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Effect of Acidic pH on the Structure and Lipid Binding Properties of Porcine Surfactant Protein A

POTENTIAL ROLE OF ACIDIFICATION ALONG ITS EXOCYTIC PATHWAY*

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Miguel L. F. Ruano, Jesus Pérez-Gil, and Cristina Casals‡

From the Department of Biochemistry and Molecular Biology, Faculty of Biology, Complutense University of Madrid, 28040 Madrid, Spain

Pulmonary surfactant protein A (SP-A) is synthesized by type II cells and stored intracellularly in secretory granules (lamellar bodies) together with surfactant lipids and hydrophobic surfactant proteins B and C (SP-B and SP-C). We asked whether the progressive decrease in pH along the exocytic pathway could influence the secondary structure and lipid binding and aggregation properties of porcine SP-A. Conformational analysis from CD spectra of SP-A at various pH values indicated that the percentage of α-helix progressively decreased and that of β-sheet increased as the pH was reduced. The protein underwent a marked self-aggregation at mildly acidic pH in the presence of Ca^{2+}, conditions thought to resemble those existing in the trans-Golgi network. Protein aggregation was greater as the pH was reduced. We also found that both neutral and acidic vesicles either with or without SP-B or SP-C bound to SP-A at acidic pH as demonstrated by co-migration during centrifugation. However, the binding of acidic but not neutral vesicles to SP-A led to 1) a striking change in the CD spectra of the protein, which was interpreted as a decrease of the level of SP-A self-aggregation, and 2) a protection of the protein from endoproteinase Glu-C degradation at pH 4.5. SP-A massively aggregated acidic vesicles but poorly aggregated neutral vesicles at acidic pH. Aggregation of dipalmitoylphosphatidylcholine (DPPC) vesicles either with or without SP-B and/or SP-C strongly depended on pH, being progressively decreased as the pH was reduced and markedly increased when pH was shifted back to 7.0. At the pH of lamellar bodies, SP-A-induced aggregation of DPPC vesicles containing SP-B or a mixture of SP-B and SP-C was very low, although SP-A bound to these vesicles. These results indicate that 1) DPPC binding and DPPC aggregation are different phenomena that probably have different SP-A structural requirements and 2) aggregation of membranes induced by SP-A at acidic pH is critically dependent on the presence of acidic phospholipids, which affect protein structure, probably preventing the formation of large aggregates of protein.

Pulmonary surfactant is a mixture of approximately 80% phospholipids, 10% other lipids, and 5–10% surfactant-specific proteins that lines the alveolar space and is essential for breathing (for reviews, see Refs. 1–3). The alveolar type II cell is the sole cell type in the lung that produces all components of pulmonary surfactant. The surfactant apolipoproteins (SP-A,[1] SP-B, and SP-C) and all of the surfactant phospholipids are stored intracellularly in lamellar bodies and are secreted as a complex (4–7). The release of surfactant to the alveolar lumen occurs by exocytosis of lamellar body content in response to secretagogue stimulation (8, 9). The regulated secretory pathway of the type II cell is atypical because the lamellar body not only functions as a classic secretory granule, but it also intersects with the endocytic pathway (10, 11). Like the storage granules in other secretory cells, lamellar bodies have an acidic internal environment (pH 5.5) (12) and high calcium content, bringing their intravesicular free Ca^{2+} concentration to a 2-10 mM range (13). It is well recognized that during the process of secretory granule formation, proteins of the granule content are segregated from proteins that are released from the cell by the constitutive secretory pathway. Morphological studies indicate that sorting of nascent secretory proteins into the regulated *versus* constitutive pathway occurs in the trans-Golgi network (TGN) (14) and in immature secretory granules (15). Biochemical studies indicate that selective aggregation of proteins plays an important role in sorting of proteins destined for secretory granules (16–18). This protein aggregation is triggered as proteins encounter mildly acidic pH and high calcium in the TGN lumen. Constitutively secreted proteins do not aggregate under these ionic and pH conditions and are excluded from such aggregates (17, 18).

SP-A is the major protein in the alveolar compartment (for reviews, see Refs. 2, 3, and 19). It is a multifunctional protein capable of binding several ligands including phospholipids, carbohydrates, and Ca^{2+}, and it belongs to the Ca^{2+}-dependent lectin family. SP-A is a large oligomeric protein of approximately 650 kDa, composed of 18 nearly identical subunits. Each SP-A subunit contains an amino-terminal collagen-like domain and a carboxyl-terminal carbohydrate recognition domain that are linked by a more hydrophobic domain (neck). Nearly all alveolar SP-A is complexed with phospholipids. The carbohydrate recognition domain and the neck region are important protein domains involved in SP-A/lipid interactions (20–22). The structural properties of SP-A and its interaction with phospholipids have been studied to date mainly at neutral pH. At this pH, SP-A preferentially interacts with dipalmitoylphosphatidylcholine (DPPC) (23, 24), the main surfactant

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‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Faculty of Biology, Complutense University of Madrid, 28040 Madrid, Spain. Tel.: 34-91-3944261; Fax: 34-91-3944672; E-mail: casals@solea.quim.ucm.es.

