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Effect of Cys⁸⁵ on biochemical properties and biological function of human SP-A variants

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Abstract

Four “core” amino acid differences within the collagen-like domain distinguish the human surfactant proteins A1 (SP-A1) variants from the SP-A2 variants. One of these, cysteine 85 that could form intermolecular disulfide bonds, is present in SP-A1 (Cys⁸⁵) and absent in SP-A2 (Arg⁸⁵). We hypothesized that residue⁸⁵ affects both structure and function of SP-A1 and SP-A2 variants. To test this, wild type (WT) variants, 6A² of SP-A1 and 1A⁰ of SP-A2, and their mutants (6A^{2(C85R)} and 1A^{0(R85C)}), were generated and studied. We found: 1) Residue⁸⁵ affected the binding ability to mannose and the oligomerization pattern of SP-As. The 1A^{0(R85C)} and 6A^{2(C85R)} patterns were similar and/or resembled those of WT 6A² and 1A⁰, respectively. 2) Both SP-A WT and mutants differentially induced rough LPS and *Pseudomonas aeruginosa* aggregation in the following order: 1A⁰ > 6A² > 6A^{2(C85R)} > 1A^{0(R85C)} for Re-LPS aggregation, and 1A⁰ > 6A² = 6A^{2(C85R)} = 1A^{0(R85C)} for bacterial aggregation. 3) SP-A WT and mutants enhanced phagocytosis of *P. aeruginosa* by rat alveolar macrophages. Their phagocytic index order was: 6A^{2(C85R)} > 1A⁰ > 6A² = 1A^{0(R85C)}. The activity of mutant 1A^{0(C85R)} was significantly lower from WT 1A⁰ but similar to 6A². Compared to WT 6A², the 6A^{2(C85R)} mutant exhibited a significantly higher activity. These results indicate that SP-A variant/mutant with Arg⁸⁵ exhibits higher ability to enhance bacterial phagocytosis than that with Cys⁸⁵. Residue⁸⁵ plays a important role in the structure and function of SP-A, and is a major factor for the differences between SP-A1 and SPA2 variants.

Keywords

Aggregation; Macrophage; Oligomerization; Phagocytosis; *Pseudomonas aeruginosa*; Surfactant protein A (SP-A); Variant

Human surfactant protein A (hSP-A) is encoded by two functional genes, *SP-A1* and *SP-A2*. More than 30 variants have been characterized (1), where 10 of them (four *SP-A1* and six *SP-A2*) appear with >1% frequency in humans (2). The SP-A variants are classified on the basis of nucleotide differences in the coding sequences. Functional differences among these variants have been documented, including differences in their ability to stimulate THP-1 cells to produce TNF- α (3–5), inhibit surfactant secretion (6), enhance bacterial phagocytosis by rat

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and human alveolar macrophages (7,8), and bind carbohydrates (9), as well as in oligomer pattern formation (6,10).

Voss, *et al* (11) has suggested that native SP-A from bronchoalveolar lavage (BAL) fluid is an octadecamer consisting of six trimers, with each trimer consisting of two SP-A1 molecules and one SP-A2 molecule. However, the SP-A1 to SP-A2 ratio at the mRNA level (12) and at the protein level (13) differed from the 2:1 proposed ratio. Given the observed functional differences between SP-A1 and SP-A2 *in vitro* expressed variants, it is possible that the overall SP-A activity in the lung depends on the relative levels of SP-A1 and SP-A2 rather than the total SP-A content. Therefore, it is important to understand the basis for these functional differences, as well as mechanisms underlying regulatory differences between SP-A1 and SP-A2.

SP-A is a C-type lectin or collectin, possessing four domains: an N-terminal region, a collagen-like domain, a neck region, and the carbohydrate recognition domain (14). Human SP-A1 and SP-A2 variants collectively differ among themselves by 10 amino acid residues. However, all SP-A1 variants are distinguished from all SP-A2 variants at four “core” amino acid residues located within the collagen-like domain. One of these residues is a cysteine at position 85 present in SP-A1 and absent in SP-A2 (which has an arginine instead) (15). Our previous work has revealed that the pattern of oligomerization of SP-A2 variants (1A, 1A⁰, 1A¹) determined by PAGE gel under nonreducing conditions and native conditions exhibits remarkable differences from that of SP-A1 (6A, 6A², 6A⁴), although differences among SP-A1 or SP-A2 variants were minimal (6). Furthermore, different structural and lipid-binding properties have also been observed between human SP-A1 and SP-A2 variants *in vitro* expressed from insect cells (10). Moreover, changes of oligomerization of SP-A has been observed in patients of pulmonary diseases. These include allergy to birch pollen (16), cystic fibrosis (17), acute respiratory disease syndrome (18), and alveolar proteinosis (19). We hypothesized that cysteine⁸⁵, one of the four “core” amino acids that distinguish SP-A1 from SP-A2 variants, plays a role in SP-A oligomer formation, and leads to structural and functional differences between SP-A1 and SP-A2 variants.

The goal of the present study was to determine whether cysteine⁸⁵ affects SP-A structure and function. To address this goal mutant constructs of the two most commonly found SP-A1 (6A²) and SP-A2 (1A⁰) variants were generated. The cysteine⁸⁵ in SP-A1 and the arginine⁸⁵ in SP-A2 were switched, while leaving the genetic background of the particular variant intact. Stably transfected cell lines were then generated with each of the four constructs, SP-A1 wild type (WT) 6A² and mutant 6A²(C85R), and SP-A2 WT 1A⁰ and mutant 1A⁰(R85C). SP-A proteins were purified from each of the cell lines and studied with regards to their biochemical properties and their biological function. Gel electrophoresis under several conditions (reducing, nonreducing, and native) was used to assess oligomer pattern formation. Spectrometric assay was used to determine whether the SP-A variants differentially mediate Re-LPS and *P. aeruginosa* aggregation. Light microscopy was used to assess the ability of the variant SPAs to enhance association of *P. aeruginosa* with rat alveolar macrophages.

Materials and Methods

Cell lines and cell culture conditions

A mammalian cell line (T-REx-CHO) purchased from a commercial source (Invitrogen, Carlsbad, CA) was used in this study. The T-REx-CHO cell line, is a CHO cell line that has been stably transfected and expresses the tetracycline repressor protein. In this Tetracycline-Regulated Expression System (T-REx System), a heterologous protein gene was cloned into the expression cassette of vector pcDNA5/TO, transfected into T-REx-CHO cells, and the

heterologous protein was expressed through a tetracycline inducible mechanism. All four SPA variants used in this study were produced using this *in vitro* tetracycline inducible system.

