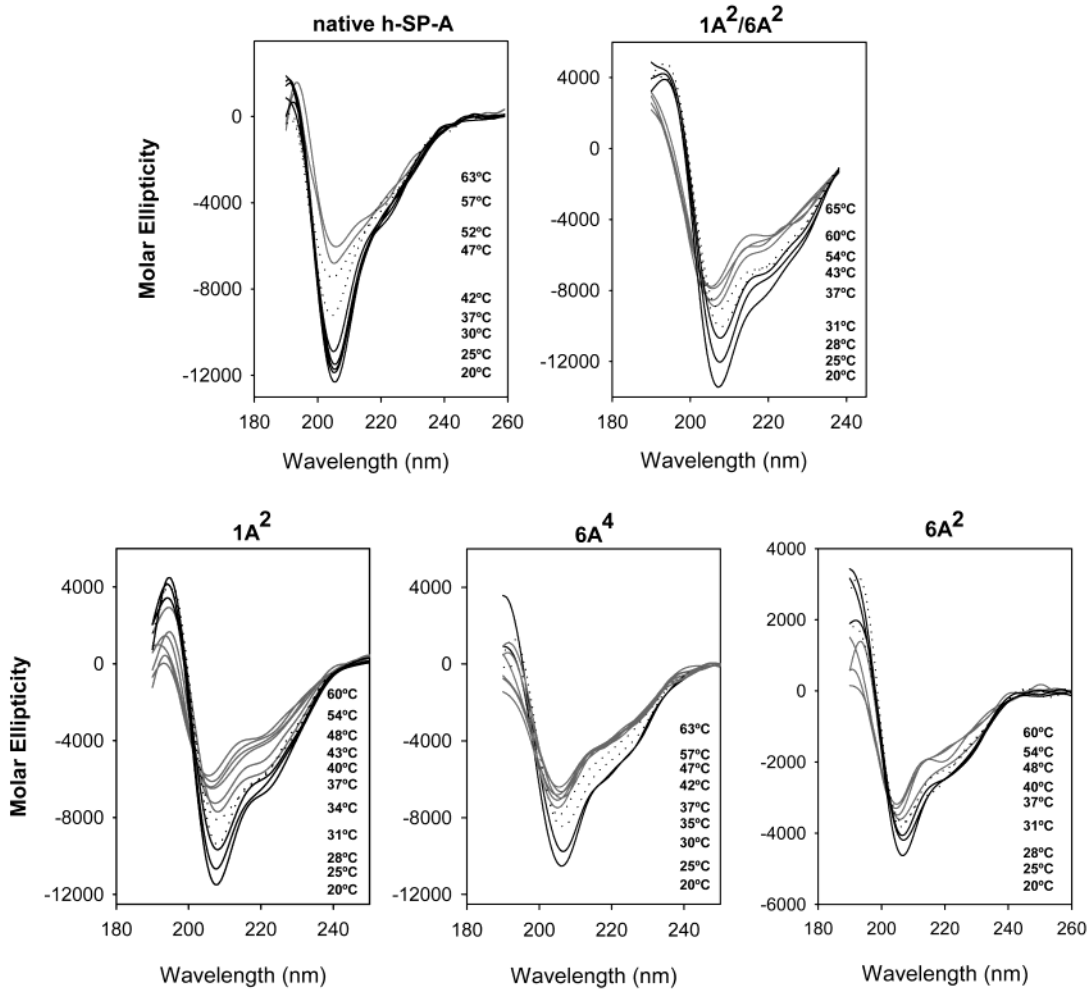

FIGURE 2: Circular dichroic spectra of native human SP-A and recombinant human SP-A variants at different temperatures. All measurements were performed in 5 mM Tris-HCl buffer, pH 7.2. Protein concentrations in all cases were 80 μg/mL, as determined by amino acid analysis.

deg·cm^2·dmol^{-1} in all of the samples studied, and the values for $6A^4$ variant were around $-10\,000$ deg·cm^2·dmol^{-1}. In contrast, these values were equal to or greater than $-12\,000$ deg·cm^2·dmol^{-1} for $1A^2$ (SP-A2), coexpressed $1A^2/6A^2$, and native human SP-A.

Heat treatment of native human SP-A (80 μg/mL) resulted in a pronounced decrease in the magnitude of the negative band at 207 nm without any shift of this band. The molar ellipticity at 207 nm as a function of temperature is shown in Figure 3. Melting curves were monitored at 207 nm, in the same quartz cells (1 mm) but at higher protein concentrations (120 μg/mL), while the sample temperature was raised from 20 to 65 °C. The native human SP-A had a T_m of 48 °C, which is lower than that for alveolar proteinosis-human SP-A (about 52 °C) (*40*) and for canine (51.5 °C) and porcine (51 °C) SP-A (*24*). Of potential relevance, human SP-A from one patient with alveolar proteinosis consisted mainly of the SP-A2 gene product (*19*). On the other hand, heat treatment of recombinant human SP-A (80 μg/mL) also resulted in a pronounced decrease in the magnitude of the negative band at 207 nm, with blue shift of the minimum at temperatures higher than their respective midpoint melting temperatures (Figure 2). Recombinant SP-As expressed in the baculovirus system denature at low temperature (30−33 °C) (Figure 3). Midpoint melting temperatures of recombinant SP-As were reduced by about 15−18 °C with respect to that of native SP-A.

