Surfactant Protein A without the Interruption of Gly-X-Y Repeats Loses a Kink of Oligomeric Structure and Exhibits Impaired Phospholipid Liposome Aggregation Ability†

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ABSTRACT: Pulmonary surfactant protein A (SP-A) belongs to the collectin subgroup of the C-type lectin superfamily. SP-A oligomerizes as an octadecamer, which forms a flower bouquet-like structure. A collagen-like domain of human SP-A consists of 23 Gly-X-Y repeats with an interruption near the midpoint of this domain. This interruption causes a kink, but its role remains unknown. To define the importance of the kink region of SP-A, two mutated proteins were constructed to disrupt the interruption of Gly-X-Y repeats: SP-A^{DEL}, which lacks the Pro^{47}-Cys^{48}-Pro^{49}-Pro^{50} sequence at the interruption, and SP-A^{INS}, in which two glycines were introduced to insert Gly-X-Y repeats (*Gly*-Pro^{47}-Cys^{48}-*Gly*-Pro^{49}-Pro^{50}). Electron microscopy using rotary shadowing revealed that both mutants form octadecamers that lack a bend in the collagenous domain. Electrophoretic analysis under nondenaturing conditions and gel filtration chromatography demonstrated that SP-A^{INS} consisted of a large assembly of oligomers whereas SP-A^{DEL} formed mainly octadecamers. Both SP-A^{DEL} and SP-A^{INS} mutants as well as wild-type SP-A bound to liposomes containing dipalmitoylphosphatidylcholine and galactosylceramide at equivalent levels, but the abilities of the mutants to induce phospholipid liposome aggregation were significantly less developed than that of the wild type. The mutants SP-A^{DEL} and SP-A^{INS} augmented liposome uptake by alveolar type II cells and inhibited secretion of phospholipids from type II cells at a level comparable to that of wild-type SP-A. These results indicate that the interruption of Gly-X-Y repeats in the SP-A molecule is critical for the formation of a flower bouquet-like octadecamer and contributes to SP-A's capacity to aggregate phospholipid liposomes.

Lung surfactant is a mixture of phospholipids and proteins which is secreted into air spaces by alveolar type II cells and Clara cells. Surfactant is essential for reducing surface tension to keep alveoli from collapsing during expiration. Surfactant proteins specific to this complex play important roles in the surfactant functions of host defense and modulation of the biophysical properties of the lung. The most abundant hydrophilic surfactant protein is surfactant protein A (SP-A),[1] which plays important roles in innate immune functions of the lung (*1*, *2*). In addition to its immunomodulatory functions, the contributions of SP-A to surfactant homeostasis have been suggested by its in vitro activities. SP-A is a Ca^{2+}-dependent phospholipid binding protein which specifically binds to dipalmitoylphosphatidylcholine (DPPC) (*3*), an essential phospholipid for reducing surface tension at the air−liquid interface, and galactosylceramide (GalCer) (*4*). SP-A initiates aggregation of liposomes containing DPPC in the presence of Ca^{2+} (*5*). The specific interaction of SP-A with alveolar type II cells has been documented, and SP-A inhibits the secretion of surfactant phospholipids and enhances the uptake of liposomes by type II cells, suggesting that SP-A has roles in the regulation of surfactant phospholipid pools in alveolar spaces (*6*, *7*). On the other hand, the precise role of SP-A in pulmonary surfactant homeostasis in vivo is still controversial. Mouse strains with null alleles for SP-A appear to have only modest alterations in surfactant properties and exhibit normal survival and resting respiration, despite defective formation of surfactant tubular myelin (*8*).

SP-A belongs to the collectin subgroup of the C-type lectin superfamily, along with surfactant protein D, mannose-binding lectin (MBL), bovine conglutinin, and the protein CL43. These proteins possess characteristic primary structures that are organized into four domains (*9*): (1) a cysteine-

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[1] Abbreviations: SP-A, surfactant protein A; DPPC, dipalmitoylphosphatidylcholine; GalCer, galactosyl ceramide; MBL, mannose-binding lectin; CRD, carbohydrate recognition domain; wt SP-A, recombinant wild-type human SP-A; PC, phosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; TPA, 12-*o*-tetradecanoyl phorbol 13-acetate.

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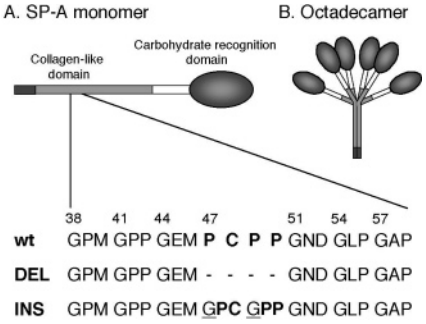

FIGURE 1: Schematic representation of the recombinant SP-A proteins. (A) A monomeric form of SP-A is illustrated. The dark gray region is the cysteine-rich N-terminal domain, the light gray region the collagen-like domain consisting of Gly-X-Y repeats, the white region the neck domain, and the globe region the carbohydrate recognition domain. The sequences in recombinant SP-As are as follows: wt, wild type with the interruption of Gly-X-Y repeats (bold); DEL, SP-A^{DEL} in which amino acids 47−50 were deleted; and INS, SP-A^{INS} in which two glycines were introduced (underlined) to insert Gly-X-Y repeats. (B) A flower bouquet-like octadecamer of SP-A.

containing short amino-terminal segment, (2) a collagen-like sequence, (3) a neck domain, and (4) a carbohydrate recognition domain (CRD). Trimeric association occurs by folding of collagenous domains into triple helices (*10*) and coiled-coil bundling of α-helices in the neck (*11*). Fully assembled SP-A, like MBL and C1q, is a flower bouquet-like octadecamer consisting of six trimeric subunits that are stabilized by the N-terminal sequences and disulfide bonds (*12*). A collagenous domain of human MBL is comprised of 19 repeating Gly-X-Y triplets with an interruption at the eighth triplet that is presumed to form a kink in the extended structure of the molecule (*13*). In human SP-A, a collagenous domain is composed of 23 repeats of the Gly-X-Y motif with a break sequence (Pro^{47}-Cys^{48}-Pro^{49}-Pro^{50}) between the 13th and 14th triplets (*9*). Although this interruption causes a hinge in the SP-A molecule, its precise role remains to be clarified.