T-REx-CHO cells, before transfection with each SP-A variant, were maintained in Glasgow's modified Eagle's Medium with glutamine (GMEM) plus 10% fetal bovine serum at 37°C in an atmosphere of 5% CO₂, as suggested by the vendor. After transfection with the SP-A variant, the stably transfected T-REx-CHO cell lines were cultured in GMEM plus 10% fetal bovine serum, hygromycin (400 µg/ml), and blasticidin (20 µg/ml) at 37°C in an atmosphere of 5% CO₂. For each SP-A variant production, the cells were grown to a confluence of 80%. Then, the media were replaced with expression media containing tetracycline and ascorbic acid but without FBS, harvested after 5 days, and the collected media were either immediately column purified or frozen at -20°C. In this tetracycline inducible system, following induction of expression with tetracycline, the cell clones could produce a higher yield of SP-A protein compared to that of other expression systems (i.e. pEE14 vector-mediated expression system) (6).

Preparation of SP-A recombinant constructs for expression in T-Rex-CHO cells

Vector pcDNA 5/TO (Invitrogen, Carlsbad, CA) was used along with the T-REx tetracycline-inducible system. Each human SP-A1 (6A²) and SP-A2 (1A⁰) variant was cloned into pcDNA 5/TO vector using restriction enzymes EcoR V and Xho I sites. In brief, 1.34 kb cDNA fragments of human SP-A1 (6A²) and SP-A2 (1A⁰) were amplified with primers 1248 and 1249 (see Table 1) from the respective SP-A cDNA plasmids, available in our laboratory (15). The 1.34kb fragment contained the entire SP-A coding region (about 0.74kb) plus about 0.1 kb of 5'-UTR and 0.5 kb of 3'-UTR. PCR was performed in 1x Buffer, 0.0625 mM of each dNTP, 1 ng/µl of each of the primers, and 3.5 units of Expand high fidelity PCR system (Roche, Mannheim, Germany) in 50 µl final volume under the following conditions, at 94 °C for 2 min, and 30 cycles at 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 1.5 min. The final extension step was at 72 °C for 5 min. The PCR product was digested with EcoR V/Xho I and cloned into pcDNA 5/TO, which had been digested with the same restriction enzymes, to generate recombinant constructs that contained the CMV promoter, two tetracycline operator sequences (TetO₂), and the SP-A cDNA variant (Figure 1). Because the sequences of two tetracycline operators (TetO₂) have been inserted between the TATA box and the transcriptional start site of CMV promoter, the transcriptional activity of hSP-A was inhibited in the absence of tetracycline. When tetracycline was added into the culture medium, human SP-A was expressed in the stably transfected cells by depressing the transcription mechanism. Recombinant DNA was prepared according to standard methods.

Generation of mutant constructs

Two recombinant constructs (pcDNA 5/TO-1A⁰, and pcDNA 5/TO-6A²) were mutated using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Primers listed in Table 1 were synthesized in the Macromolecular Core Facility of the Pennsylvania State University College of Medicine. The mutation processes were carried out according to the manufacture's protocol (Stratagene, La Jolla, CA). The resulting plasmid DNAs were transformed into *E. coli*. The mutated sequences in the plasmid were confirmed by DNA sequencing in the Core Facility of the Pennsylvania State University College of Medicine.

Transfection and selection of stably transfected cell lines

To obtain plasmid DNA for transfection, a large-scale purification of plasmid DNA was performed using the Qiagen plasmid maxi kit (Qiagen, Valencia, CA). Each of the four recombinant constructs was transfected into T-REx-CHO cells with the lipofectamine Plus reagent kit (Invitrogen, Carlsbad, CA) as described by Wang et al (5). Positive clones were selected in the GMEM medium containing 10% fetal bovine serum, hygromycin (400 µg/ml),

and blasticidin (20 µg/ml). Each of the positive clones was isolated and cultured in a separate well of a 6-well plate. SP-A expression for each clone was determined by Western-blot analysis. For subsequent experimentation, we chose clones with a middle level of SP-A expression.

Purification of SP-A variants from *in vitro* expression media and human SP-A

Media from *in vitro* expressed SP-A was defrosted and centrifuged at 150×g for 10 minutes to remove cell fragments and pooled. CaCl₂ (1 M stock solution) was added into the SP-A medium to obtain a final concentration of 2 mM CaCl₂ solution. Each SP-A variant was purified using mannose-affinity chromatography method, as previously outlined (20), and concentrated using Amicon Centriprep concentrators (Amicon, Austin, TX). All procedures were performed either at 4°C or on ice. Human SP-A from BAL fluid obtained from alveolar proteinosis patient were prepared as described previously (6). Protein concentration was determined by Micro-BCA (21) and RNase A was used as the standard. Purified SP-A was stored at -20°C until use.

Gel electrophoresis under reduced, nonreduced, and native conditions

In vitro expressed SP-A variants and native human SP-A from BAL fluid (used as control) were subjected to gel electrophoresis under reduced, nonreduced, and native conditions. Samples were reduced with loading buffer consisting of 0.1 M dithiothreitol, 1 M β-mercaptoethanol, 2% SDS, 0.1 M Tris/HCl (pH 6.8), and 10% glycerol, then denatured for 10 minutes at 95°C and run in 10% polyacrylamide gels for 90 minutes at 90 Volt. For non-reducing SDS-PAGE, samples were combined with loading buffer containing 2% SDS, 0.1 M Tris/HCl (pH 6.8), and 10% glycerol, and denatured for 10 minutes at 95°C. Samples were then loaded into 4–15% polyacrylamide gels and electrophoresed at 90 Volt for one hour, then at 100 Volt for 5 hours. Native PAGE samples were prepared in loading buffer of 0.2 M Tris/HCl (pH 7.5) and 40% glycerol, and electrophoresed at 4°C on 4–20% polyacrylamide gels at 50 Volt for one hour, followed by 36 hours at 110 Volt.