Fluorescence Spectroscopic Analysis of Recombinant Human SP-A Expressed in Insect Cells. The fluorescence emission spectrum of native and recombinant human SP-A on excitation at 275 nm is characterized by a fluorescence emission maximum at 330 nm, which is the midpoint between the two emission maxima at 326 and 337 nm,

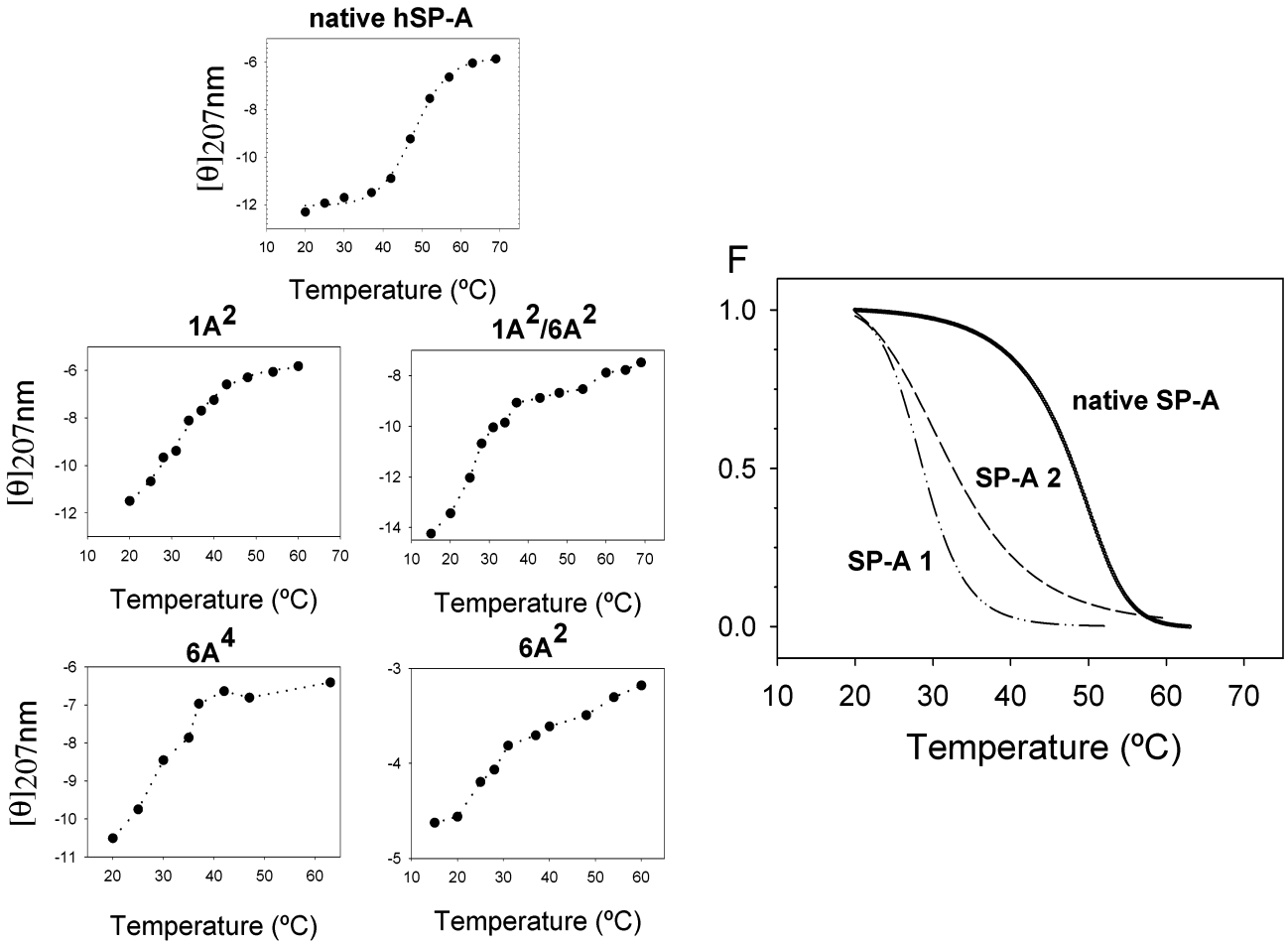

FIGURE 3: (Left) Ellipticities at 207 nm of native and recombinant SP-A variants as a function of temperature. (Right) Degree of helicity F as a function of temperature. F was calculated from melting curves monitored at 207 nm, at 120 or 80 μg/mL SP-A, while the sample temperature was raised from 20 to 65 °C. $F = ([\theta] - [\theta]_u)/([\theta]_n - [\theta]_u)$,where $[\theta]$ is the observed mean residue molar ellipticity at 207 nm, and $[\theta]_n$ and $[\theta]_u$ are the mean values for native and unfolded SP-A, respectively.