[1] The abbreviations used are: SP-A, -B, and -C, surfactant protein A, B, and C, respectively; TGN, trans-Golgi network; DPPC, dipalmitoylphosphatidylcholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; PAGE, polyacrylamide gel electrophoresis; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PG, 1,2-diacyl-*sn*-glycero-3-phosphoglycerol; P_{II}, poly(L-proline II)-type.

lipid (1). The interaction of DPPC with SP-A leads to a conformational change on the protein molecule (24) and a marked protection of SP-A from trypsin degradation (25). The interaction of SP-A with phospholipid vesicles induces vesicle aggregation in the presence of Ca^{2+} (25–27). SP-A also induces aggregation of liposomes containing SP-B or SP-C or both hydrophobic proteins (27, 28). The association of SP-A with lipids appears essential for the conversion of lipid aggregates from multilamellar forms present in the exocytic granule to dispersed ordered arrays known as tubular myelin (29). This physical transformation also requires the hydrophobic surfactant protein SP-B (30). SP-A enhances adsorption of lipids along the air/liquid interface in a concerted action with SP-B (31). In addition, SP-A is involved in surfactant homeostasis (2, 3, 19) and participates in lung-specific host defense (32). With respect to SP-A biosynthetic routing in alveolar type II cells, the protein is synthesized in the endoplasmic reticulum and transported together with the precursors of hydrophobic surfactant proteins SP-B and SP-C through the same pathway from Golgi complex to lamellar bodies (7). Its correct routing and secretion is independent of glycosylation (6, 33). SP-A is a protein rich in negatively charged amino acids with isoelectric points varying between pH 4.5 and 5.2 (3, 27). The question that we address here is whether the progressive decrease in pH along the exocytic pathway could influence the structure and the lipid binding and aggregation properties of SP-A.

The present study analyzes the structural properties of SP-A and its interaction with phospholipids at acidic pH in the presence and absence of the hydrophobic surfactant proteins SP-B and SP-C. We found that the secondary structure of SP-A was changed by lowering the pH and that the protein underwent a rapid aggregation at mildly acidic pH in the presence of Ca^{2+}, conditions thought to resemble those existing in the TGN and along the exocytic pathway. The binding of acidic but not neutral vesicles to SP-A, at acidic pH, affected the secondary structure of the protein. Vesicle aggregation induced by SP-A at acidic pH was critically dependent on the presence of negatively charged phospholipids in the composition of the vesicle.

EXPERIMENTAL PROCEDURES

Isolation of SP-A, SP-B, and SP-C—Pulmonary surfactant was prepared from pig bronchoalveolar lavage as described previously (34). SP-A was purified from isolated surfactant using sequential butanol and octyl glucoside extractions (31). SP-B and SP-C were isolated directly from pig lungs by minor modifications of the method of Curstedt *et al.* (35) described elsewhere (36). The purity of SP-A, SP-B, and SP-C was checked by one-dimensional SDS-PAGE (12 and 16% acrylamide for SP-A and for SP-B and SP-C, respectively) under reducing conditions (50 mM dithiothreitol). Quantification of the proteins was carried out by amino acid analysis in a Beckman System 6300 high performance analyzer. The protein hydrolysis was performed with 0.2 ml of 6 M HCl, containing 0.1% (w/v) phenol in evacuated and sealed tubes at 108 °C for 24 h. Norleucine was added to each sample as an internal standard. In the course of this work, eight different preparations of porcine SP-A and four preparations of porcine SP-B and SP-C were used. Some of the experiments were repeated with canine SP-A with identical results.

CD Measurements—CD spectra were obtained on a Jasco J-715 spectropolarimeter fitted with a 150-watt xenon lamp. Quartz cells of 1-mm path length were used, and the spectra were recorded in the far-uv region (190–260 nm) at 50 nm/min scanning speed and at room temperature. Five scans were accumulated and averaged for each spectrum. The acquired spectra were corrected by subtracting the appropriate blank runs (of water or phospholipid vesicle solutions), subjected to noise reduction analysis and presented as molar ellipticities (degrees·cm^2·$dmol^{-1}$), assuming 110 Da as the average molecular mass per amino acid residue. At least three independent preparations of SP-A were measured. Final SP-A concentration was 80–100 μg/ml. Measurements at acidic or neutral pH were done in 5 mM acetate, 5 mM Tris/HCl buffer, pH 4.5 or 7.2, respectively. pH titration was started at neutral pH in 5 mM Tris, 5 mM MES, 5 mM acetate buffer, pH 7.2, in the presence or absence of 5 mM $CaCl_2$. From the starting pH, SP-A was titrated to pH 4.2 by the addition of 1–2-μl aliquots of 0.05 or 0.1 M HCl solution. During titration, the medium pH was monitored with a micro-pH electrode. The spectrum of SP-A at each pH was recorded 10 min after each change of pH.

SP-A Self-aggregation Assays—Self-aggregation of SP-A induced by H^+ was studied at 37 °C by measuring the change in absorbance at 360 nm in a Beckman DU-640 spectrophotometer. Both sample and reference cuvettes were first filled with 5 mM acetate, 5 mM Tris/HCl buffer, pH 4.5. After a 10-min equilibration at 37 °C, SP-A (20 μg/ml, final concentration) was added to the sample cuvette, and the turbidity change at 360 nm was monitored at 1-min intervals over 10 min. Self-aggregation of SP-A was reversed by shifting back the pH to 7.2. pH-induced protein aggregation was also determined by centrifugation at 12,000 × *g* in a Hettich microliter centrifuge for 15 min. The entire pellet fractions and supernatants were then subjected to SDS-PAGE under reducing conditions followed by staining of the gels with Coomassie Blue. The variation of SP-A aggregation as the pH was reduced was studied at 37 °C in the presence or the absence of 5 mM Ca^{2+}. Samples containing SP-A (5.5 μg) in 350 μl of 5 mM Tris/HCl, 5 mM MES, 5 mM acetate buffer, pH 7.2, were titrated to the indicated pH values by adding 1–2-μl aliquots of 0.05 or 0.1 M HCl while vortexing. During titration, the medium pH was monitored. The absorbance at 360 nm was registered once it was stabilized after each change of pH.

Preparation of Lipid Vesicles and Liposomes Containing SP-B and/or SP-C—Synthetic phospholipids, DPPC, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) were purchased from Avanti Polar Lipids (Birmingham, AL); egg 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC) and 1,2-diacyl-*sn*-glycero-3-phosphoglycerol (PG) were from Sigma, and their homogeneity was routinely tested on thin layer chromatography. The organic solvents (methanol and chloroform) used to dissolve lipids and to isolate and store hydrophobic surfactant proteins were high pressure liquid chromatography grade (Scharlau, Barcelona, Spain).