Detection of sugars in glycoprotein by an enzyme immunoassay

To examine the glycosylation pattern of SP-A isoforms, SP-A WT and mutants were subjected to separation by electrophoresis under nonreducing gel condition. The protein was then transferred onto PVDF membrane. The sugars in glycoprotein were then examined according to the experimental protocol of a DIG Glycan Detection kit (Roche Applied Science, Indianapolis, IN 46250). This method is able to detect all sugars of the glycoprotein through oxidation of the adjacent hydroxyl groups in sugars of glycoconjugates to aldehyde groups, and subsequent covalent attachment of digoxigenin (DIG) to the aldehydes. The DIG was recognized by anti-DIG antibody that was conjugated with alkaline phosphatase. The activity of alkaline phosphatase was detected through staining with 4-Nitro-blue tetrazolium chloride (NBT)/5-Bromo-4-chloro-3-indolyl-phosphate (X-phosphate) solution.

Silver staining and Western blot analysis of SP-A protein

SP-A purity was confirmed by silver staining (22). Western blots were performed after transfer of proteins to PVDF membranes (0.2µm pore size, Bio-Rad, Hercules, CA). Blots were incubated with primary rabbit antibody (IgG) to human SP-A at a 1:1000 dilution (4) followed by a secondary incubation with goat anti-rabbit IgG (AP conjugated) at a 1:2000 dilution, and developed with alkaline phosphatase buffer, BCIP, and NBT (Bio-Rad, Hercules, CA).

Lipopolysaccharide aggregation induced by SP-A variants

Rough lipopolysaccharide (Re-LPS) from *Salmonella minnesota* (serotype 595) was ordered from Sigma-Aldrich Chemical Com. (St. Louis, MO). LPS aggregation assay was performed according to a previous protocol (10,23) with a few modifications. In brief, LPS (80 µg/ml) was hydrated for 1 hour at 41°C in 5 mM Tris-HCl buffer (pH 7.2) containing 150 mM NaCl

and 0.1 mM EDTA. The LPS solution was mixed by vortexing for 5 min, and was then sonicated for 2 min. The LPS preparation was further diluted to 20 µg/ml with the same above buffer just before use.

SP-A-induced LPS aggregation was examined at 37°C by measuring the changes in absorbance at 400 nm using a kinetic program of a SpectraMax M2 Microplate reader (Molecular Devices Corporation, Sunnyvale, CA). A 96-well plate was used for this assay. Each well of the plate contained 100 µl of LPS sample or control buffer, and optical density at 400 nm was monitored for 21 min period with 1 min interval for each condition. The plate was agitated, via the kinetic program, briefly before each measurement. The procedure of the assay included four steps: 1) LPS solution was equilibrated and measured at 37°C for 21 min; 2) SP-A (final concentration 10 µg/ml) was added into the sample well and optical density at 400 nm was monitored for 21 min; 3) Ca⁺⁺ (final concentration = 2.5 mM) was added to each of the sample well and the control well, optical density at 400 nm was monitored for 21 min; 4) EDTA (final concentration = 5 mM) was added into each of the sample well and the control well, and same measurement was performed.

SP-A-induced bacterial aggregation assay

A nonmucoid *P. aeruginosa* strain (ATCC 39018) was purchased from the American Type Culture Collection (ATCC, Rockville, MD). Bacterial culture and preparation were carried out as described previously (7). In brief, bacteria were grown on tryptic soy agar plates overnight at 30°C. Bacterial cells on the plates were collected using a sterile bacteriological loop and suspended in a saline buffer (150 mM NaCl, 0.1 mM EDTA, 25 mM Tris-HCl, pH 7.2). After vortex-mixing, agar debris and large bacterial aggregates in the bacterial solution was removed through centrifugation at 250 × g for 5 min. The supernatant was then diluted with the Tris-buffer saline to OD₇₀₀ = 1.0. Bacterial aggregation induced by SP-A variants was evaluated by measuring the change in absorbance in 700 nm using a SpectraMax M2 Microplate reader. Each well of the 96-well plate contained 150 µl of sample or control buffer. Optical density at 700 nm was monitored for 90 min with 3 min interval. To avoid sediment of bacterial aggregates the plate was agitated before each measurement.

Preparation of rat alveolar macrophages

Alveolar macrophages were obtained from male pathogen-free Sprague-Dawley rats (Harlan, Indianapolis, IN) as described previously (7). The animal protocol was approved by the Penn State University Institutional Animal Care and Use Committee. Rats were anesthetized with an IM injection of ketamine (Ketaset, Fort Dodge Animal Health) and xylazine (XYLA-JECT, Phoenix Pharmaceuticals, St. Joseph, MO), then bled, and their tracheas were cannulated and their lungs were lavaged with sterile saline three times. Lavage fluid was collected in tubes on ice, and macrophages were isolated from BAL by centrifugation at 250 × g for 3 minutes at 4°C. Cell pellets were washed three times with RPMI medium at 250 × g for 3 minutes each time. Cells were then counted on a hemocytometer and suspended at 1 × 10⁶ cells/ml in ice-cold RPMI medium.

Association of *P. aeruginosa* with rat alveolar macrophages

The cell-association assay was performed as described by Mikerov et al, (7) a modification of earlier described procedures (24,25). Briefly, 50 µl of both alveolar macrophages (10⁶ cells/ml) and bacteria (10⁸ CFU/ml) in RPMI medium were mixed, and SP-A was immediately added, to a final concentration of 5 or 33 µg SP-A/ml. This mixture was then incubated under “end-over-end” shaking conditions for one hour at 37°C to ensure thorough mixing and prevent adherence of macrophages to the interior of the test tubes. Following incubation, 1 ml of cold PBS was added to stop the phagocytosis and the mixture was centrifuged at 250 g for 8 minutes. To remove unbound bacteria, samples were washed twice more with 1 ml cold PBS and

centrifuged at 250 g for 8 minutes. The alveolar macrophages were then resuspended in 200 μ l of PBS and applied to glass slides using a cytocentrifuge. Slides were stained with the Hema-3 Stain Kit (Fisher Scientific, Pittsburgh, PA) for light microscopy and 200 randomly selected macrophages were assessed on each slide. The phagocytic index was used to describe cell association (7) and was calculated as follows: percentage of bacteria-positive macrophages $\times$ average number of bacteria per bacteria-positive macrophage (7). For each experiment the phagocytic index was expressed as the percentage of the negative control (no SP-A present), with the negative control being set to 100%, and the data expressed as the percentage of the negative control. The same comparisons were made for the number of bacteria-positive alveolar macrophages and for the average number of bacteria per bacteria-positive cells.