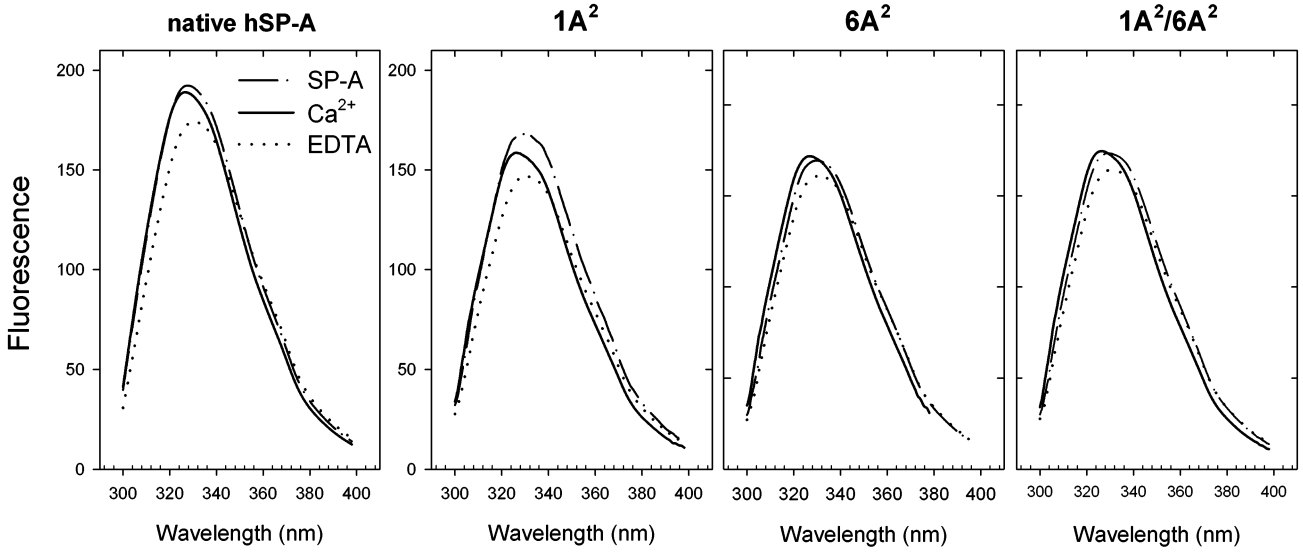

FIGURE 4: Fluorescence emission spectra of native human SP-A, SP-A2 ($1A^2$), SP-A1 ($6A^2$), and coexpressed SP-A1/SP-A2 ($1A^2/6A^2$) at 25 °C. The dashed−dotted lines represent the emission spectra on excitation at 275 nm, in 5 mM Tris-HCl buffer (pH 7.2). The solid lines correspond to repeat emission spectra 10 min after the addition of Ca^{2+} to a final concentration of 2 mM. The dotted lines represent repeat emission spectra 10 min after the addition of EDTA (4 mM final concentration).

previously reported for alveolar proteinosis-human SP-A (*34*) (Figure 4).

Addition of 2 mM Ca^{2+} led to a blue shift in the wavelength of the emission maximum from 330 to 326 nm. This indicates that the binding of calcium led to a conformational change in the protein, affecting the polarity in the environment of the tryptophan residues. Recombinant SP-A derived from one gene (*SP-A1* or *SP-A2*) or two genes

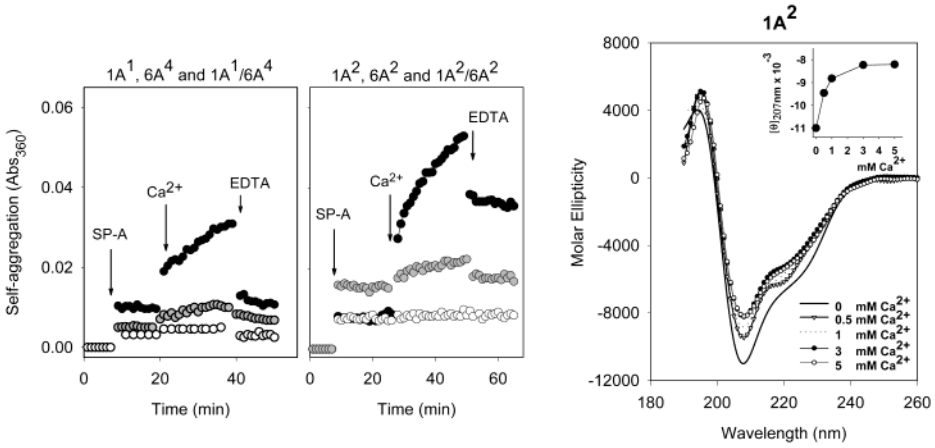

FIGURE 5: (Left) Kinetics of Ca^{2+}-dependent self-aggregation of SP-A variants. Recombinant SP-A (50 μg/mL) was added to the sample cuvette filled with 5 mM Tris-HCl buffer (pH 7.2). The turbidity change at 360 nm was monitored at 20 °C at 1-min intervals. After stabilization, 5 mM Ca^{2+} (final concentration) was added to both the sample and reference cuvettes and the turbidity changes were monitored again. Addition of EDTA (10 mM final concentration) dissociated SP-A aggregates induced by Ca^{2+}. (●) SP-A2 ($1A^1$, $1A^2$); (○) SP-A1 ($6A^2$, $6A^4$); (gray circle) coexpressed SP-A ($1A^1/6A^4$, $1A^2/6A^2$). (Right) Changes in the circular dichroic spectrum of SP-A2 ($1A^2$) as a function of Ca^{2+} concentration. Experiments of Ca^{2+} titration were performed at 20 °C at a protein concentration of 80 μg/mL. Inset: Ellipticity at 207 nm of SP-A2 as a function of Ca^{2+} concentration.

showed the same blue shift in the wavelength of the emission maximum upon addition of calcium. Although the samples were stirred continuously during the course of experiments, a small decrease in fluorescence emission intensity of native human SP-A and SP-A2 occurred upon addition of calcium. The observed decrease in fluorescence intensity is likely related to self-aggregation of these proteins induced by calcium (*24*). This was significant for native human SP-A and SP-A2 ($1A^2$) but not for SP-A1 ($6A^2$) and coexpressed $1A^2/6A^2$ (Figure 5). Figure 4 also shows that chelating calcium with EDTA reversed the spectral changes induced by calcium. However, addition of EDTA caused not only a red shift in the fluorescence emission maximum (from 326 to 330 nm) but also a decrease in the fluorescence emission intensity of both native and recombinant SP-As, which probably implies a higher level of quenching of the tryptophan fluorescence by polarizable groups in the proximity of these residues. Removing Ca^{2+} also caused additional important changes in the CD spectrum of SP-A, resulting in a marked loss of ellipticity (*24*).