Throughout all experiments, unilamellar vesicles of DPPC, DPPG, or DPPC/other (7:3, w/w) were used. The different lipid vesicles were prepared at a phospholipid concentration of 1 or 3 mg/ml by hydrating dry lipid films in a buffer containing 150 mM NaCl, 0.1 mM EDTA, 25 mM Tris/HCl, 25 mM acetate, pH 7.2 or 4.5, and allowing them to swell for 1 h at a temperature above the phase transition temperature of the corresponding phospholipid. Next, the lipid dispersion (1 ml) was sonicated at the same temperature (typically above 45 °C) during 2 min at 390 watts/cm^2 (bursts of 0.6 s, 0.4 s between bursts) in a UP 200S sonifier with a 2-mm microtip. All vesicles were prepared freshly each day, just before starting the experiment. The phospholipid concentration was assessed by phosphorus determination according to Rouser *et al.* (37). For vesicle-size analysis in solution, quasielastic light scattering was used as described previously (24). Vesicle diameter at 37 °C was 135 nm for DPPC vesicles and around 60 nm for DPPC/DPPG (7:3, w/w) vesicles.

Reconstitution of SP-B or SP-C or both hydrophobic proteins in DPPC or DPPC/DPPG (7:3, w/w) vesicles was performed as described previously (38) at a protein:lipid ratio of either 1:10 or 1:20. Briefly, appropriate amounts of phospholipids dissolved in chloroform/methanol (2/1, v/v) were mixed with the desired amount of SP-B and/or SP-C stored in chloroform/methanol (2/1, v/v). Dry protein/lipid samples were hydrated in the appropriate buffer at 65 °C for 1 h with occasional mixing. Sonication was done at the same temperature under conditions described above.

Phospholipid Binding—The binding of SP-A to phospholipid vesicles at acidic pH was determined by coflotation of protein and lipids after discontinuous sucrose density gradient centrifugation. SP-A (25 μg/ml final concentration) was added to 150 mM NaCl, 25 mM acetate buffer, pH 4.5 with or without 377 μg/ml phospholipid vesicles. The mixture was incubated during 15 min at room temperature and then carefully placed in a centrifuge tube over a 1-ml cushion of 30% sucrose in the same buffer. Centrifugation was conducted at 25 °C in a Beckman SW-65 rotor at 129,000 × *g* for 2 h. The supernatants were then transferred to concentrating tubes (Millipore Microcon 100®) and centrifugated in order to eliminate salts. The presence of SP-A in pellet and supernatant fractions was determined by one-dimensional SDS-PAGE, under reducing conditions. Phospholipid content in both fractions was assessed by phosphorus analysis (37). Control experiments where liposomes or SP-A were deleted from the incubation were performed. After centrifugation, more than 97% of phospholipids were recovered in the supernatant, and all SP-A sedimented in the absence of phospholipid vesicles.

Phospholipid Vesicle Aggregation Assay—The assay was performed

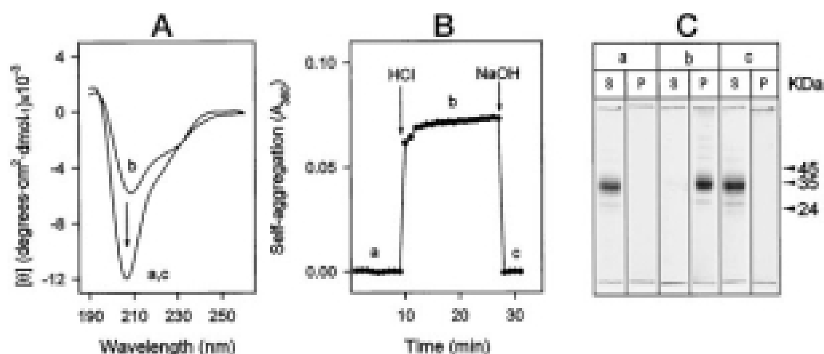

FIG. 1. **Reversible pH-dependent change of secondary structure and self-aggregation of SP-A.** *A,* Far-uv circular dichroism spectra of SP-A. *a,* spectra of SP-A (0.08 mg/ml) in 5 mM acetate, 5 mM Tris/HCl buffer, pH 7.2; *b,* spectra of the protein in the same buffer but at pH 4.5; *c,* spectra of SP-A after adding NaOH to change pH from 4.5 to 7.2. *B,* H^+-induced self-aggregation of SP-A. In this experiment, sample and reference cuvettes were first filled with 20 μg/ml SP-A in 5 mM acetate, 5 mM Tris/HCl buffer, pH 7.2 (*a*). After a 10-min equilibration at 37 °C, HCl was added to the sample cuvette to shift the pH from 7.2 to 4.5. The turbidity change at 360 nm was monitored at 1-min intervals over 20 min (*b*). When the pH was shifted back to 7.2 by the addition of NaOH, SP-A aggregates completely dissociated (*c*). *C,* SP-A is pelleted by centrifugation at acidic but not at neutral pH. SP-A concentration in the assays was 8 μg/ml. *a,* in 5 mM acetate, 5 mM Tris/HCl buffer, pH 7.2; *b,* in the same buffer after changing pH to 4.5; *c,* after the addition of NaOH to change back pH to 7.2. After centrifugation of these samples, the pellets (*P*) and the supernatants (*S*) were subjected to SDS-PAGE, and the gel was stained with Coomassie Blue. Molecular mass markers in kDa are indicated to the *right* of the gel. In *A, B,* and *C,* a representative one of four experiments from four different porcine SP-A preparations is shown.

at acidic or neutral pH by measuring the absorbance at 400 nm in a Beckman DU-640 spectrophotometer at 37 °C as described previously (25). Briefly, phospholipid vesicles (40 μg) with or without hydrophobic proteins were added to both the sample and the reference cuvette in a total volume of 0.5 ml of 150 mM NaCl, 0.1 mM EDTA, 25 mM Tris, 25 mM acetate buffer, pH 7.2 or 4.5. After a 10-min equilibration at 37 °C, 4 μg of SP-A were added to the sample cuvette, and the change in optical density at 400 nm was monitored at 1-min intervals. Next, Ca^{2+} (1 mM or 50 μM, final concentration) was added to both the sample and the reference cuvette, and the change in absorbance was monitored again.