Statistics

Statistical analysis was performed using SigmaStat version 2.03. Data for each variant was compared to the other variants and positive and negative controls by one-way ANOVA or *t*-test. Results were considered significant when $P < 0.05$.

Results

Production of hSP-A variants and mutants in the T-REx CHO System

Recombinant constructs, transfection, expression, and purification of recombinant WT and mutant SP-A proteins were done as described in the Methods section. The amino acid differences among the four recombinant proteins under study are shown in Table 2, and SP-A domains and variation of amino acid residues between $1A^0$ and $6A^2$, as well as between $1A^{0(R85C)}$ and $6A^{2(C85R)}$, are shown in Figure 2. In order to generate stably transfected T-REx CHO cell lines, more than twenty stably transfected cell clones from each WT and mutant constructs, i.e. $1A^0$, $6A^2$, $1A^{0(R85C)}$, $6A^{2(C85R)}$ were determined for SPA expression by Western blot analysis. The clones with an appropriate SP-A yield for each SP-A WT and mutants were used for the following SP-A production. Both SP-A WT and mutants could be purified through mannose-affinity chromatography, indicating that both WT and mutants retain the ability to bind to mannose. We also observed that the $6A^2$ protein binds mannose with lower affinity compared to $1A^0$, as shown previously (9). The rate of SP-A recovery after purification through mannose-affinity chromatographic method was about 80% (pure SP-A/total protein in the conditioned medium) for $1A^0$, and about 50% for $6A^2$. Moreover, the mannose binding affinity of $1A^{0(R85C)}$ protein was similar to that of $6A^2$, and differed from that of $6A^{2(C85R)}$, which was similar to $1A^0$. These differences indicate that binding characteristics between $1A^0$ and $6A^2$ are inversely maintained in the respective mutants.

Characteristics of oligomerization of SP-A WT and mutants

As expected, on reduced gels, all four variants produced a major band at approximately 33 kDa, which represents monomers (Fig 3A). A similar size band is present in the hSP-A control. These experiments as well as those with nonreduced and native gels (see below) were performed at least three times. In all cases, consistent and reproducible results were observed in the patterns of oligomerization.

The oligomer pattern observed following electrophoresis of the proteins in nonreduced (Fig. 3B) and native (Fig. 3C) gels indicates differences between the WT variants, as shown previously (6) as well as between the mutants. Under both conditions, the mutants acquired oligomer band patterns that were shared by the WT variant they were changed to at amino acid 85. For example, under nonreducing conditions, the pattern of mutant $1A^{0(R85C)}$ is similar to that of $6A^2$ (Fig. 3B). A 9X oligomer (triangle) present in $6A^2$ and absent in $1A^0$ is detectable in the $1A^{0(R85C)}$ mutant. Three types of oligomers (2X, 3X, 4X and 6X) are present in all variants both WT and mutants (arrow).

Under native conditions, the pattern of mutant $1A^{0(R85C)}$ is similar to that of $6A^2$ (Fig. 3C). A number of different size (9X and higher size) oligomer bands (triangles) present in $6A^2$ and absent in $1A^0$ are now detectable in the $1A^{0(R85C)}$ mutant. The mutant $6A^{2(C85R)}$ acquired a prominent band, representing a 12X oligomer (star); this size band is present in $1A^0$ and absent from the $6A^2$ and $1A^{0(R85C)}$. The $6A^{2(C85R)}$ mutant also differs from the $1A^0$ (as well as the $6A^2$ and $1A^{0(R85C)}$). A high molecular weight band with high intensity (black dot) is lost from the $6A^{2(C85R)}$ mutant, but is present in the other three variants. Other less prominent similarities between $1A^0$ and $6A^{2(C85R)}$ include bands of low intensity (e.g. ~6X and >12X) present in both. These data indicate that Cys⁸⁵ plays an important role in oligomer pattern formation.

Glycosylation patterns of SP-A WT and mutants

To examine the glycosylation patterns of SP-A isoforms of WT $1A^0$ and mutant $1A^{0(R85C)}$, WT $6A^2$ and $6A^{2(C85R)}$, SP-A protein was separated by SDS-PAGE gel electrophoresis under nonreducing condition, and the sugars in SP-A protein were then examined by an enzyme immunoassay. The results shown in Fig. 3D, indicated that all the oligomers of SP-A WT and mutants under nonreducing conditions are glycosylated. Moreover, no major difference was observed in the pattern of glycosylated oligomers (Fig. 3D) when compared to the silver stained gel in Fig. 3B.

Re-LPS aggregation induced by SP-A WT and mutants

To study SP-A-induced LPS aggregation, we used the same type of Re-LPS from *Salmonella Minnesota* (serotype 595) and a similar protocol as described previously (10,23). Figure 4A depicts the pattern of absorbance at 400 nm of Re-LPS (20 µg/ml) solution before and after adding sequentially human SP-A (final concentration at 10 µg/ml), Ca⁺⁺ containing buffer, and EDTA containing buffer. The results showed that human SP-A could induce aggregation at a significantly higher level than the control (no SP-A), indicating the validity of the experimental system. Then, we examined the capacity of SP-A1 and SP-A2 WT and mutants to induce Re-LPS aggregation using the same procedure (Fig. 4B). The SP-A WT and mutants exhibited different capacities in their ability to induce Re-LPS aggregation. In order to compare the activity of the SP-A WT and mutants statistically, the OD values of absorbance at one time point (before adding the EDTA containing buffer) of the SP-A WT and mutants from three independent experiments were analyzed, as shown in Figure 4C. The order of activity among the four variants is: $1A^0 > 6A^2 > 6A^{2(C85R)} > 1A^{0(R85C)}$. These data indicate that Cys⁸⁵ of SP-A has a remarkable effect on SP-A-mediated RE-LPS aggregation.