The fact that both recombinant SP-As and native SP-A have similar positions of the fluorescence emission maxima of tryptophan residues and similar spectral changes with calcium and EDTA indicates that the polarity in the environment of tryptophan residues is identical in these proteins ($1A^2$, $6A^2$, and $1A^2/6A^2$) and there are not structural differences in the calcium-binding region of these SP-A variants. However, the $6A^4$ allele has different fluorescence characteristics (data not shown). The functional significance of $Arg^{219} \rightarrow Trp^{219}$ substitution in the $6A^4$ allele requires further investigation.

Ca^{2+}-Dependent Self-Aggregation of Recombinant Human SP-A Expressed in Insect Cells. The comparison of Ca^{2+}-dependent self-aggregation activity of SP-A variants is shown in Figure 5. SP-A2 variant ($1A^1$ and $1A^2$) aggregated upon addition of calcium, whereas SP-A1 ($6A^4$ and $6A^2$) did not detectably aggregate at a concentration of 50 μg/mL and at 20 °C. Coexpressed SP-A1/SP-A2 ($1A^1/6A^4$ and $1A^2/6A^2$) weakly aggregated under these conditions. Recombinant SP-A2 and coexpressed SP-A1/SP-A2 were only able to self-aggregate at 20 °C but not at 37 °C, and at a protein concentration much higher than that required for native human SP-A (Figure 8). These results are consistent with the fact that SP-A from insect cells exhibits a lower melting temperature of the collagen-like domain compared to native human SP-A, and the Ca^{2+}-dependent self-aggregation process depends on the collagen domain (*24*). Figure 5 also shows the change in the circular dichroic spectrum of SP-A2 as a function of Ca^{2+} concentration. CD is very sensitive to aggregation phenomena that affect intensity of the dichroic signal and band position. We have previously shown that protein self-aggregation could be followed by a decrease in the CD signal of SP-A (*24*). The molar ellipticity at 207 nm (θ_{207}) as a function of the Ca^{2+} concentration is shown in the inset of Figure 5. The Ca^{2+}-dependent decrease of the SP-A2 molar ellipticity at 207 nm (θ_{207}) reached a maximum at around 2 mM Ca^{2+} in the absence of salts. Higher concentrations of calcium (about 5 mM) are needed to saturate this process in the case of native human SP-A (*24*).

Ca^{2+}-Dependent Lipopolysaccharide Aggregation Induced by Recombinant Human SP-A Expressed in Insect Cells. Data on the ability of recombinant human SP-A variants to induce rough LPS aggregation in the presence of calcium are shown in Figure 6. These amphiphilic molecules are able to aggregate in buffers containing calcium. We show here that SP-A1 ($6A^2$) weakly induced a further aggregation of rough LPS in the presence of calcium or even inhibited (in the case of $6A^4$) the aggregation of rough LPS induced by calcium in the absence of any protein, indicating that the $6A^4$ allele could bind but not aggregate rough LPS. By contrast, SP-A2 ($1A^1$ and $1A^2$) was able to foster further aggregation of the rough LPS. However, the ability of SP-A2 to induce aggregation of rough LPS in the presence of calcium was lower than that of native human SP-A (Figure 8).

Figure 6 also shows that the presence of SP-A1 ($6A^4$ and $6A^2$) in coexpressed products ($1A^1/6A^4$ and $1A^2/6A^2$, respectively) reduced the ability of coexpressed SP-A to aggregate rough LPS, compared with that of SP-A2. On the other hand, unfolding of the collagen-like domain of SP-A