The objectives of this study were (1) to determine whether the interruption of Gly-X-Y repeats of SP-A is critical for the formation of a bend in the molecule and (2) to determine whether the SP-A kink region is required for oligomerization and interaction with lipids and alveolar type II cells. In this study, we expressed two mutant SP-As that do not possess the break in Gly-X-Y repeats and assessed the effects of the mutations on the formation of the oligomeric structure and interaction with lipids and type II cells.

EXPERIMENTAL PROCEDURES

Lipids. DPPC was obtained from Avanti (Alabaster, AL). Phosphatidylcholine (PC) from egg yolk, phosphatidylinositol (PI) from bovine liver, phosphatidylglycerol (PG) from egg yolk, GalCer, phosphatidylserine (PS), and cholesterol were purchased from Sigma (St. Louis, MO). 1-Palmitoyl-2-[^{3}H]-palmitoyl-L-3-phosphatidylcholine ([^{3}H]DPPC) was purchased from Amersham (Piscataway, NJ).

DNA Construction of Recombinant SP-A Molecules. The isolation of the 1.13 kb cDNA for recombinant wild-type human SP-A (wt SP-A) was described previously (*14*). The amino acid sequences of two mutant SP-As were designed as shown in Figure 1. In the mutant SP-A^{DEL}, amino acids 47−50 were deleted to remove an interruption in the Gly-X-Y repeat structure. In the mutant SP-A^{INS}, two Gly residues were inserted before Pro^{47} and Pro^{49} to restore the motif of Gly-X-Y repeats. The mutated cDNAs were generated by site-directed mutagenesis (QuickChange mutagenesis kit, Strategene, Cedar Creek, TX). For SP-A^{DEL}, PCR was performed using the mutagenic complementary primers (5′-ATGGGTCCACCTGGAGAAATGGGAAATGATGGGCTGCCTGGA) and wt SP-A cDNA in the pVL1392 vector as a template. The cDNA for SP-A^{INS} was constructed by PCR using the mutagenic complementary primers (5′-CCTGGAGAAATGGGGCCATGTGGGCCTCCTGGAAATGATGGG) and wt SP-A cDNA in the pEE14 vector as a template and inserted into the pVL1392 vector using the *Nco*I sites.

Expression and Isolation of Recombinant Proteins. The recombinant proteins were expressed in the baculovirus-insect cell system, as described by O'Reilly et al. (*15*). *Spodoptera frugiperda* (Sf9) cells were cotransfected with linearized virus DNA (BaculoGold, Pharmingen, San Diego, CA) and the plasmid pVL1392 vector containing the cDNAs for SP-A molecules. Plaques were isolated, and virus titers were amplified three times to approximately $2-6 \times 10^8$ plaque-forming units/mL. The recombinant proteins were expressed in *Trichoplusia ni* (Tni) cells that had been infected at a multiplicity of 2. After incubation for 4−5 days, the recombinant proteins were purified from the culture media by using a mannose-Sepharose 6B affinity column, as described previously (*16*).

Polyacrylamide Gel Electrophoresis. Four micrograms of the proteins was resolved by 13% SDS−PAGE under nonreducing or reducing (1% β-mercaptoethanol) conditions and visualized by Coomassie brilliant blue staining. For native conditions, 5 μg of the proteins was subjected to a NativePAGE Novex 4 to 16% Bis-Tris gel (Invitrogen, Calsbad, CA) and electrophoresed at 4 °C for 2 h.

Electron Microscopy. Recombinant SP-A samples were diluted to 10 μg/mL in 50% glycerol and 20 mM ammonium bicarbonate, sprayed onto freshly cleaved mica, and rotary-shadowed with platinum at an angle of 6° (*17*). The rotary-shadowed molecules were observed under a JEOL JEM 1010 electron microscope operated at 75 kV.

Gel Filtration Chromatography. Size fractionation of the recombinant proteins was performed by gel filtration chromatography using an Econo-Column (1.5 cm × 100 cm) (Bio-Rad Laboratories, Hercules, CA) of 6% agarose beads (Iberagar, Coina, Portugal) in 5 mM Tris buffer (pH 7.4) containing 2 mM $CaCl_2$. Blue dextran (2000 kDa) and ferritin (440 kDa) were used as molecular mass standards. The amount of SP-A proteins in each fraction (1.85 mL) was determined by an ELISA as described below. Fifty microliters of each fraction was coated onto microtiter wells, and nonspecific binding was blocked with phosphate-buffered saline containing 3% (w/v) skim milk and 0.1% (v/v) Triton X-100. Polyclonal antibody raised against human SP-A (*18*) (10 μg/mL, 50 μL/well) was added to the wells and incubated at 37 °C for 1 h. After the wells had been washed, horseradish peroxidase-labeled anti-rabbit IgG (1:1000) was added and incubated at room temperature for 1 h. The peroxidase reaction was finally developed using *o*-phenylenediamine as a substrate, and the absorbance was measured at 492 nm.