Bacterial aggregation induced by SP-A WT and mutants

To study SP-A-mediated bacterial aggregation, an SP-A dose-course experiment was first performed as described in the Materials and Methods. We examined the change of the absorbance of *P. aeruginosa* bacterial solution at 700 nm with human SP-A at three concentrations (5, 10, 25 µg/ml) for a 90-min period with 3 min interval. The pattern of change of absorbance of the *P. aeruginosa* bacterial solution showed a gradual decrease of the OD values during the 90 min period in an SP-A concentration-dependent manner (Fig. 5A), indicating that *P. aeruginosa* aggregation may depend on SP-A concentration. The OD value at a 90-min time point with human SP-A 5, 10, 25 µg/ml decreased by 5.2%, 15.9% and 18.2%, respectively, compared with that at the starting point. To avoid SP-A self-aggregation at high SP-A concentrations, we chose an SP-A concentration of 10 µg/ml to further study SP-A WT and mutants. In each experiment, $6A^2$ and $6A^{2(C85R)}$, $1A^0$ and $1A^{0(R85C)}$, hSP-A from BAL (as a positive control), and no SP-A (as a negative control) were investigated. Three independent experiments were carried out. The results from a representative experiment are shown in Figure 5B. The results indicated that all SP-A WT and mutants could induce *P. aeruginosa* aggregation. To compare this capacity statistically, the OD values at the 90-min

time point were analyzed. The results revealed that SP-A WT and mutants, as well as hSP-A had a significantly higher activity than the control (no SP-A) ($p < 0.01$); and $1A^0$ exhibited the highest activity compared to all ($p < 0.05$). Although, no significant difference was observed among the other four SP-As (including hSP-A), $6A^2$ showed higher activity than hSP-A or $1A^{0(R85C)}$ ($p < 0.05$), when all 30 time points were considered in the statistical analysis.

Association of *P. aeruginosa* with rat alveolar macrophages in the presence of human WT and mutant SP-A variants

For each experiment four variants and two controls were used: SP-A1 ($6A^2$ and $6A^{2(C85R)}$), SP-A2 ($1A^0$ and $1A^{0(R85C)}$), human SP-A (hSP-A) from BAL (positive control), and RPMI medium with no SP-A (negative control). Based on our previous work (7,8) two concentrations of SP-A, i.e. 5 and 33 $\mu\text{g/ml}$ were examined in this study. Patterns of phagocytic activity were similar at both concentrations although phagocytic index (PI) at 5 $\mu\text{g/ml}$ of SP-A was lower than those at 33 $\mu\text{g/ml}$ of SP-A (data not shown). The PI from three independent experiments are shown in Figure 6. In these experiments, a concentration of SPA protein of 33 $\mu\text{g/ml}$ was used. hSP-A (positive control) from BAL fluid and each *in vitro* expressed variant enhanced phagocytosis of *P. aeruginosa* by rat alveolar macrophages at a significantly higher level than that of the negative control ($p < 0.01$). hSP-A from BAL fluid also exhibited a significantly higher activity than each of the four variants studied ($p < 0.01$). The PI of SP-A variants and controls are: no SP-A (as negative control) = 100; hSP-A (as positive control) = 1056 ± 237 ; $1A^0 = 582 \pm 77$; $1A^{0(R85C)} = 363 \pm 51$; $6A^2 = 347 \pm 55$; $6A^{2(C85R)} = 737 \pm 138$. When Arg^{85} of the $1A^0$ variant was replaced with Cys^{85} , the ability of the mutant, $1A^{0(R85C)}$, to enhance phagocytosis was significantly lower compared to that of the WT, $1A^0$ ($p < 0.01$). However, the $6A^{2(C85R)}$ mutant where its Cys^{85} was changed to Arg^{85} , exhibited a significantly higher ability than that of WT, either $6A^2$ ($p < 0.01$) or $1A^0$ ($p < 0.05$) (Fig. 6). The phagocytic index order for the WT variants and mutants was: $6A^{2(C85R)} > 1A^0 > 6A^2 = 1A^{0(R85C)}$.

Discussion

SP-A is involved in lung host defense and surfactant-related functions. Previously, we have observed functional (4–8) and structural (3,5,6,10) differences between SP-A1 and SP-A2 variants and have postulated that residue⁸⁵ of the SP-A precursor protein, which is either arginine (SP-A2) or cysteine (SP-A1), accounts for the differences noted. In this study, we show the direct effects of arginine/cysteine⁸⁵ on both structure and function of human SPA. Following the study of two WT, one SP-A1 ($6A^2$) and one SP-A2 ($1A^0$), and two mutant ($1A^{0(R85C)}$, $6A^{2(C85R)}$) SP-A proteins, we observed that, a) the oligomer pattern of each mutant differed from its parent WT variant. It became either nearly identical to the WT variant to which residue⁸⁵ had changed to, as it was the case for the $1A^{0(R85C)}$, or shared oligomer pattern characteristics, as it was the case for the $6A^{2(C85R)}$; b) with regards to SP-A-induced Re-LPS aggregation the SP-A2 $1A^0$ protein was more active than the SP-A1 $6A^2$ (10), and the $6A^{2(C85R)}$ mutant protein was more active than the $1A^{0(R85C)}$; c) although SP-A-induced aggregation of *P. aeruginosa* was higher with $1A^0$ compared to $6A^2$, no significant differences were observed between $1A^{0(R85C)}$ and $6A^{2(C85R)}$; and d) the $6A^{2(C85R)}$ mutant protein showed higher phagocytic activity than $1A^{0(R85C)}$ and WT $1A^0$. Because the only difference between WT SP-A1 or SP-A2 WT and each of their corresponding mutant proteins was amino acid 85, we conclude that the differences (in activity and structure) that distinguish SP-A1 and SP-A2 variants are primarily attributable to residue⁸⁵, and that amino acid differences other than residue⁸⁵ modulate the magnitude and/or quality of the structural and functional differences imparted by residue⁸⁵.

The $1A^0$ and $6A^2$ variants studied here are identical in both the carbohydrate recognition domain (CRD) and the neck region (2) (see Fig. 2A). Thus, differences in structure and function

between 1A⁰ and 6A² and their mutants are due to amino acid differences in the collagen-like domain and/or the N-terminal region. The only amino acid difference in the N-terminal region is in amino acid 19. After the signal peptide cleavage, most SP-A molecules lack residue¹⁹ (i.e. Ala¹⁹ or Val¹⁹), and only a small portion of SP-A molecules contain residue¹⁹, as observed in several independent studies (6,10,26,27). The chemical structures and properties of Ala and Val are similar. Therefore, it is expected that structural and/or functional differences due to Ala/Val¹⁹ should be minimal, and the differences observed in structure and function between SP-A1 and SP-A2 and their corresponding mutants are likely to be due to changes in the collagen-like domain.