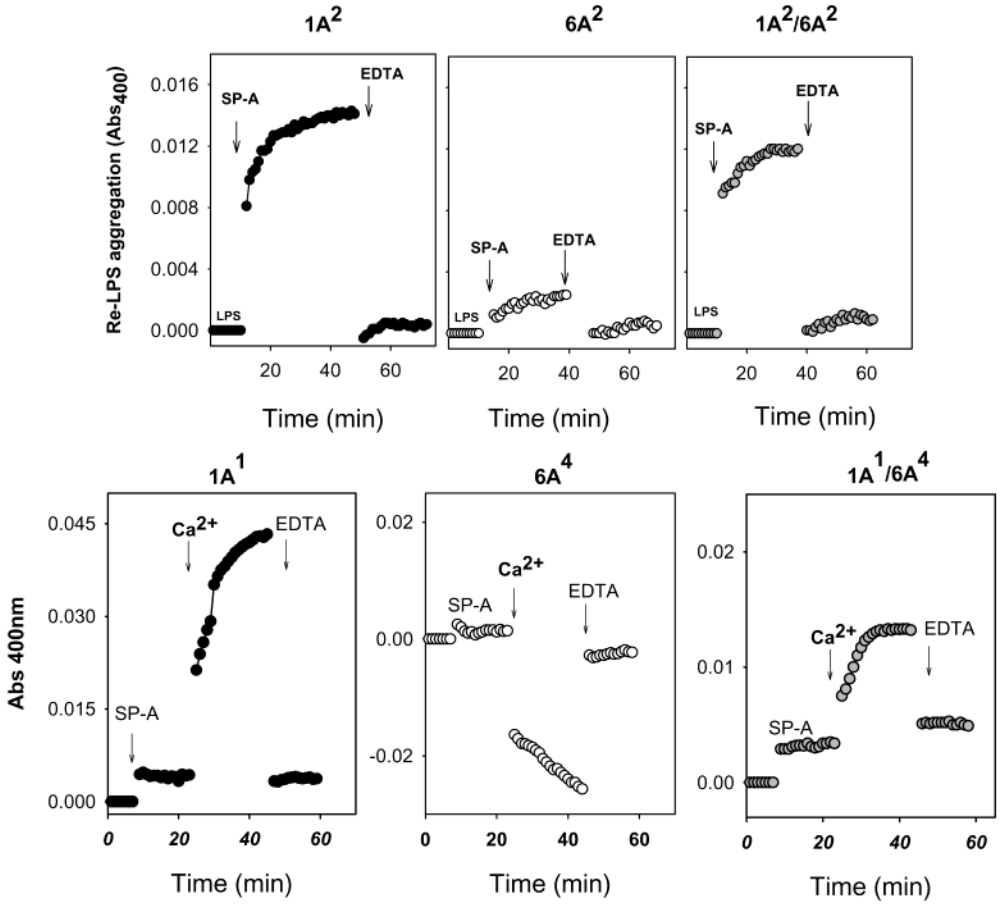

FIGURE 6: LPS aggregation induced by human SP-A variants expressed in insect cells. Bottom panels: sample and reference cuvettes were first filled with LPS (40 or 20 μg/mL final concentration) in 25 mM Tris, 150 mM NaCl, and 0.2 mM EDTA buffer at pH 7.2. After a 10-min equilibration at 25 °C, SP-A (20 fg/mL) was added to the sample cuvette, and the change in optical density at 400 nm was monitored. Next Ca^{2+} (2.5 mM) was added to both the sample and reference cuvettes, and the change in absorbance was monitored again. Ca^{2+}-dependent LPS aggregation was reversed by adding EDTA (5 mM). Upper panels: sample and reference cuvettes were first filled with Re-LPS and 2.5 mM Ca^{2+}. After a 10-min equilibration, SP-A was added to the sample cuvette and the change in optical density at 400 nm was monitored.

variants at 37 °C abolished the activity of recombinant SP-A variants in inducing rough LPS aggregation (data not shown).

Ca^{2+}-Dependent Phospholipid Vesicle Aggregation Induced by Recombinant Human SP-A Expressed in Insect Cells. Figure 7 indicates that the ability of SP-A2 variants ($1A^1$ and $1A^2$) to induce DPPC/DPPG vesicle aggregation was greater than that of coexpressed SP-A1/SP-A2 variants ($1A^1/6A^4$ and $1A^2/6A^2$), which in turn was greater than that of SP-A1 ($6A^4$ and $6A^2$). Subtle differences between SP-A2 alleles ($1A^1$ and $1A^2$) and SP-A1 alleles ($6A^2$ and $6A^4$) also exist. In contrast to self-aggregation and LPS aggregation activities, phospholipid vesicle aggregation mediated by SP-A variants occurred at both 25 and 37 °C. A potential explanation for this result comes from the studies of Bi et al. (*41*), who indicated that the combined presence of both DPPC and calcium confers a remarkable thermal stability upon SP-A studied by infrared transmission spectroscopy. The frequencies of the two dominant bands at 1653 and 1636 cm^{-1}, arising from the collagen domain of native porcine SP-A, remain essentially unchanged from 25 to 70 °C in the presence of both DPPC and calcium but not in the absence of any of these components (either DPPC or Ca^{2+}).

Native Human SP-A. Figure 8 shows the ability of native human SP-A to self-aggregate (panel A) and to induce DPPC/DPPG aggregation (panel B) and Re-LPS aggregation (panel C) for comparison with recombinant SP-A variants. All of these experiments were performed at 37 °C, at lower protein concentration, and at higher lipid/SP-A weight ratio (10:1). The ability of native SP-A to self-aggregate and to aggregate phospholipid vesicles and Re-LPS was higher than that of SP-A2 variants ($1A^1$ and $1A^2$).

DISCUSSION

In humans there are two functional genes (*SP-A1* and *SP-A2*) (*42*, *43*) corresponding to two different SP-A cDNA sequences (*44*). The nucleotide sequence differences between the two human genes that result in amino acid changes are located in the signal peptide and collagen-like and CRD domains of the resulting proteins (*5*−*7*). Once the signal peptide sequence is cleaved during the N-terminal processing of SP-A, the "core" amino acid differences between SP-A1 and SP-A2 alleles are located in the collagen domain (*7*). The functional importance of having two distinct chain types in the collagen-like region of SP-A is undefined. The production of recombinant SP-A derived from single genes (*SP-A1* or *SP-A2*) and coexpressed SP-As derived from both genes presents a unique opportunity to explore changes in collagen structure and protein activities as a result of a lack or overabundance of one of the functional human SP-A polypeptide chains.