Liposome Binding. The binding of the proteins to multilamellar liposomes was performed as described previously

(*16*). The mixture, composed of DPPC, PG, and cholesterol (7:2:1, w/w/w) or of GalCer, PS, and cholesterol (7:2:1, w/w/w), or PI was dried under nitrogen and hydrated in 20 mM Tris buffer (pH 7.4) containing 0.1 M NaCl at 37 °C for 1 h and vortexed vigorously for 5 min to prepare multilamellar liposomes. The multilamellar liposomes (100 μg) and the protein solution [0.2 μg in 50 μL of 20 mM Tris buffer (pH 7.4) containing 0.1 M NaCl, 5 mM $CaCl_2$, and 20 mg/mL bovine serum albumin (binding buffer)] were separately centrifuged at 12 000 rpm and room temperature for 10 min. The supernatant of the protein solution was added to the liposome pellet. The mixtures were suspended and incubated at room temperature for 1 h. After cooling on ice for 15 min, the mixtures were centrifuged at 12 000 rpm and 4 °C for 10 min. The supernatant (50 μL) was stored, and the pellet was washed once with 50 μL of ice-cold binding buffer and centrifuged again. The supernatant was combined, and the pellet was finally suspended with 100 μL of the binding buffer. The amount of SP-A protein in each fraction was determined by a sandwich ELISA using ELSIA-F300 (Sysmex, Kobe, Japan). Control experiments without liposomes were also performed to obtain specific sedimentation.

Liposome Aggregation. Liposome aggregation was performed by a modified method based on that described by Hawgood et al. (*5*). Unilamellar liposomes composed of DPPC, egg PC, and PG (7:2:1, w/w/w) were prepared by probe sonication. The liposomes (300 μg/mL) and the proteins (20, 40, or 80 μg/mL) in 10 mM Tris buffer (pH 7.4) containing 0.15 M NaCl were mixed and preincubated for 90 s. $CaCl_2$ was then added to a final concentration of 5 mM, and the absorbance at 400 nm was measured at room temperature after 10 min had elapsed.

Uptake of Phospholipid Liposomes. Alveolar type II cells were isolated from the lungs of male Sprague-Dawley rats by tissue dissociation with elastase digestion and purification on metrizamide gradients (*19*). Uptake of phospholipids by freshly isolated type II cells was performed by the method described by Wright et al. (*20*). Type II cells (2×10^6 cells) were incubated with radiolabeled phospholipid liposomes (100 μg/mL) composed of DPPC, egg PC, and PG (7:2:1, w/w/w) and 1 μCi of [^{3}H]DPPC in the presence of 5 or 20 μg/mL proteins at 37 °C for 1 h in 0.5 mL of Dulbecco's modified Eagle's medium containing Hepes (pH 7.4). After incubation, the cells and media were separated by centrifugation at 1500 rpm and 4 °C for 5 min. The cells were washed three times with 1 mL of ice-cold phosphate-buffered saline containing 1 mg/mL bovine serum albumin. Before the final centrifugation, the cell suspension was transferred to a fresh tube. The radioactivity associated with the cells was finally counted.

Inhibition of Secretion of Lipid from Alveolar Type II Cells. Rat alveolar type II cells (2×10^6 cells) were incubated overnight with 0.5 μCi/mL of [^{3}H]choline, and secretion of radiolabeled phosphatidylcholine was performed using the purified proteins as antagonists of 12-*o*-tetradecanoyl phorbol 13-acetate (TPA) (10^{-7} M)-stimulated surfactant secretion as described previously (*21*). Following incubation for 3 h, radiolabeled phospholipids were extracted from the cells and media, and radioactivity was counted. Surfactant secretion was expressed as percent secretion (radioactivity in medium/radioactivity in medium plus cells × 100).

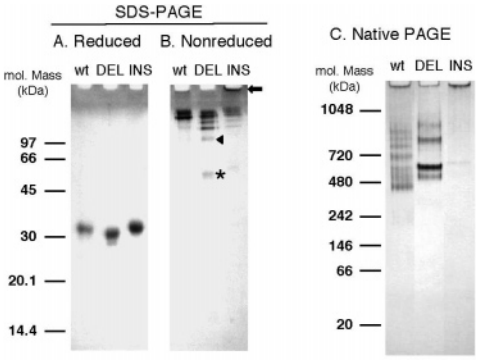

FIGURE 2: Electrophoretic analysis of recombinant SP-As. Recombinant proteins were subjected to 13% SDS−PAGE under reducing (A) or nonreducing (B) conditions or to native PAGE (C). The proteins were visualized by Coomassie Brilliant Blue staining: arrow, large aggregate of SP-A^{INS}; arrowhead, trimer of SP-A^{DEL}; and asterisk, dimer of SP-A^{DEL}.

RESULTS

Effects of Mutations on Biosynthesis and Secretion of SP-As Expressed in Insect Cells. Recombinant wild-type human SP-A (wt SP-A) and the mutants SP-A^{DEL} and SP-A^{INS} in which the interruption of Gly-X-Y repeats was eliminated (Figure 1) were expressed using the baculovirus insect cell system and purified by mannose affinity chromatography. To determine the effects of these mutations on synthesis and secretion of SP-A, the levels of the purified recombinant proteins were assessed and compared. The total amount of SP-A^{DEL} or SP-A^{INS} recovered from 1 L of culture media was 3.85 or 3.73 mg, respectively. These levels were almost comparable to that of wt SP-A, of which as much as 3.01 mg/L of the media was typically obtained. These results indicated that the mutations introduced to disrupt the break in Gly-X-Y repeats did not affect protein expression or secretion of SP-A. The results were consistent with previous studies demonstrating that the recombinant MBL mutants, which do not contain the interruption in the Gly-X-Y sequence, were secreted into media at levels almost equivalent to that of wild-type MBL (*22*, *23*).