Protease Digestion of SP-A at Acidic pH—To determine the protease sensitivity of lipid-free and membrane-bound SP-A, the endoproteinase Glu-C from *Staphylococcus aureus V8* (Boehringer Mannheim GmbH, Germany) was used. This serine protease hydrolyzes peptide and ester bonds specifically at the carboxylic side of Glu at pH 4.0 or both Glu and Asp at pH 8.0–8.5. SP-A (6.7 μg) was added to 150 mM NaCl, 25 mM acetate buffer, pH 4.5, either in the presence or absence of 60 μg of phospholipid vesicles prepared in the same buffer. After 10 min of incubation at 37 °C, 13.4 μg of freshly prepared enzyme solution (2:1 enzyme/protein, w/w) were added. The final incubation volume was 110 μl. The mixture was incubated during 4 h at 37 °C, and the digestion was stopped by freezing the samples at −20 °C. Samples were analyzed by SDS-PAGE under reducing conditions followed by Western blot analysis of SP-A as described previously (39) because the electrophoretic band of V8 protease (30–35 kDa) overlapped with that of SP-A (28–36 kDa). The primary antibody was kindly supplied by Dr. J. A. Whitsett (University of Cincinnati).

RESULTS

Reversible pH-dependent Change of the Secondary Structure of SP-A and Aggregation of the Protein—CD spectra of SP-A at neutral and acidic pH are shown in Fig. 1*A*. At neutral pH, spectra were characterized by a shoulder at 220 nm and a strong negative extreme at 207 nm as previously reported (24, 40). The change of pH from 7.2 to 4.5 led to a change in the shape of SP-A spectrum and markedly reduced the contribution of the 207-nm minimum to the spectrum. This change in the CD spectra of SP-A was reversed when the pH was changed back to 7.2. On the other hand, pH-dependent aggregation of SP-A was studied by measuring H^+-induced turbidity changes (Fig. 1*B*), and the level of aggregation was determined by centrifugation (Fig. 1*C*). SP-A rapidly aggregated at pH 4.5, and all of the protein was recovered in the pellet. When the pH was changed back to 7.2, the turbidity at 360 nm vanished and all of SP-A was recovered in the supernatant fraction, indicating that pH-dependent self-aggregation of SP-A was reversible. The CD spectrum of SP-A at pH 4.5 corresponded to the H^+-induced aggregated form of the protein. To determine whether protein aggregation might affect CD measurements, CD spectra of SP-A at pH 4.5 were recorded at different protein concentrations. No significant differences were observed in the range of 40–140 μg of SP-A/ml tested.

The estimation of secondary structure fractions of porcine SP-A at neutral and acidic pH was performed by the SELCON program (the self-consistent method) recently modified by Sreerama and Woody (41). This program determines the contribution of α-helix, β-structure, β-turn, and poly(L-proline II)-type (P_{II}) conformation to the spectra of proteins. The P_{II} conformation is a left-handed extended helix with three residues per turn and is favored in proline-rich polypeptides due to the limited conformational flexibility of the proline ring (41). The P_{II} conformation was found in collagen and in short segments of some globular proteins. According to the SELCON program, porcine SP-A at neutral pH contained 19% α-helix, 26% β-sheet, 25% β-turn, 14% P_{II} conformation, and 17% unordered form. The self-aggregated SP-A at pH 4.5 contained 10% α-helix, 39% β-sheet, 22% β-turn, 12% P_{II} conformation, and 18% unordered form, indicating that at acidic pH the content of β-sheet conformation increased and that of α-helix decreased, whereas the content of P_{II} conformation did not change.

SP-A Aggregation Increases as the pH Is Reduced: This Effect Is Greater in the Presence of Ca^{2+}—Fig. 2*A* shows the effect of lowering pH on self-association of the protein in the presence and absence of 5 mM Ca^{2+}. SP-A underwent pH-dependent aggregation in the absence of Ca^{2+}. Protein aggregation started at pH below 6.0 and reached a maximum value at pH 4.5. The ionization p*K* calculated from the plot of the dependence of SP-A aggregation on the pH was 5.26 ± 0.05. In the presence of Ca^{2+}, a marked aggregation occurred in the pH range of TGN (6–6.5) and was greater as the pH was reduced. The ionization p*K* calculated in the presence of Ca^{2+} was 5.55 ± 0.15. At pH 4.5, protein aggregation was not influenced by Ca^{2+} as shown in Fig. 2*B*. At neutral pH, self-association of SP-A was absolutely dependent on 1-8 mM concentrations of Ca^{2+} (25, 42). The Ca^{2+} concentration needed for half-maximal self-aggregation of pig SP-A was 2.36 ± 0.15 mM (25).

The effect of lowering pH on the CD spectra of SP-A was analyzed in the absence (Fig. 3*A*) and the presence (Fig. 3*B*) of Ca^{2+}. Fig. 3*A* shows a progressive decrease of the negative ellipticity and a progressive shift of the negative maximum from 207 to 209 nm as the pH was reduced. Conformational analysis by the SELCON program indicated that the percent-