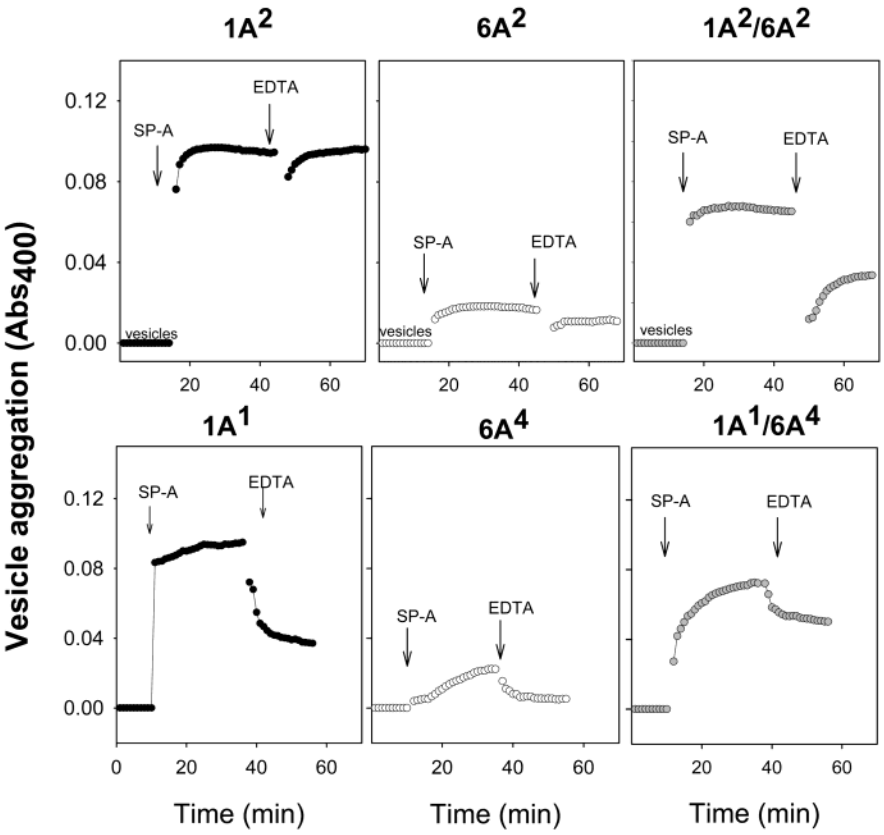

FIGURE 7: Vesicle aggregation induced by human SP-A variants. Sample and reference cuvettes were first filled with DPPC/DPPG (7:3 w/w) vesicles (100 μg/mL) in a buffer containing 2.5 mM Ca^{2+}, 150 mM NaCl, and 25 mM Tris-HCl buffer (pH 7.2). After a 10-min equilibration, SP-A (20 fg/mL) was added to the sample cuvette and the change in optical density at 400 nm was monitored.

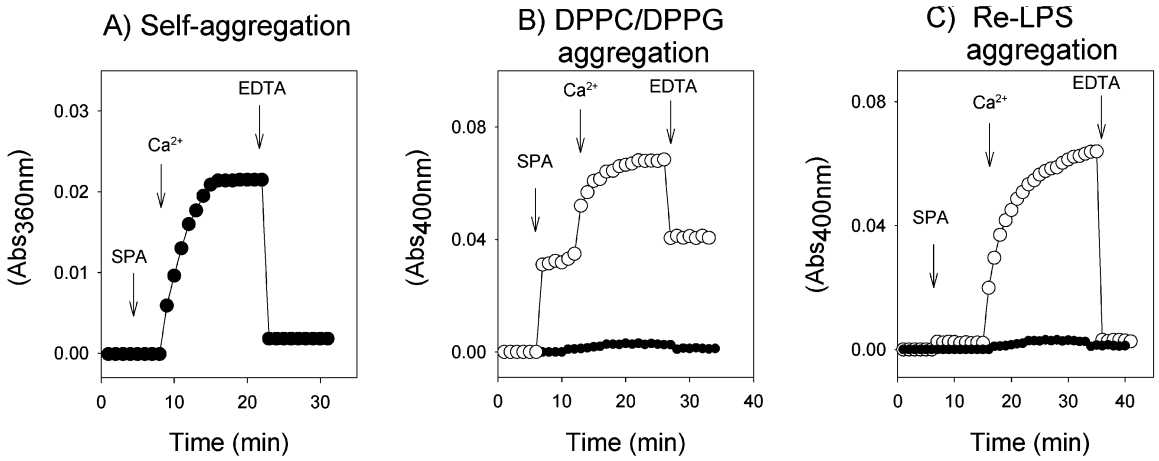

FIGURE 8: Self-aggregation and vesicle and Re-LPS aggregation induced by native human SP-A. (A) SP-A (20 μg/mL) was added to the sample cuvette filled with 5 mM Tris-HCl buffer (pH 7.2). The turbidity change at 360 nm was monitored at 37 °C at 1-min intervals. After stabilization, 5 mM Ca^{2+} (final concentration) was added to both the sample and reference cuvettes, and the turbidity changes were monitored again. Addition of EDTA (10 mM final concentration) dissociated SP-A aggregates induced by Ca^{2+}. (B) Sample and reference cuvettes were first filled with DPPC/DPPG vesicles (50 μg/mL final concentration) in 25 mM Tris, 150 mM NaCl, and 0.2 mM EDTA buffer at pH 7.2. After a 10-min equilibration at 37 °C, SP-A (5 μg/mL) was added to the sample cuvette, and the change in optical density at 400 nm was monitored. Next, Ca^{2+} (2.5 mM) was added to both the sample and reference cuvettes, and the change in absorbance was monitored again. Ca^{2+}-dependent vesicle aggregation was reversed by adding EDTA (5 mM). (C) SP-A-induced Re-LPS aggregation assays were performed as described above for vesicle aggregation. The final concentrations of native human SP-A, Re-LPS, Ca^{2+}, and EDTA were 5 μg/mL, 50 μg/mL, 2.5 mM, and 5 mM, respectively.

SP-A1, SP-A2, and coexpressed SP-A1/SP-A2 variants under native gel electrophoresis conditions have different patterns of oligomerization, with SP-A1 found mainly in trimers (3X) and hexamers (6X) and SP-A2 found in dimers (2X), tetramers (4X), nonamers (9X), and higher size forms. Coexpression of both gene products generated mainly oligomers with 18 polypeptide chains, suggesting that in vitro coexpressed gene products favor largely formation of higher size forms. The relative contribution of SP-A1 and SP-A2 in the coexpressed SP-A variants is currently unknown and requires further investigation However, although the functional significance of SP-A1 and SP-A2 oligomers remains