Effects of Mutations on Multimer Formation. The purified recombinant SP-As were analyzed by SDS−polyacrylamide gel electrophoresis. When analyzed under reducing conditions, wt SP-A migrated as a band with an apparent molecular mass of 32−34 kDa (Figure 2A). SP-A^{DEL} or SP-A^{INS} were slightly more or less mobile, respectively, when compared with wt SP-A. These differences in molecular mass could be explained by the deletion or insertion of amino acids in the mutants. Consistent with the previous study (*14*), the recombinant SP-As produced in insect cells typically migrated faster than SP-A derived from lung lavage (data not shown), possibly due to the differences in glycosylation between mammalian cells and insect cells and the lack of hydroxylation of proline residues (*24*). Analysis under nonreducing conditions demonstrated that wt SP-A, SP-A^{DEL}, and SP-A^{INS} migrated as oligomers (Figure 2B). Interestingly, SP-A^{INS} mutant appeared to form higher-order multimers than wt SP-A, since some of the SP-A^{INS} proteins did not even enter into the dissolving gel (Figure 2B, arrow). In contrast, the SP-A^{DEL} preparation contained lower-order oligomers, including dimers (Figure 2B, asterisk) and trimers (Figure 2B, arrowhead) under nonreducing conditions. The oligo-

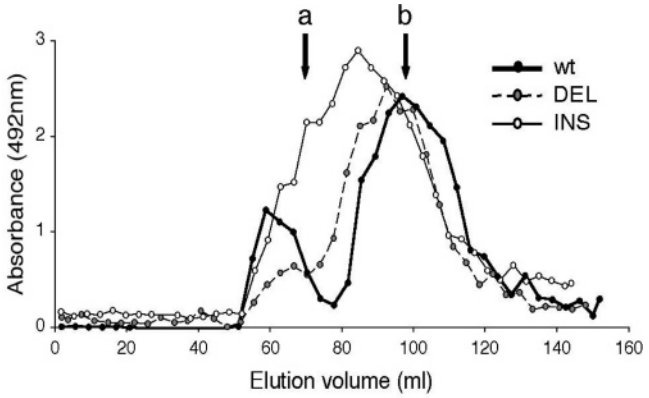

FIGURE 3: Gel filtration chromatography of recombinant SP-As. Wild-type SP-A (●), SP-A^{DEL} (gray circles), and SP-A^{INS} (○) were subjected to a 6% agarose beads column (1.5 cm × 100 cm). The amount of SP-A in each fraction (1.85 mL) was determined by an ELISA as described in Experimental Procedures, and the absorbance at 492 nm is shown. The molecular mass standards are blue dextran (2000 kDa) (a) and ferritin (440 kDa) (b).

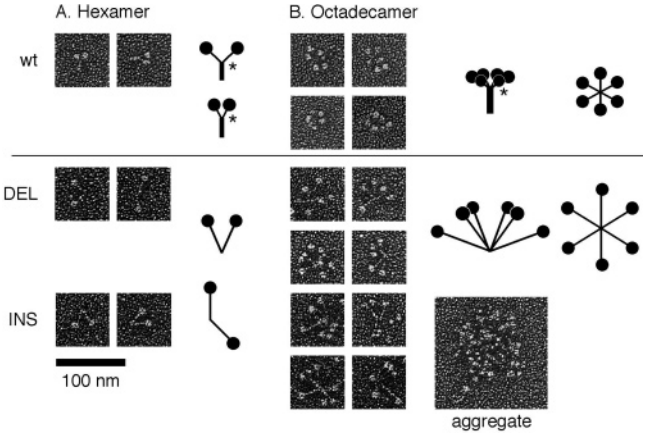

FIGURE 4: Electron micrographs of rotary-shadowed recombinant SP-A molecules. The hexameric form of wt SP-A represents a "Y"-like structure, whereas those of SP-A^{DEL} and SP-A^{INS} represent "V"-like structures. SP-A^{DEL} and SP-A^{INS} form an octadecamer composed of straight-shaped collagenous stalks: asterisk, a kink region; and aggregate, large aggregate observed in the SP-A^{INS} preparation.

meric states of the proteins were further assessed by native PAGE (Figure 2C). Although multiple noncovalently associated oligomers were observed in wt SP-A and SP-A^{DEL} preparations, most of the SP-A^{INS} proteins could not enter into the native gel, suggesting that SP-A^{INS} consisted of a large assembly of the oligomers. A similar large assembly together with multiple orders of oligomerization (430−900 kDa) was observed in the wt SP-A preparation. On the other hand, most of the SP-A^{DEL} proteins entered into the native gel and migrated mainly as a band at approximately 600−630 kDa.

We next determined the oligomeric states of the recombinant proteins by gel permeation chromatography. As shown in Figure 3, two different peaks were observed when wt SP-A was eluted from a column of 6% agarose beads. The peak with a large or small molecular mass was recognized at a position near that of blue dextran or ferritin, respectively. The large assembly of wt SP-A appears to correspond to the proteins that could not enter into the native gel (Figure 2C). The mutant SP-A^{DEL} eluted mainly at a position of an approximate molecular mass of 700 kDa, which seems to be consistent with the result obtained from native PAGE. When compared with wt SP-A, the SP-A^{DEL} preparation contained a significantly smaller amount of large oligomers that were eluted at a position near that of blue dextran. On the other hand, SP-A^{INS} exhibited a unique elution profile. The mutant SP-A^{INS} eluted broadly from the position ahead of blue dextran through the position behind ferritin, suggesting that the protein consists of multiple orders of oligomeric assembly. The estimated molecular mass at the main elution peak of SP-A^{INS} was approximately 1100 kDa, which was significantly larger than those of wt SP-A and SP-A^{DEL}. These results demonstrated that the SP-A^{INS} oligomers formed aggregates. The results appeared to correlate well with the results obtained from native PAGE analysis and SDS−PAGE analysis under nonreducing conditions. Taken together, our findings suggest that the mutations introduced at the interruption in Gly-X-Y repeats may have altered the self-assembly of the SP-A molecules.