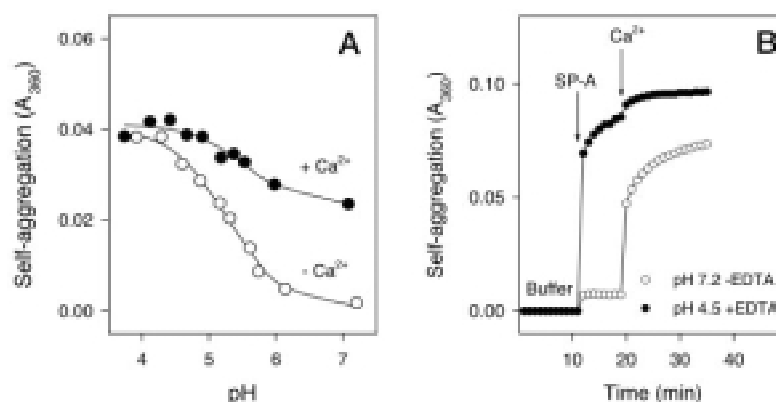

FIG. 2. **SP-A self-aggregation increases as the pH is reduced.** The experiments were done at 37 °C as described under "Experimental Procedures." *A*, pH titration started at pH 7.2 in 5 mM Tris/HCl, 5 mM MES, 5 mM acetate buffer either with or without 5 mM Ca^{2+}. A representative one of six experiments is shown. Three different preparations of porcine SP-A were used. *B,* comparison of the kinetics of self-association of SP-A at pH 4.5 and 7.2. A representative one of four experiments from two different porcine SP-A preparations is shown.

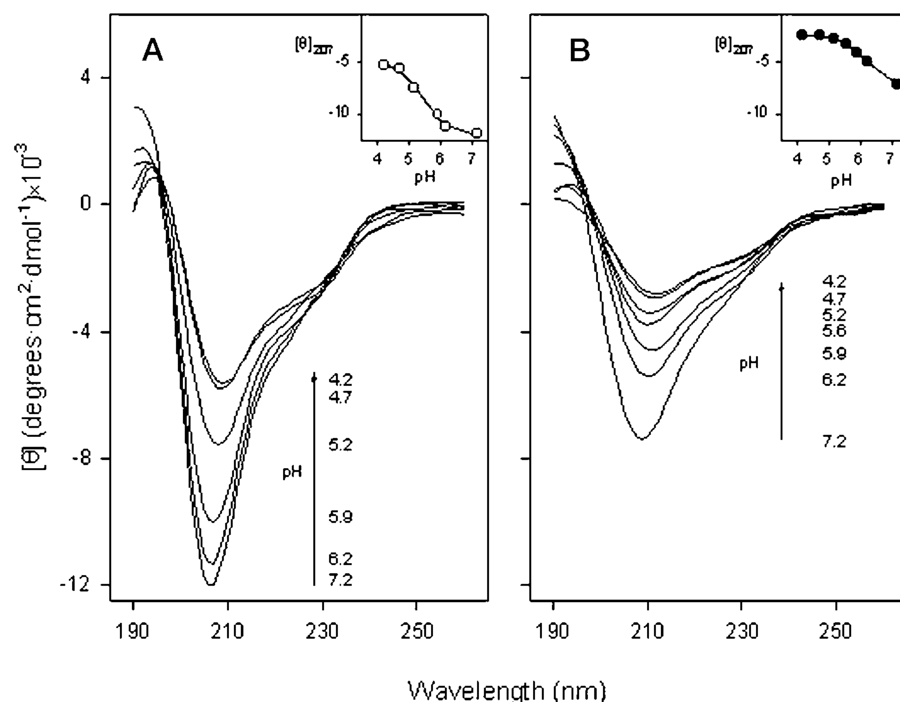

FIG. 3. **The secondary structure of SP-A changes as the pH is reduced.** pH titration experiments were performed as described under "Experimental Procedures" and started at pH 7.2 in 5 mM Tris/HCl, 5 mM MES, 5 mM acetate buffer in the absence (*A*) and the presence (*B*) of 5 mM Ca^{2+}. *Insets,* ellipticities at 207 nm of porcine SP-A as a function of pH. A representative one of three experiments from three different porcine SP-A preparations is shown.

age of α-helix progressively decreased (from 20 to 8%) and that of β-sheet progressively increased (from 26 to 41%) without significant variation in the content of P_{II} and unordered conformation. On the other hand, from data of the ellipticity at 207 nm *versus* pH (Fig. 3*A, inset*) we calculated a p*K* of 5.46 ± 0.1 for the H^+-induced change of the CD spectrum of SP-A. The similarity between p*K* values calculated from protein aggregation assays and CD measurements supports the concept that the change of CD spectra of SP-A as a function of the pH reflects the conformational change of SP-A at different levels of self-association.

Fig. 3*B* shows the CD spectra of the Ca^{2+}-induced aggregated form of SP-A at neutral pH. Conformational analysis estimated 11% α-helix, 38% β-sheet, 22% β-turn, 12% P_{II} and 18% unordered form. Ca^{2+} ions at pH 7.0 seemed to produce a similar effect on the CD spectra of SP-A as that found for mildly acidic pH in the absence of Ca^{2+}. However, the changes in the CD spectra of SP-A at acidic pH were entirely reversible when the pH was shifted back to 7.0 (Fig. 1), but those changes produced by 5 mM Ca^{2+} were not reversed when the protein was dissociated by the addition of EDTA (data not shown). When the pH of samples containing Ca^{2+} was titrated from 7.2 to below 5.0, the negative ellipticity was reduced, and the negative maximum progressively shifted from 208 to 211 nm. The pH-dependent change of ellipticity at 207 nm, shown in the *inset* of Fig. 3*B*, and the pH-dependent increase of SP-A aggregation in the presence of Ca^{2+}, shown in Fig. 2*A*, were similar.

SP-A-induced Lipid Aggregation—Fig. 4 shows liposome aggregation induced by SP-A at neutral and acidic pH. The addition of SP-A to acidic vesicles (DPPC/DPPG or DPPC/PG) at pH 4.5 in the presence of EDTA resulted in a marked increase in light absorbance due to lipid aggregation. Light absorbance slightly increased after the addition of Ca^{2+}. This additional increase was completely reversed by adding EDTA. Interestingly, little or no change in light absorbance was observed after the addition of SP-A and Ca^{2+} to DPPC or DPPC/PC vesicles, indicating that SP-A-induced aggregation of neutral vesicles markedly decreased at that acidic pH. In contrast, at neutral pH, SP-A aggregated neutral vesicles in the presence of micromolar concentrations of Ca^{2+} (25). On the other hand, the extent of aggregation of disaturated phospholipid vesicles (DPPC or DPPC/DPPG), which at 37 °C were in the gel state, was higher than that of unsaturated vesicles (DPPC/PC or DPPC/PG), supporting previous results concerning the influence of the physical state of the vesicles on this process (24).