The collagen-like region of human SP-A includes amino acids 28 to 100 and consists of a total of 23 Gly-X-Y units and a four-amino acid “kink” unit. Variants 1A⁰ and 6A² differ by a single amino acid at position 66, 73, 81, 85, and 91 (2), and these five amino acid differences reside within triplets 13, 14, 17, 18, and 20 (also see Fig. 2). We have previously speculated (10) that Cys⁸⁵ in triplet 18 of 6A² (Gly-Glu-Cys⁸⁵) provides local “micro-unfolding” and compromises the thermal stability of SP-A1 variants compared to SP-A2 variants (10) and that this may have an impact on SP-A structure and function (3–8). The present data support this hypothesis and indicate that indeed residue⁸⁵ affects structural and functional characteristics of SP-A. For example, the oligomerization pattern of the 1A⁰(R85C) mutant was nearly identical to that of 6A² and their phagocytic activities were similar indicating a structure-function relationship. The 6A²(C85R) mutant, on the other hand, shares characteristics with the oligomer pattern of the 1A⁰ variant, but with distinct differences observed both in the oligomerization patterns and phagocytic activity. The latter indicates that other amino acid differences may modify the quality and magnitude of at least some of the changes imparted by residue⁸⁵. In addition to Gly-X-Y units, 13, 14, 17, and 18, that contain the “core” amino acid differences between SP-A1 and SP-A2, triplet 20 also contains an amino acid difference between variants 1A⁰ and 6A². This is Gly-Glu-Ala⁹¹ and Gly-Glu-Pro⁹¹ for 1A⁰ and 6A², respectively (2). There is a significant difference between Ala and Pro chemical structure and their effect on the collagen triple helix (28–30). Collagen peptide containing hydroxylated Pro at the Y position of Gly-X-Y exhibited a remarkably higher thermal stability (*T_m* = 47.3 °C) than that with Ala (*T_m* = 40.9 °C) (28). Although currently it is unknown whether this difference contributes to SP-A structural and/or functional differences observed, future experimentation of SP-A2 variants 1A and 1A⁰ could shed light on this, as these two variants (after the cleavage of the signal peptide) differ only within amino acid 91 (1).

The importance of the collagen-like region in SP-A function has been noted previously. SP-A^{-/-}ΔG8-P80 transgenic mice (that lacked the collagen-like domain) could not restore tubular myelin formation, or provide resistance of isolated surfactant to serum protein inhibition (31). In the present study, amino acid changes within the collagen domain contributed to functional differences. This may occur either directly, via changes of the collagenous tails and their ability to bind receptors, such as Calreticulin/CD91 complex (32,33), or indirectly. In the latter, structural changes in the collagen-like domain could bring about changes in the ability of the CRD domain to bind cell surface receptors, for instance, TLR4 (34), SIRPα (33). Although SP-A interacts with other cell surface receptors on the macrophage, such as C1qRp (CD93) (35,36), SPR210 (37,38), and TLR2 (39) to promote microbial or ligand phagocytosis, it is currently unknown which domain(s) of SP-A are involved in these processes.

In other systems (40), a specific sequence of two Gly-X-Y triplets, GEKGEP, just below the kink in the collagen-like domain of the mannose binding lectin (MBL) has been identified as a critical site on MBL for enhancement of phagocytosis. This sequence mediates enhancement of phagocytosis via C1qRp (40). Following comparison of other defense collagens a consensus motif, GE(K/Q/R)GEP, was identified as playing an important role in phagocytosis (40). Although the specific motif is present in the SP-A1, WT (6A²) and mutant (6A²(C85R))

sequences studied here (See Fig. 2B) (and absent from SP-A2), their function is not advantaged by the presence of the motif. In fact, the SP-A1 molecules exhibited lower activity with regards to their ability to enhance phagocytosis compared to SP-A2. The GEKGEP in SP-A1 is located above the kink whereas in MBL is below the kink. It is possible that a) the location of this motif within the collagen-like domain plays a more important role than the sequence itself, b) the motif identified in MBL, although important for function, is specific for that protein, and/or c) other factors in SP-A1 counteract the effect of the motif.

In a previous study, we had observed that *in vitro* expressed SP-A variants from insect cells exhibited different capacities in their ability to induce Re-LPS and lipid aggregation (10). The present results of *in vitro* expressed SP-A variants from mammalian cells confirm the previous observation, indicating that the protein backbone plays an important role. Residue⁸⁵ in particular is critical for distinguishing SP-A1 from SP-A2 structural and/or functional differences. Although posttranslational modifications are not essential for the SP-A1 and SP-A2 differences studied, these do contribute to the function and structure of SP-A, as assessed by the requirement of considerably higher protein concentrations for insect cell expressed variants compared to those from mammalian cells (7,8,10). Although the mechanisms, as to how SP-A-induced Re-LPS aggregation or SP-A-mediated enhancement of *P. aeruginosa* phagocytosis occur, are not fully understood, one recent study observed that SP-A not only interacts with LPS but also influences the process on the binding of Re-LPS to CD14 (23). SP-A decreased the binding of Re-LPS to CD14, but not to the LPS-binding protein (LBP), indicating that SP-A modulates Re-LPS response by changing the competence of the LBP-CD14 receptor complex (23). It is possible that SP-A variants differentially affect these processes, resulting therefore in functional differences and presumably in differences in the susceptibility to infection.

The degree of oligomerization of collectins including SP-A, SP-D, and MBL is important for the binding of these molecules to carbohydrate substances, and for their biological activity (26,41–44). Previous studies have provided evidence of the importance of SP-A oligomerization in its function (26). An SP-A1^{ΔAVC,C6S} *in vitro* expressed mutant from CHO cells, in which three amino acid residues within the N-terminal region (residues 18, 19, and 20 of the precursor SP-A molecule) had been deleted and a cysteine at residue 26 was changed to serine, yielded only SP-A trimers. No higher size oligomers were observed. These trimers exhibited a significant decrease of the collagen triple helix thermal stability and lacked the ability to induce Re-LPS aggregation, self aggregation, and to enhance SP-B/SP-C surfactant adsorption (26). Moreover, the collagenase-resistant fragment (only trimer) of SP-A has been identified with a significantly reduced ability to bind to TLR4 and MD-2, indicating the importance of a supratrimeric oligomerization pattern of SP-A in the immunomodulatory function (34). Similarly, several disease-associated genetic variants of MBL were investigated *in vitro* for their oligomeric pattern and function. The variations of these genetic variants were located in the collagen-like region of MBL (45). Most of these genetic variants, even those with a single amino acid change, formed lower size oligomers and had a markedly lower mannan-binding capacity and biological activity compared with the wild-type MBL (45). In the present study, the oligomerization patterns of the SP-A molecules differed as did their function pointing to a structure/function correlation. Together these indicate that oligomerization patterns of lectins and SP-A, in particular, have a significant impact on biological function.