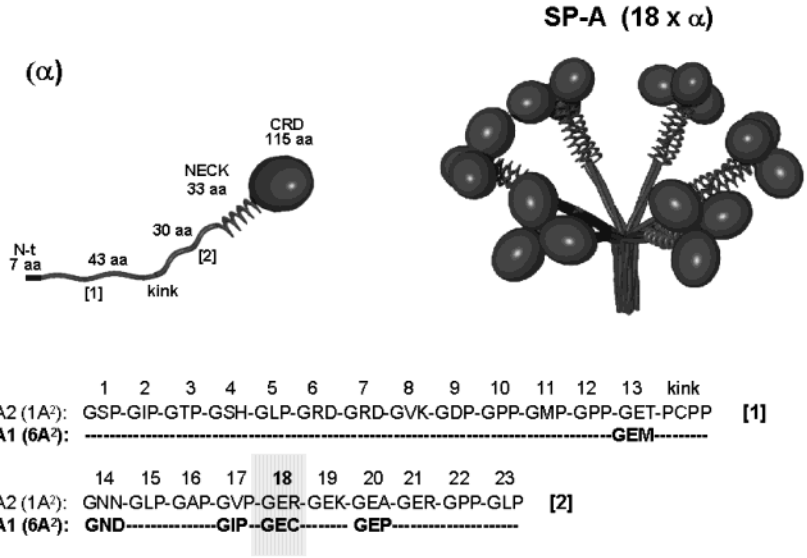

FIGURE 9: Schematic model and GXY triplet sequences of the collagen domain of SP-A1 and SP-A2. A three-dimensional model of the oligomeric form of SP-A (left) and a polypeptide chain of the protein (right) are shown. Trimers of SP-A are each built up by the association of three polypeptide chains, the collagen regions of which intertwine to form a collagen triple helix. A total of six trimers may associate by the microfibril end piece to form the characteristic "bunch of tulips". Human SP-A may be a heterooligomer of both SP-A1 and SP-A2 homotrimers; alternatively, human SP-A might be a homooligomer of heterotrimers composed of two different polypeptide chains, SP-A1 and SP-A2. The collagen domain of both polypeptide chains consists of 23 GXY triplets with a sequence irregularity (kink) between triplets 13 and 14. Differences in GXY triplets between SP-A1 and SP-A2 are shown.

to be determined, functional differences between SP-A1 and SP-A2 alleles have been observed (*22*, *23*) and loss of higher size oligomers has been associated with disease (*20*).

Circular dichroic spectroscopy was used for the examination of triple helical structures. The spectra of SP-A1 and SP-A2 gene products had similar shapes, but differences in the midpoint transition "melting" temperature between recombinant human SP-A1 and SP-A2 variants were observed, with SP-A2 being apparently structurally more stable than SP-A1. Recombinant human SP-A variants from insect cells exhibited a lower melting temperature of the collagen-like domain compared to native human SP-A, a likely consequence of the defect in posttranslational hydroxylation of proline residues. Rat SP-A expressed from baculovirus-mediated insect cells lack proline hydroxylation (*45*). The T_m values determined by CD analysis were around 30, 33, and 48 °C for recombinant SP-A1 and SP-A2 produced in the baculovirus system and native SP-A, respectively. The apparent melting temperature of coexpressed SP-A1/SP-A2 produced in insect cells is around 30 °C, indicating that thermal stability is not influenced by oligomerization but mainly depends on hydroxylation of proline residues. Lamberg et al. (*46*) reported that the T_m of recombinant type III collagen produced in the baculovirus system increased from 32−34 to 40 °C after coexpression with both the α and β subunits of prolyl 4-hydroxylase, indicating that when the prolyl hydroxylation is enhanced to a normal level, the T_m of recombinant type III collagen increases. In a comparable situation, the formation of trimeric, disulfide cross-linked type XII minicollagen was considerably increased in a baculovirus expression system after the coexpression of both subunits of propyl 4-hydroxylase (*47*). These data are of importance for considering production of recombinant SP-A in the baculovirus system for use either as a part of surfactant therapy or as a drug itself. Recombinant SP-A would be very unstable at physiological temperatures since the T_m of recombinant SP-A expressed in insect cells is lower than body temperature. Recent studies (*48*) show that the collagen-like region of SP-A is required for correction of surfactant structural and functional defects in SP-A deficient mice.

Examination of the collagen-like sequence of SP-A suggests that the distribution of amino acids between positions X and Y in the Gly-X-Y repeats does not occur by chance. This could be due to the nonequivalence of the X and Y positions in the triple helix, in terms of solvent exposure and interactions with neighboring chains (*49*, *50*). Thus, it is useful to consider the Gly-X-Y triplet as the basic unit, rather than individual amino acids. Figure 9 shows the sequence of Gly-X-Y triplets for the collagen-like domains of SP-A1 and SP-A2. The major differences between the SP-A1 and SP-A2 alleles are in the collagen triplets above the kink. The fibrillike end pieces of SP-A1 and SP-A2 are almost similar. The collagen-like domains of both SP-A1 and SP-A2 contain 13% of the hydrophobic residues. Leu, Val, and Ile (90% of the hydrophobic residues) are exclusively in the X position of Gly-X-Y triplets. The placement of hydrophobic residues in the X position is also found in different collagen types (*51*). The preference for hydrophobic amino acids in the X position could favor intermolecular hydrophobic interactions since the X position has greater solvent accessibility (*51*). On the other hand, hydrophobic residues do not stabilize the triple-helical conformation. Taken together, we reasoned that the change in triplet 17 [Gly-Val-Pro (SP-A2)/Gly-Ile-Pro (SP-A1)] probably does not have any effect. However, the change in triplet 18 [Gly-Glu-Arg (SP-A2)/Gly-Glu-Cys (SP-A1)] would likely decrease the local stability of the SP-A1 triple helix above the kink.