The effect of the ionic strength on lipid aggregation is shown in Fig. 5. At low or physiological ionic strength, the aggregation of DPPC at pH 4.5 is very low. To restore the extent of aggre-

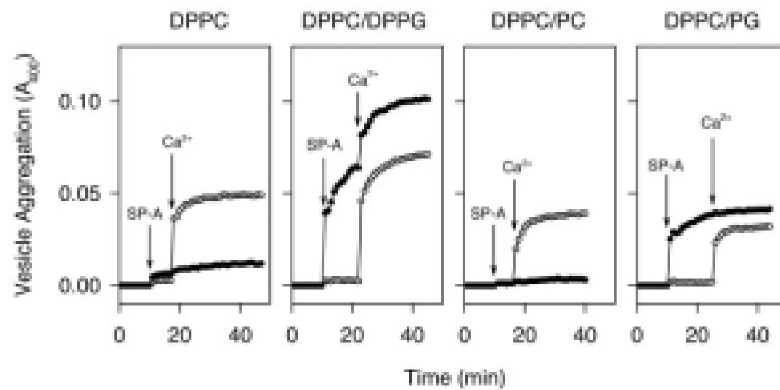

FIG. 4. **Vesicle aggregation induced by SP-A at acidic and neutral pH.** Neutral vesicles of DPPC or DPPC/PC (7:3, w/w) and acidic vesicles of DPPC/DPPG (7:3, w/w) or DPPC/PG (7:3, w/w) were prepared in 150 mM NaCl, 0.1 mM EDTA, 25 mM acetate, 25 mM Tris/HCl buffer at either pH 7.2 (○) or 4.5 (●). Sample and reference cuvettes were filled with phospholipid vesicles (80 μg/ml), and after a 10-min equilibration at 37 °C, SP-A (8 μg/ml) was added to the sample cuvette. At pH 4.5 (●), vesicle aggregation did not depend on Ca^{2+} ions and started upon SP-A addition. At neutral pH (○), SP-A-induced vesicle aggregation depended on the addition of 1 mM Ca^{2+}. These experiments were also done with 50 μM free Ca^{2+} at both neutral and acidic pH with identical results. Four different preparations of porcine SP-A were used. A representative experiment is shown.

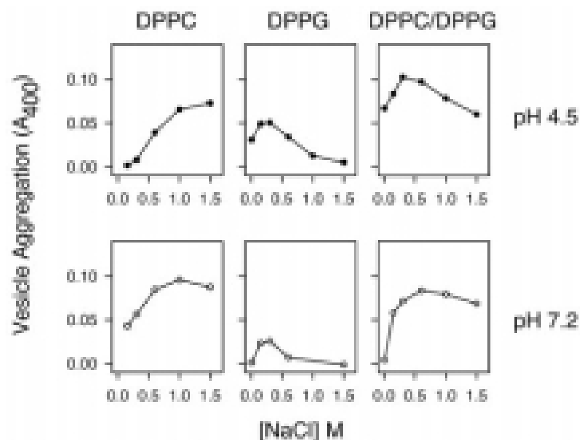

FIG. 5. **Effect of the ionic strength on vesicle aggregation induced by SP-A at acidic and neutral pH.** Concentrated vesicle suspensions (3 mg/ml) of DPPC, DPPG, and DPPC/DPPG (7:3, w/w) were prepared in 100 mM NaCl, 0.1 mM EDTA, 25 mM acetate, 25 mM Tris/HCl buffer at either pH 7.2 (○) or 4.5 (●). Vesicle aggregation experiments were done at 37 °C as described under "Experimental Procedures." Final concentrations of phospholipids, SP-A, and free Ca^{2+} were 85 μg/ml, 8.5 μg/ml, and 50 μM, respectively. Sample turbidity was continuously monitored at 400 nm. The NaCl concentration was increased in the medium by the addition of repeated small aliquots of a concentrated NaCl solution. The absorbance at 400 nm was registered once it was stabilized after each change of NaCl concentration. The experiment started at a NaCl concentration of 2.7 mM because lipid vesicles were always prepared in the presence of salts. Results presented are from a representative one of three experiments using three different preparations of porcine SP-A.

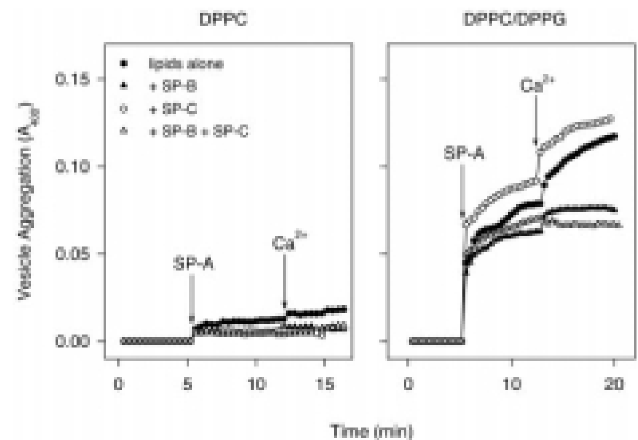

FIG. 6. **SP-A-induced aggregation of liposomes containing SP-B and/or SP-C at acidic pH.** Reconstitution of SP-B or SP-C or both hydrophobic proteins in DPPC or DPPC/DPPG (7:3, w/w) vesicles was performed, as described under "Experimental Procedures," in 150 mM NaCl, 0.1 mM EDTA, 25 mM acetate, 25 mM Tris/HCl buffer, pH 4.5. Vesicle aggregation experiments were performed as in Fig. 5. Final concentrations of SP-A, phospholipids, and Ca^{2+} were 7 μg/ml, 70 μg/ml and 1 mM, respectively. The SP-B or SP-C to lipid weight ratio was 1:20. The results shown are from a representative one of three experiments. Four experiments using vesicles containing 10% (w/w) of either SP-B or SP-C were also performed with identical results.