In summary, the results show that residue⁸⁵ plays a key role in the function and structure of SP-A. In specific, this single residue change has a major impact on SP-A oligomerization structure and on the ability of the various SP-A molecules to stimulate LPS aggregation, and phagocytosis of *P. aeruginosa*. Residue⁸⁵ provides the basis of the structural and functional differences observed between SP-A1 and SP-A2 variants. The data also show that amino acid

changes other than residue⁸⁵ can modify the degree and/or quality of the changes brought about by residue⁸⁵. These observations indicate that residue⁸⁵ plays a prominent and an essential role in the gene-specific differences, and that quantitative and/or qualitative differences among the variants of each gene may exist due to other amino acid differences. These changes collectively under certain circumstances may explain individual differences in disease susceptibility.

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Abbreviations

BAL	Bronchoalveolar lavage
BML	mannose binding lectin
CHO cell line	Chinese Hamster Ovary cell line
CRD	carbohydrate recognition domain
Re-LPS	rough lipopolysaccharide
GMEM	Glasgow's modified Eagle's medium
PC	phosphatidylcholine
SP-A	Surfactant protein A
WT	wild type

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Figure 1. Schematic representation of recombinant constructs of rpcDNA5/TO-SP-A

A tetracycline-inducible vector, pcDNA5/TO, was used in this study for expression of human SP-A variants. Two wild-type SP-A constructs (pcDNA5/TO-1A⁰ or pcDNA5/TO-6A²) and two mutant constructs (pcDNA5/TO-1A⁰(R85C) or pcDNA5/TO-6A²(C85R)) were generated as described in the section of Materials and Methods. In this T-Rex system, hSP-A expression was driven by the CMV promoter and the transcriptional activity of hSP-A was induced in the presence of tetracycline.

Figure 2. Schematic presentation of SP-A domains, amino acids, and Gly-X-Y triplets of SP-A variants and mutants

Panel A depicts the domains of the mature SP-A molecule (i.e. after removal of the signal peptide). The signal peptide consists of amino acids, 1 through 18/19/20. The carboxy terminal site of the signal peptide cleavage in *in vitro* expressed hSP-A variants from CHO cells varies, as shown in our previous study (6); the cleavage could occur after amino acid 18, 19, or 20. The four SP-A domains/regions present in the mature molecule are: 1) an N-terminal region (from amino acid 19/20/21 to 27); 2) a collagen-like domain (amino acids 28 -100); 3) a coiled-coil neck region (amino acids 101 - 133); 4) a carbohydrate recognition domain (amino acids 134 - 248). In addition to the four “core” amino acids (66, 73, 81, 85, solid arrows) that distinguish SP-A1 and SP-A2 variants, differences among the 1A⁰, 6A², 1A⁰(R85C), and 6A²(C85R) variants under study exist at amino acids positions 19 and 91 shown by dotline arrows.

Panel B depicts a portion of the collagen-like region that contains the four “core” amino acids. The amino acid sequence of Gly-X-Y triplets 12 -23 is shown for WT 1A⁰ and 6A², and mutants 1A⁰(R85C) and 6A²(C85R). Amino acid differences are noted by the amino acid number of the precursor SP-A. There are a total of 23 Gly-X-Y triplets in the SP-A collagen-like region and a four-residue (PCPP) “kink” between triplets 13 and 14 (in box). Five Gly-X-Y triplets (13, 14, 17, 18, and 20) each contains a different amino acid (shown in bold). The GEKGEP sequence in 6A² and 6A²(C85R) is shown by underline.

Figure 3. Oligomerization and glycosylation patterns of SP-A variants and mutants

SP-A variants (WT and mutants) were expressed from stably transfected T-REx-CHO cell lines and were purified by mannose-affinity chromatographic method. hSP-A purified from human BAL fluid of an alveolar proteinosis (AP) patient was used as a positive control.

Panel A: Patterns of SP-A variants and mutants and hSP-A under reducing conditions. The four SP-A variants (WT, $1A^0$, $6A^2$, and their corresponding mutants, $1A^{0(R85C)}$, $6A^{2(C85R)}$) and AP hSP-A were reduced and subjected to 10% SDS-PAGE electrophoresis, and then protein was detected by silver staining. Numbers on the left denote molecular mass. The arrow on the right indicates SP-A monomer.

Panel B: Patterns of SP-A oligomerization under nonreducing conditions. The four SP-A variants ($1A^0$, $6A^2$, $1A^{0(R85C)}$, $6A^{2(C85R)}$) and two mixed samples of SP-A WT and mutant ($1A^0 + A^{0(R85C)}$, $6A^2 + 6A^{2(C85R)}$) were subjected to 4–15% SDS-PAGE electrophoresis at 4°C at 100 V for 5 hours under nonreducing conditions (2% SDS, heat at 95°C for 10 min) and then followed by silver staining. Numbers on the left denote molecular mass. Designations on the right denote oligomer size. Triangles (Δ) denote the similarity of bands between $6A^2$ and $1A^{0(R85C)}$; arrows ($\leftarrow$) denote similarity of bands in the four SP-A variants.

Panel C: Patterns of SP-A oligomerization under native conditions. The four SP-A variants ($1A^0$, $6A^2$, $1A^{0(R85C)}$, $6A^{2(C85R)}$) were subjected to 4–20% gradient PAGE electrophoresis under native conditions. The protein samples were not reduced by chemicals and heating. Electrophoresis was performed at 4°C at 50 V for 1 hour and 110 V for 36 hours. The proteins were detected by silver staining. Numbers on the left denote molecular mass. Designations on the right denote oligomer size. Triangles (Δ) denote the similarity of bands between $6A^2$ and $1A^{0(R85C)}$; star (*) denotes the band similarity between $1A^0$ and $6A^{2(C85R)}$; and black dot ($\bullet$) denotes the absence of a high intensity band from the $6A^{2(C85R)}$ and the presence of this band in the other 3 variants.