Electron Microscopic Observation of the Recombinant Proteins. The interruption of Gly-X-Y repeats in the collagenous domain has been considered to contribute to formation of the kink of SP-A (*12*). To determine whether the interruption of Gly-X-Y repeats is critical for the flower bouquet-like structure of SP-A, electron microscopic observation of the recombinant proteins was performed using the rotary shadowing technique. In the preparation of wt SP-A as well as mutant SP-A^{DEL} and SP-A^{INS}, heterogeneous particles, presumably consisting of two, four, and six trimers, were observed. The representatives of hexamers and octadecamers are shown in Figure 4. As we had expected, the bend in collagenous domains of SP-A^{DEL} and SP-A^{INS} seemed to be abrogated and the mutants had straight-shaped collagenous domains that were laterally assembled at the N-terminus. The octadecamer of wt SP-A formed a flower bouquet-like structure whose diameter was approximately 28 nm, which was consistent with the diameter of a normal subpopulation of SP-A oligomers isolated from patients with alveolar proteinosis (*25*). In contrast, SP-A^{DEL} and SP-A^{INS} formed a radially and widely spread octadecamer whose diameter was estimated to be approximately 60 nm. The results clearly defined the interruption of Gly-X-Y repeats as the kink region of SP-A. In addition, multimerized large oligomers or aggregates were frequently observed in SP-A^{INS} preparations (Figure 4, aggregate) but rarely seen in wt SP-A or SP-A^{DEL} preparations. A quantification of the different visualized species of SP-A^{INS} resulted in approximately 8.8% supra-octadecameric aggregates (15 aggregates in 170 variants). In contrast, only one aggregate (0.2%) was observed in 465 variant species of wt SP-A, and two aggregates (0.6%) were recognized in 308 species of SP-A^{DEL}. These results are consistent with the current data obtained from electrophoretic analysis and gel filtration analysis (Figures 2 and 3) and indicate that more populations of SP-A^{INS} oligomers exist as large aggregates than of wt SP-A and SP-A^{DEL}.

SP-A^{DEL} and SP-A^{INS} Exhibit Impaired Abilities To Induce Liposome Aggregation. We next examined the abilities of the mutants SP-A^{DEL} and SP-A^{INS} to interact with lipids. The binding of the recombinant proteins to multilamellar liposomes containing DPPC, GalCer, or PI was first investigated by the sedimentation method. The mutants SP-A^{DEL} and SP-A^{INS} cosedimented DPPC liposomes to nearly the same extent as wt SP-A, such that 94.5 ± 3.9% (mean ± standard error;

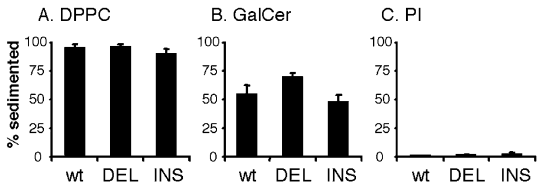

FIGURE 5: Binding of recombinant SP-As to multilamellar liposomes. Multilamellar liposomes (100 μg) containing DPPC, PG, and cholesterol (7:2:1) (A), GalCer, PS, and cholesterol (7:2:1) (B), or PI (C) were mixed with the recombinant proteins (0.2 μg). The amount of SP-As cosedimented with liposomes was determined by an ELISA. The results show specific sedimentation from which values obtained in the absence of liposomes were subtracted. The data are means ± the standard error of three experiments.

$n = 3$) of wt SP-A, 95.9 ± 2.2% of SP-A^{DEL}, and 89.7 ± 4.5% of SP-A^{INS} were recovered in the pellet fraction (Figure 5A). Similarly, the sedimentation with GalCer liposomes was not significantly different among the proteins: 54.7 ± 8.1% of wt SP-A, 70.0 ± 4.0% of SP-A^{DEL}, and 48.1 ± 6.3% of SP-A^{INS} cosedimented with GalCer liposomes (Figure 5B). PI liposomes cosedimented none of wt SP-A, SP-A^{DEL}, or SP-A^{INS} (Figure 5C). These results clearly demonstrate that SP-A^{DEL} or SP-A^{INS} retains the ability to bind liposomes containing DPPC or GalCer. Disruption of the Gly-X-Y repeat interruption does not alter the lipid binding specificity of SP-A. This conclusion seemed reasonable since the C-terminal region of CRD is critical for lipid interaction of SP-A (*16*, *26*).

Since SP-A causes Ca^{2+}-dependent aggregation of phospholipid liposomes containing DPPC, the abilities of the mutants to induce liposome aggregation were also investigated. As shown in Figure 6, wt SP-A induced phospholipid vesicle aggregation in the presence of Ca^{2+} in a time- and concentration-dependent manner, as assessed by the change in absorbance at 400 nm. The mutants SP-A^{DEL} and SP-A^{INS} also caused liposome aggregation, but the maximal turbidity induced by SP-A^{DEL} or SP-A^{INS} was significantly reduced when compared to that induced by wt SP-A. The calculated equilibrium end points for light scattering of SP-A^{DEL} at concentrations of 20, 40, and 80 μg/mL were 49, 60, and 66% of that for wt SP-A, respectively (0.070, 0.150, and 0.235 A_{400} unit vs 0.144, 0.250, and 0.356 A_{400} unit, respectively; $p < 0.005$). The maximal turbidities at SP-A^{INS} concentrations of 20, 40, and 80 μg/mL were 35, 62, and 51% of that of wt SP-A, respectively (0.050, 0.154, and 0.182 A_{400} unit vs 0.144, 0.250, and 0.356 A_{400} unit, respectively; $p < 0.005$). The rate of aggregation, that is, the increase in absorbance at 400 nm per second, was also calculated during the first 60 s after the addition of Ca^{2+} (Table 1). The rate induced by SP-A^{DEL} at concentrations of 20, 40, and 80 μg/mL was significantly decreased compared to that of wt SP-A. Similarly, the rate induced by SP-A^{INS} was significantly reduced at 40 and 80 μg/mL compared to that of wt SP-A. These data indicated that the activity of SP-A in mediating liposome aggregation was attenuated by introducing the mutations at the interruption of Gly-X-Y repeats. Since SP-A^{DEL} and SP-A^{INS} mutants exhibited DPPC bindings at a level comparable to that of wt SP-A (Figure 5), the results were not due to the reduced level of binding of the mutants to liposomes. The loss of a bend in collagenous domains may impair the cross-linking of liposomes that was associated with the globular heads of the mutants.