gation found at neutral pH, high ionic strength was needed. At pH 7.2, the aggregation of DPPC vesicles was independent of ionic strength because it occurred at very low NaCl concentrations (2.7 mM). However, the extent of aggregation was enhanced as the concentration of NaCl was increased. In the case of DPPG vesicles or vesicles containing DPPG, higher levels of vesicle aggregation occurred at very low ionic strength at pH 4.5 than at pH 7.2. At neutral pH, aggregation of acidic vesicles induced by SP-A was abrogated at very low NaCl and Ca^{2+} concentrations, probably due to electrostatic repulsion between the negative charge of phospholipids and the negative surface charge on the protein (24). However, at pH 4.5, the protonation of carboxyl groups of SP-A would reduce the negative surface charge on the protein. On the other hand, aggregation of DPPG vesicles at pH 4.5 was progressively decreased as NaCl concentration increased, suggesting that the interaction of SP-A with DPPG at acidic pH was ionogenic. Aggregation of DPPC/DPPG vesicles slightly decreased as the ionic strength increased due to the presence of DPPC in the vesicle, which might interact with SP-A by hydrophobic interactions.

SP-A is stored intracellularly in acidic lamellar bodies together with surfactant lipids and mature SP-B and SP-C (6, 7). It was of interest to determine whether the presence of SP-B or SP-C or both hydrophobic proteins modified the lipid aggregation activity of SP-A at acidic pH. Therefore, SP-B and SP-C were reconstituted in DPPC or DPPC/DPPG vesicles (protein: lipid weight ratio, 1:20 or 1:10). DPPC aggregation induced by SP-A was essentially negligible at pH 4.5 when DPPC vesicles contained SP-B, SP-C, or a mixture of both proteins (Fig. 6). DPPC/DPPG vesicles either with or without SP-B or SP-C markedly aggregated at pH 4.5 upon the addition of SP-A in a Ca^{2+}-independent manner. Aggregation of acidic vesicles containing SP-B or SP-C at acidic pH was higher than that at neutral pH (data not shown). Vesicles containing SP-B or a

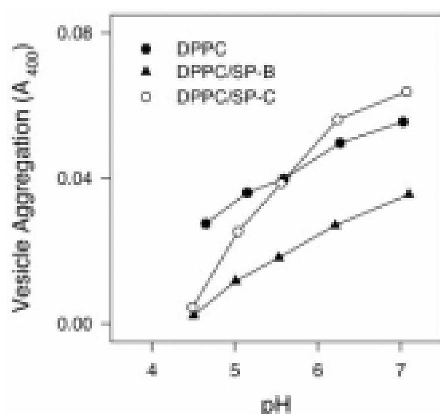

FIG. 7. **SP-A-induced aggregation of DPPC vesicles with or without SP-B or SP-C as a function of pH.** DPPC vesicles with or without SP-B or SP-C (protein:lipid weight ratio, 1:10) were prepared in 150 mM NaCl, 0.1 mM EDTA, 25 mM acetate, 25 mM MES, 25 mM Tris/HCl buffer at various pH values. Experiments were done at the indicated pH at 37 °C as described before. The final turbidity change at 400 nm after the addition of SP-A and 1 mM Ca^{2+} is shown as a function of pH. Results are from a representative one of three experiments. Two different preparations of SP-A, SP-B, and SP-C were used.

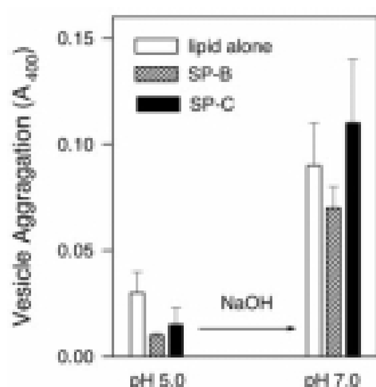

FIG. 8. **Effect of the shift of pH from 5.0 to 7.0 on SP-A-induced aggregation of DPPC vesicles containing SP-B or SP-C.** DPPC vesicles (1 mg/ml) were prepared with or without SP-B or SP-C (protein: lipid weight ratio, 1:10) in 150 mM NaCl, 0.1 mM EDTA, 25 mM acetate, 25 mM Tris/HCl buffer at pH 5.0. Sample turbidity as a consequence of DPPC aggregation at pH 5.0 was monitored at 400 nm at 37 °C. Final concentrations of SP-A, phospholipids, and Ca^{2+} were 8 μg/ml, 80 μg/ml, and 1 mM, respectively. The pH shift from 5.0 to 7.0 was done by adding a small aliquot of a concentrated solution of NaOH. After pH shift, the change in turbidity at 400 nm was monitored again over 10 min. Values are expressed as means ± S.D. of four experiments.

mixture of both hydrophobic proteins showed less aggregation than those vesicles with SP-C or without proteins.

Next, we analyzed the ability of SP-A to aggregate DPPC vesicles at various pH values (Fig. 7). DPPC aggregation increased as the pH was raised to pH 7.0. Aggregation of DPPC vesicles at pH higher than 5.0 was dependent on the presence of Ca^{2+} ions. At the pH of lamellar bodies (about 5.5), SP-A mediated aggregation of DPPC vesicles containing SP-B or a mixture of hydrophobic proteins (data not shown) is lower than that found with DPPC vesicles alone or containing SP-C.