Panel D: Glycosylation patterns of SP-A isoforms under nonreducing conditions. The four SP-A variants ($1A^0$, $6A^2$, $1A^{0(R85C)}$, $6A^{2(C85R)}$) were subjected to 4–15% SDS-PAGE electrophoresis under nonreducing conditions and then the protein was transferred onto DVPF membrane. The components of glycojugates were detected by a DIG Glycan detection method as described in the Materials and Methods. The results indicated that all the oligomers of SP-A WT and mutants under nonreducing conditions are glycosylated. No major difference was observed in the pattern of glycosylated oligomers when compared to the silver stained gel in Fig. 3B. Numbers on the left denote molecular mass. Designations on the right denote oligomer size.

LPS aggregation by human SP-A

LPS aggregation by SP-A variants

Figure 4. SP-A-induced LPS aggregation; comparison among wild type variants and mutants Rough lipopolysaccharide (Re-LPS) (20 $\mu\text{g/ml}$) was prepared in 5 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA buffer (pH 7.2). SP-A-induced LPS aggregation was examined at 37°C using a 96-well plate in a SpectraMax M2 Microplate reader. Each well contained 100 μl of Re-LPS sample or control buffer. The OD value at 400 nm was monitored for 21 min period with 1 min interval for each condition according to the following order: a) basal level of Re-LPS buffer, b) adding SP-A (final 10 $\mu\text{g/ml}$), c) adding Ca^{++} containing buffer (final 2.5 mM), d) adding EDTA containing buffer (final 5 mM). **Panel A:** The pattern of OD₄₀₀ absorbance of Re-LPS aggregation induced by native hSP-A and a control (no SP-A). **Panel B:** The pattern of OD₄₀₀ absorbance of Re-LPS aggregation by the four SP-A variants (1A⁰, 6A², 1A⁰(R85C), 6A²(C85R)). **Panel C:** Comparison of OD₄₀₀ absorbance of Re-LPS aggregation by the SP-A variants (1A⁰, 6A², 1A⁰(R85C), 6A²(C85R)) and native hSP-A at a single time point just before EDTA addition. The data were obtained from three independent experiments. The overall order of the SP-A capacity to induce Re-LPS aggregation is: (1A⁰ > 6A² > 6A²(C85R) = hSP-A > 1A⁰(R85C) > No SP-A).

Bacterial aggregation induced by human SP-A (dose-course experiment)

Bacterial aggregation induced by SP-A variants

Figure 5. SP-A-induced bacterial *P. aeruginosa* aggregation; comparison among wild type variants and mutants

Bacterial cells of *P. aeruginosa* were prepared from an overnight culture on a tryptic soy agar plate. The cells were suspended and diluted to $OD_{700} = 1.0$ with Tris-buffer saline (150 mM NaCl, 0.1 mM EDTA, 25 mM Tris-HCl, pH 7.2). Bacterial aggregation induced by native hSP-A and SP-A variants was evaluated by measuring the OD change at 700 nm using a SpectraMax M2 Microplate reader with shaking. Each well of the 96-well plate contained 150 μ l of sample or control buffer. Optical density at 700 nm was monitored for 90 min with 3 min interval. To avoid sedimentation of bacterial aggregates the plate was agitated before each measurement.

Panel A: The change of OD_{700} absorbance of bacterial *P. aeruginosa* aggregation induced by native hSP-A at 5, 10, 25 μ g/ml. **Panel B:** comparison of OD_{700} absorbance of *P. aeruginosa* aggregation by each of the four SP-A variants (1A⁰, 6A², 1A⁰(R85C), 6A²(C85R)), native hSP-A (10 μ g/ml), and a negative control (no SP-A).

Figure 6. Comparison of SP-A-mediated phagocytic activity among wild-type SP-A variants and mutants

The ability of the four SP-A variants to enhance phagocytosis of *P. aeruginosa* by rat alveolar macrophages was examined as described in methods. A significantly higher level ($p < 0.01$) of phagocytic activity by macrophages is observed in the presence of each of the four SP-A variants (1A⁰, 6A², 1A⁰(R85C), 6A²(C85R)) compared to the negative control (no SP-A), indicating that the *in vitro* expressed SP-A variants functional. The level of activity of *in vitro* expressed SPA variants was lower than that of hSP-A ($p < 0.01$), which was used as positive control. The 1A⁰(R85C) exhibits significantly lower activity than that of the wild type 1A⁰, whereas the mutant 6A²(C85R) exhibits significantly higher level of activity compared to wild type 6A². The overall order of the phagocytic index is, 6A²(C85R) > 1A⁰ > 6A² = 1A⁰(R85C).

Table 1

Primers used in this study

Primer	Sequence (5' to 3')	Comments	
1248	GGGCCGgatacCTGGAGGCTCTGTGTGTGGG	For hSP-A variant cloning into vector pcDNA5/TO, sense	EcoR V
1249	GGGCCCctcgagCTGCCACAGAGACCTCAGAG	For hSP-A variant cloning into vector pcDNA5/TO, antisense	Xho I
1419	CTGGTGTCCTGGAGAGTGTGGAGAGAAGG	Change Arg85 to Cys85 in 1A ⁰ , sense	
1420	CCTTCTCTCCACACTCTCCAGGGACACCAG	Change Arg85 to Cys85 in 1A ⁰ , antisense	
1421	CTGGTATCCCTGGAGAGCGTGGAGAGAAGG	Change Cys85 To Arg85 In 6A ² , sense	
1422	TCCTCTCTCCACGCTCTCCAGGGATACCAG	Change Cys85 to Arg85 in 6A ² , antisense	

¹ Lower case indicates additional sequences present in the given primers. These sequences are restriction enzyme recognition sites.

² Bold letters in the primers show nucleotides that have been changed and mismatched sequence of human SP-A variants.

Table 2

Amino acid differences among SP-A variants and mutants

Variant/mutant	AA19*	AA66	AA73	AA81	AA85	AA91
I ^A ₀	Ala	Thr	Asn	Val	Arg	Ala
I ^A ₀ R85C	Ala	Thr	Asn	Val	Cys	Ala
6A ²	Val	Met	Asp	Ile	Cys	Pro
6A ² C88R	Val	Met	Asp	Ile	Arg	Pro

* Amino acid 19; the numbering of the amino acid position is based on the SP-A precursor protein molecule.