Interactions of the Mutants with Alveolar Type II Cells. SP-A promotes association of phospholipids with alveolar type II cells in vitro (*20*). We therefore examined whether the mutants SP-A^{DEL} and SP-A^{INS} could mediate liposome uptake. As shown in Figure 7, the association of liposomes with type II cells was significantly promoted in the presence of wt SP-A [specific uptake of 5.5 ± 1.1% at 5 μg/mL and 20.9 ± 2.2% at 20 μg/mL (mean ± standard error; $n = 3$)]. The uptake of liposomes into type II cells was 5.8 ± 1.1 or 20.5 ± 3.7% in the presense of 5 or 20 μg/mL SP-A^{DEL} and 5.6 ± 1.0 or 18.6 ± 1.1% in the presense of 5 or 20 μg/mL SP-A^{INS}, respectively. These results indicate that the activties of the mutants were clearly comparable to that of wt SP-A. The mutants SP-A^{DEL} and SP-A^{INS} retained the ability to mediate uptake of liposomes by type II cells, although their effects on liposome aggregation were significantly reduced (Figure 6).

SP-A inhibits secretion of phospholipids from alveolar type II cells, and the inhibitory activity of SP-A correlates well with the receptor binding activity of SP-A (*27*). As shown in Figure 8, wt SP-A significantly inhibited TPA-stimulated secretion of lipid from type II cells in a concentration-dependent manner. The mutants SP-A^{DEL} and SP-A^{INS} inhibited lipid secretion at levels almost equivalent to that of wt SP-A; the level of TPA-stimulated lipid secretion was decreased to 34.8 ± 4.5, 36.1 ± 1.9, and 28.7 ± 3.9% (mean ± standard error; $n = 3$) in the presense of 1 μg/mL wt SP-A, SP-A^{DEL}, and SP-A^{INS}, respectively. The deletion of the kink in collagenous domains may not alter the SP-A−type II cell interaction.

DISCUSSION

The collagenous domain of the collectins is recognized from the amino acid sequence with its characteristic Gly-X-Y repeats, where X and Y can be any amino acid but are frequently prolines and hydroxyprolines. This study provides the evidence that the Pro^{47}-Cys-Pro-Pro sequence at the interruption of Gly-X-Y repeats is responsible for the formation of a kink in the collagenous domain of SP-A. The mutants SP-A^{DEL} and SP-A^{INS}, in which the interruption in Gly-X-Y repeats was disrupted, were composed of the straight-shaped collagenous domains under electron microscopic observation.

Recombinant SP-A synthesized in insect cells contained little or no hydroxyproline in the collagen-like region (*24*). Hydroxylation of proline residues is required for perfect oligomerization of SP-A and for thermal stability in the interaction with lipid (*24*). However, recombinant SP-A produced in insect cells avidly binds to the mannose affinity column, interacts with alveolar type II cells, inhibits the secretion of phospholipid from alveolar type II cells, and aggregates lipid vesicles at 20 °C in a manner independent of the presence of N-linked carbohydrates (*24*). By using wild-type SP-A and various mutants expressed in insect cells, many studies from this and other laboratories have clarified the required region of SP-A for lipid binding and type II cell interaction (*14*, *16*, *24*, *26*, *28*−*31*). The possibility that neither proline hydroxylation nor the addition of attached carbohydrate is essential for lipid aggregation, receptor binding, or the inhibition of secretion of phospholipid from alveolar type II cells is considered. Therefore, we used an insect expression system for this study.

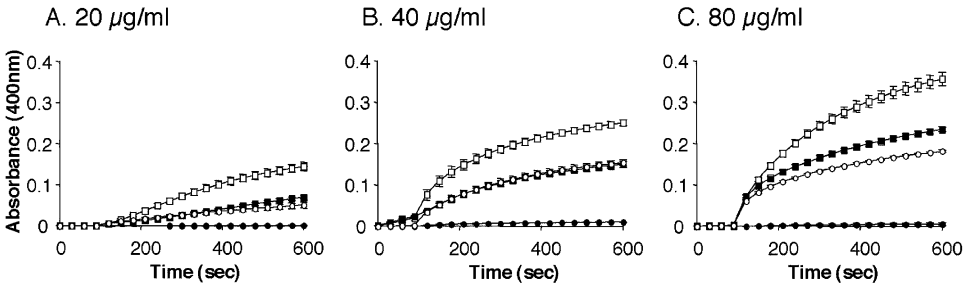

FIGURE 6: Ability of SP-A^{DEL} and SP-A^{INS} to aggregate DPPC liposomes which was weaker than that of wt SP-A. Unilamellar liposomes containing a mixture of DPPC, egg PC, and PG (7:2:1) (300 μg/mL) were preincubated with the recombinant SP-As at concentrations of 20 (A), 40 (B), and 80 μg/mL (C), and $CaCl_2$ (5 mM) was added after 90 s. The change in absorbance at 400 nm was measured at room temperature: (□) wt SP-A, (■) SP-A^{DEL}, (○) SP-A^{INS}, and (●) no protein. Values are means ± the standard error of three experiments.