The data set on stability of individual tripeptide units is now large enough to be useful in predicting local triple-helix stability for triple helix-containing proteins (*51*−*55*). Studies on host−guest triple-helical peptides with a single Gly-X-Y

embedded within Ac(Gly-Pro-Hyp)$_3$-Gly-X-Y-(Gly-Pro-Hyp)$_4$Gly-Gly-NH_2, with double substitutions in both X and Y positions of the guest triplet, point to a high stability of the host−guest peptide containing the guest triplet Gly-Glu-Arg (*52*, *53*). It is reasonable to think that the $Arg^{85} \rightarrow Cys^{85}$ substitution in SP-A1 results in local destabilization on the SP-A1 triple helix above the kink. Cys, in its reduced form, has moderate stability and yet is never found in fibril-forming collagens, except in pathological conditions (*56*). We postulate that $Arg^{85} \rightarrow Cys^{85}$ substitution in SP-A1 affects local stability on the triple helix above the kink. Regions of lower stability may undergo "microunfolding", defined as reversible local structural perturbations (*54*). The analysis of SP-A activities that depend on the collagen domain is consistent with this hypothesis. Native human SP-A undergoes self-aggregation in the presence of calcium (*57*). Temperature-dependent experiments indicate that a structurally intact collagen-like domain is required for Ca^{2+}-induced self-aggregation of the protein (*24*). SP-A may self-aggregate by the microfibril end piece of the collagen-like domain or between globular heads of oligomers. In the latter case, a structurally intact collagen domain would be required for the grouping and orientation of globular heads in the oligomer. We found that SP-A1 hardly self-associated at 50 μg/mL, and the ability of SP-A2 to self-aggregate was greater than that of coexpressed SP-A1/SP-A2 variants. Moreover, the presence of either homotrimers of SP-A1 or heterotrimers containing SP-A1 polypeptide chains in coexpressed products reduces the self-aggregation activity of the entire oligomer. In addition, differences in CRD between SP-A2 alleles ($1A^2$ and $1A^1$) may account for differences observed between these alleles in their ability to self-aggregate.

SP-A binds rough LPS via the lipid A moiety and induces aggregation of rough LPS in the presence of calcium (*58*). Aggregation of rough LPS or Gram-negative bacteria that express the rough LPS phenotype could be important to prevent adherence of endotoxins or bacteria to the alveolar epithelium and to facilitate phagocytosis of large aggregates by alveolar macrophages. We show here that the stability of the collagen domain and the degree of oligomerization may play an important role for LPS recognition and subsequent LPS aggregation in the presence of calcium: (1) The ability of SP-A2 variants to induce LPS aggregation was slightly greater than that of coexpressed SP-A1/SP-A2 variants, which in turn was much greater than that of SP-A1. (2) SP-A-induced LPS aggregation was inhibited by unfolding of the collagen-like domain of SP-A variants at 37 °C. (3) At temperatures lower than T_m for the collagen domain of SP-A variants, the ability of these proteins to aggregate LPS was low in comparison with native human SP-A. The location of the LPS binding site of SP-A is not yet defined, but it is thought to be in the globular C-terminal domain. Oligomerization of SP-A presumably enhances its binding affinity to LPS. Our findings suggest that instabilities in the collagen domain could affect the orientation and/or flexibility of the globular heads of SP-A in relation to LPS binding and aggregation. On the other hand, SP-A1 allele differences were observed, with the $6A^4$ (SP-A1) allele even showing an inhibition of LPS aggregation. It is possible that differences in CRD account for SP-A1 allele specific differences.

The ability of SP-A variants expressed in insect cells to aggregate phospholipid vesicles was lower than that of native human SP-A. Phospholipid vesicle aggregation mediated by SP-A2 was slightly greater than that mediated by SP-A1/SP-A2 oligomers, which in turn was greater than that mediated by SP-A1. These results indicate that the stability of the collagen-like domain and the degree of oligomerization might also influence this process. However, it has been reported (*59*) that the collagen-deletion mutant ΔG8−P80 of rat SP-A expressed in the baculovirus system was as capable of aggregating phospholipid vesicles as wild-type rat $SP\text{-}A^{hyp}$, although not as effective as rat SP-A, suggesting that the collagen domain is not implicated in this process. Interestingly, these authors recently found that overexpression of the collagen-deletion mutant ΔG8−P80 of rat SP-A in the $SP\text{-}A^{-/-}$ mouse at levels that were comparable to mouse SP-A in $SP\text{-}A^{+/+}$ cannot restore tubular myelin formation (*48*), a structural form of surfactant where SP-A and phospholipids are essential components. In addition, they found that the collagen-like region of SP-A is required for avid surfactant association and for protection of surfactant from serum protein inhibition.

SP-A self-aggregation and SP-A-induced LPS and lipid aggregation depend on the binding of Ca^{2+} to the globular domains of SP-A. The two conserved Trp residues (located at positions 211 and 233) are located in a hydrophobic cluster that holds together the carbohydrate/calcium binding region. We found that the change in tryptophan fluorescence of SP-A1, SP-A2, or coexpressed variants upon addition of calcium was similar in all three and similar to that of native human SP-A, indicating that there are not structural differences in the calcium-binding region of SP-A variants.

In summary, we found that (1) recombinant human SP-A variants expressed in insect cells, derived from one gene (*SP-A1* or *SP-A2*) or both genes, differ in the relative extent and heterogeneity of oligomerization; (2) according to circular dichroic and fluorescence spectroscopic studies, there are structural differences between SP-A variants in the collagen-like domain, with SP-A2 being more stable than SP-A1, but not in the calcium-binding region; (3) the ability of SP-A to undergo self-aggregation and induce phospholipid and bacterial lipopolysaccharide aggregation is greater for SP-A2 than for coexpressed SP-A/SP-A2, which in turn is greater than that observed for SP-A1; and (4) differences to a varying degree between SP-A1 or SP-A2 alleles are evident in the assays performed. These data indicate that SP-A variants exhibit differences in their biochemical properties and these may have an impact on functional capabilities.

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