Table 1: Rates of Liposome Aggregation (ΔOD per second[a]) at 90−150 s

	20 μg/mL	40 μg/mL	80 μg/mL
wt	$(0.24 \pm 0.04) \times 10^{-3}$	$(1.49 \pm 0.10) \times 10^{-3}$	$(1.88 \pm 0.08) \times 10^{-3}$
DEL	$(0.13 \pm 0.01) \times 10^{-3}$ [b]	$(0.47 \pm 0.05) \times 10^{-3}$ [c]	$(1.59 \pm 0.08) \times 10^{-3}$ [b]
INS	$(0.19 \pm 0.04) \times 10^{-3}$	$(0.84 \pm 0.09) \times 10^{-3}$ [c]	$(1.33 \pm 0.03) \times 10^{-3}$ [c]

[a] Values are means ± the standard error of three experiments. [b] $p < 0.05$, compared with that of wt SP-A. [c] $p < 0.01$ compared with that of wt SP-A.

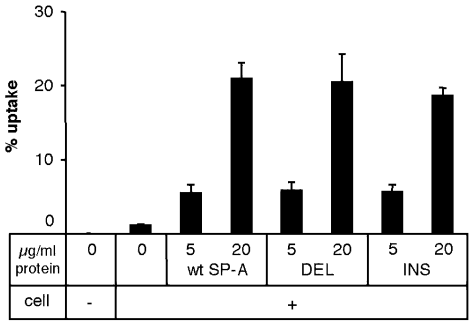

FIGURE 7: Mutants SP-A^{DEL} and SP-A^{INS} augment the uptake of lipid by type II cells at a level comparable to that of wt SP-A. Type II cells were incubated with unilamellar liposomes containing [^{3}H]DPPC in the presence of 5 or 20 μg/mL recombinant SP-As. The radioactivities associated with cells were counted. The results are expressed as a percentage of radioactivities associated with the cells in total radioactivities of the incubation mixture. The data are means ± the standard error of duplicate determinations from three separate experiments.

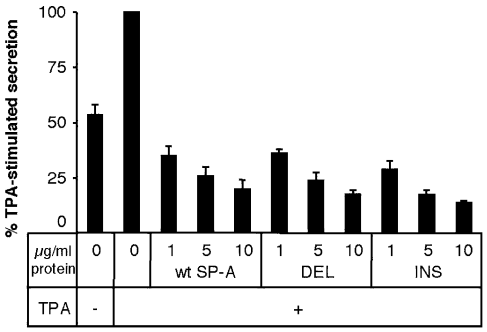

FIGURE 8: SP-A^{DEL} and SP-A^{INS} inhibit secretion of lipid from type II cells at a level comparable to that of wt SP-A. Type II cells were incubated overnight in the medium containing [^{3}H]choline and incubated for 3 h with secretagogue TPA (10^{-7} M) in the absence or presence of the indicated concentrations of recombinant SP-As. The radioactivities of phospholipids extracted from cells and medium were counted as described in Experimental Procedures. Results are expressed as a percentage of the TPA-stimulated secretion. The data are means ± the standard error of duplicate determinations in three separate experiments.

Although both mutants formed a similar octadecameric structure, the mutants SP-A^{DEL} and SP-A^{INS} exhibited different profiles of the multimer assembly. Most of the SP-A^{DEL} proteins migrated at approximately 600−630 kDa via native PAGE, but a larger assembly was observed in SP-A^{INS} preparations. The fully assembled structure of SP-A is formed by lateral association of the N-terminal segments of six trimers and stabilized by inter- and intratrimeric disulfide bonding at Cys^{-1} and Cys^6 (*12*). In addition to Cys^{-1} and Cys^6, two cysteine residues are potentially involved in the arrangement of the disulfide bonding in human SP-A: Cys^{48} within the interruption of Gly-X-Y repeats (Pro^{47}-Cys-Pro-Pro) and Cys^{65} in the collagen-like domain near the neck. This study using electrophoresis and gel filtration indicated that the mutant SP-A^{DEL}, from which Cys^{48} had been deleted, was able to form an octadecamer but contained a smaller amount of a larger assembly, when compared with wt SP-A and SP-A^{INS}. The results suggest that Cys^{48} may contribute to the formation of large aggregates of the oligomers, although it may not be required to form an octadecamer. In contrast, self-aggregation of the mutant SP-A^{INS} was frequently observed. Similar large aggregates were also found in wt SP-A preparations, especially at high concentrations of SP-A. However, significantly more aggregates were recognized in SP-A^{INS} preparations than in wt SP-A or SP-A^{DEL} preparations, at approximately the same concentrations of the proteins (100−200 μg/mL). Electron microscopic inspection of SP-A^{INS} under these conditions revealed approximately 8.8% supra-octadecameric aggregates, while 0.2 or 0.6% structures of wt SP-A or SP-A^{DEL}, respectively, were aggregated forms. The concentrations of SP-As used in this study were considered to be physiological, since the median concentration of SP-A in normal human epithelial lining fluids is presumed to be approximately 180 μg/mL (*32*). Although it is unrealistic to measure the actual SP-A concentrations in vivo, Wright (*33*) also described that the SP-A concentration in rat lung would range from 300 μg/mL to 1.8 mg/mL. The multimerized large aggregates of SP-A may be the result of sulfhydryl-dependent and -independent cross-linking (*34*). The precise mechanism by which SP-A^{INS} prefers to form large aggregates remains to be

elucidated. At present, we speculate that the self-assembly of SP-A^{INS} may be dependent on its oligomeric structure. The straight-shaped collagenous stalks of SP-A^{INS} oligomers may easily associate with each other.

The mutants SP-A^{DEL} and SP-A^{INS} exhibited DPPC and GalCer binding at a level comparable to that of wt SP-A and augmented lipid uptake by type II cells. However, the abilities of both SP-A^{DEL} and SP-A^{INS} to induce liposome aggregation were significantly weaker than that of wt SP-A. Although the mechanism by which SP-A stimulates vesicle aggregation was not clearly understood, the ability of SP-A to self-associate in the presence of calcium is considered to be involved in the mechanism (*35*). A recent study has demonstrated that the trimeric form of the cysteine mutant which had not undergone self-association was unable to induce phospholipid vesicle aggregation (*36*). The N-terminal segment of SP-A was required for supratrimeric assembly and for phospholipid aggregation (*30*, *31*). The impaired activity of the mutants SP-A^{DEL} and SP-A^{INS} to induce liposome aggregation was not due to the disability of the mutants to form supratrimeric oligomers, since approximately 90% species of SP-A^{DEL} and SP-A^{INS} as well as wt SP-A were oligomers greater than trimers when examined under the electron microscope. In addition, the self-association of SP-A^{INS} did not correlate with the activity of stimulating vesicle aggregation in our study. At present, we consider that the cross-linking of liposomes may be prevented under a "V"-like hexamer and a widely spread "starfish"-like octadecamer of the kink-eliminated mutants. Liposomes on the CRD of the mutants may fail to associate with each other due to the long distance between each trimeric head of the mutants, as observed via electron microscopy (Figure 4).

The association of SP-A with lipids is physiologically important for the conversion of lipid aggregates from multilamellar forms to a tubular myelin, a lattice-like structure that serves as the major reservoir of surfactant material in the alveoli (*37*). Formation of tubular myelin was absent in mouse strains with null alleles for SP-A (*8*). The role of the collagenous domain in tubular myelin formation has been suggested, since tubular myelin formation in the SP-A null mouse was not restored by SP-A containing a deletion of the collagenous domain (*38*). An abnormal, large aggregated form of SP-A oligomer isolated from patients with alveolar proteinosis failed to form tubular myelin structure, although it retained the ability to bind lipids (*25*). Since SP-A^{INS} exhibited large aggregates, it is suggested that normal membrane formation may not occur in this mutant.

The cell surface binding activity of SP-A is directly related to its capacity to inhibit secretion of surfactant lipids from type II cells (*27*). Although a collagenase-resistant fragment of SP-A could bind to type II cells and inhibit lipid secretion, these activities were significantly weaker than those of native SP-A (*39*). In addition, it has been suggested that the collagen-like domain is required for high-affinity receptor binding and specific inhibition of surfactant secretion (*30*). These results indicate the importance of cooperative functions of the collagenous domain and the CRD in type II cell interaction. The study presented here demonstrates that the loss of the bend in the collagenous domain failed to alter the effect of SP-A on type II cells. The results may suggest that kink-deleted SP-As can be recognized by type II cells as well as flower bouquet-like SP-As.

SP-A plays important roles in the host defense mechanism of the lung (*2*, *40*). In addition to the opsonic effects and the direct bactericidal activities, SP-A regulates immune cell functions by directly interacting with the cells. SP-A binds to several receptors such as SP-R210 (*41*, *42*), CD14 (*43*), Toll-like receptors 2 (*44*) and 4 (*18*), signal-inhibitory regulatory protein-α (*45*), and C1q receptors, including C1qR (*46*) and calreticulin (*45*), to activate phagocytosis or modulate the inflammatory cellular response. Although we could not observe significant effects of the kink deletion on the interaction with type II cells, it remains unknown whether the kink region would be required for stimulation of macrophages. Further experiments are currently under way to assess the host defense functions of the mutants SP-A^{DEL} and SP-A^{INS}.

Another collectin, MBL, forms a bouquet-like octadecamer like SP-A. The collagen-like domain of human MBL contains 19 repeats of the Gly-X-Y motif with an interruption sequence at the eighth repeat (Gly^{63}-Gln^{64}). MBL deficiency is largely explained by three single-point mutations in codons 52 (termed MBL-D), 54 (MBL-B), and 57 (MBL-C) of exon 1 in the human *MBL2* gene (*47*). These mutations are positioned at the Gly-X-Y repeats located below the kink region. MBL-B and -C interrupt the Gly-X-Y repeats in the first Gly position, and MBL-D introduces a Cys residue in the second position. Studies using artificial MBL variants revealed that the mutations compromise the assembly of higher-order oligomers, resulting in reduced ligand binding capacity and attenuated capability to activate complements (*48*). Although genetic polymorphisms at the kink region of MBL have not been reported, several studies have described the functions of the kink-deleted recombinant MBL mutants. Kurata et al. (*22*) demonstrated that the kink-deleted mutant in which Leu was inserted into the Gly^{63}-Gln-Gly sequence to restore the Gly-X-Y repeats activated complements in a manner comparable to that of wild-type MBL. Arora et al. (*23*) demonstrated that mutant A in which amino acids at the kink region were deleted could enhance FcR- and CR-1-mediated phagocytosis in a manner similar to that of wild-type MBL or C1q. Taken together, it seems that the physiological role of the kink region in MBL is still unknown.

In conclusion, we provide direct evidence that the interruption of Gly-X-Y repeats of SP-A defines the kink in the molecule, resulting in formation of a flower bouquet-like octadecamer. Disruption of the interruption of Gly-X-Y repeats causes straight-shaped collagenous stalks and results in the formation of a radially and widely spread octadecamer. The mutant SP-A^{INS} in which Gly-X-Y repeats were restored without the loss of Cys^{48} exhibited a large aggregate of oligomers. Loss of the Gly-X-Y repeat interruption impaired the ability of SP-A to induce phospholipid aggregation, indicating the importance of the kink region in liposome aggregation.

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