To determine whether a rapid shift of pH from 5.0 to 7.0 was sufficient to raise the extent of DPPC aggregation, we performed pH shift experiments in the presence and the absence of Ca^{2+}. Fig. 8 shows that the addition of NaOH to a mixture of SP-A and DPPC vesicles containing SP-B or SP-C resulted in a 7-fold increase of liposome aggregation. The extent of aggregation after pH shift increased by 3-fold for DPPC vesicles without SP-B or SP-C. The presence of Ca^{2+} ions was needed for

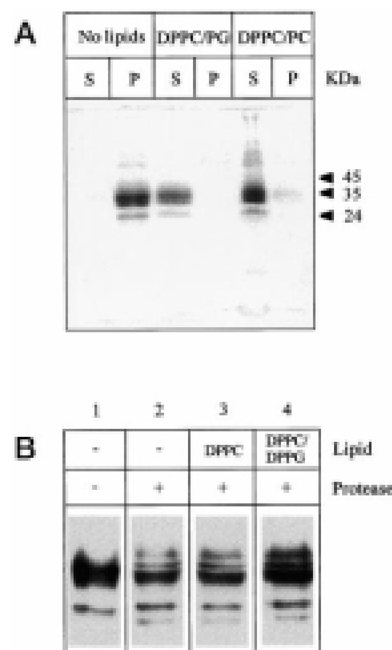

FIG. 9. **Lipid binding (*A*) and protease sensitivity (*B*) of SP-A at acidic pH.** *A,* results presented here are from vesicles of DPPC/PC and DPPC/PG (7:3, w/w) at 25 °C. Similar results were obtained from vesicles of DPPC and DPPC/DPPG (7:3, w/w) with or without SP-B and SP-C. Experiments were done as described under "Experimental Procedures." In the absence of lipids, SP-A sedimented, and all of the protein was recovered in the pellet (*P*). After incubation of SP-A with neutral or acidic vesicles, most of the protein was recovered in the supernatant (*S*) coflotating with lipids. Molecular markers in kDa are indicated to the right of the Coomassie Blue-stained SDS-PAGE gel. *B,* SP-A (6.7 μg) was digested with endoproteinase Glu-C (13.5 μg) in the absence (*lane 2*) and the presence of 60 μg of DPPC vesicles (*lane 3*) or DPPC/DPPG (7:3, w/w) vesicles (*lane 4*) in 110 μl of 150 mM NaCl, 25 mM acetate buffer, pH 4.5. A control (*lane 1*) was run with 6.7 μg of SP-A without endoproteinase Glu-C. Western blot analysis for SP-A was done as described under "Experimental Procedures." A representative one of four experiments is shown.

this reversion. In the absence of Ca^{2+}, the light scattering was almost negligible at pH 5.0 and after retitrating back to pH 7.0 (data not shown). These results suggest that pH changes are accompanied by changes in the SP-A/DPPC interactions, probably as a consequence of pH-dependent structural changes to the SP-A molecule. pH shift experiments were also performed with DPPC/DPPG vesicles with or without SP-B and SP-C. The shift of pH from 5.0 to 7.0 resulted in a small but significant decrease of the extent of aggregation of these vesicles (data not shown).

Lipid Binding and Protease Sensitivity of Membrane-bound SP-A—We next investigated whether the low aggregation activity of SP-A with neutral vesicles at acidic pH was a consequence of reduced ability of SP-A to bind DPPC vesicles at this pH. SP-A was coincubated at 25 °C with either DPPC/PC (7:3, w/w) or DPPC/PG (7:3, w/w) vesicles at pH 4.5, and binding was determined by sucrose density gradient centrifugation at the same temperature. Fig. 9*A* shows that most of the protein was recovered in the supernatant coflotating with DPPC/PC vesicles. In the absence of lipids, all of the protein was recovered in the pellet. At pH 4.5, SP-A hardly aggregated DPPC/PC vesicles at either 37 °C (Fig. 5) or 25 °C (data not shown), but it bound to these vesicles to nearly the same extent as to DPPC/PG vesicles (Fig. 9*A*). Similar results were obtained with DPPC and DPPC/DPPG, which at the temperature of the experiment were in the gel state. SP-A also bound to DPPC vesicles containing SP-B or SP-C (protein:lipid weight ratio, 1:10).

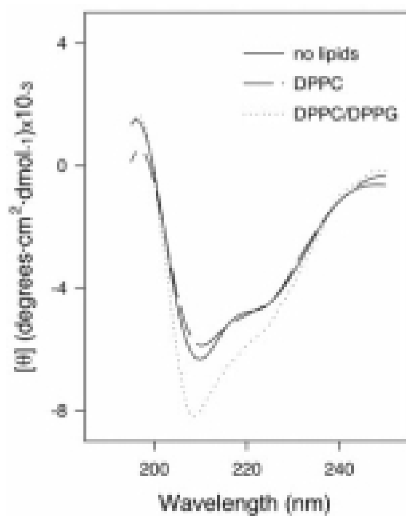

FIG. 10. **Effect of DPPC and DPPC/DPPG vesicles on circular dichroism spectra of SP-A at pH 4.5.** SP-A (0.08 mg/ml, final concentration) was added to 25 mM acetate buffer, pH 4.5, in the absence (*solid line*) or the presence of DPPC (*dashed line*) or DPPC/DPPG (7:3, w/w) vesicles (*dotted line*) (lipid:protein weight ratio, 6:1). Circular dichroism measurements were performed 10 min after the SP-A addition as described under "Experimental Procedures." CD spectra presented here are from a representative one of three experiments with different SP-A preparations.

The protease sensitivity of SP-A bound to either DPPC or DPPC/DPPG membranes at pH 4.5 was studied by means of endoproteinase Glu-C from *S. aureus V8*. Immunoblot analysis of SP-A after endoproteinase Glu-C digestion is shown in Fig. 9*B*. DPPC/DPPG-bound SP-A was more protected from protease degradation than DPPC-bound SP-A, which showed similar susceptibility to proteolysis as lipid-free SP-A. The binding of acidic vesicles to SP-A may lead to reduced accessibility of endoproteinase Glu-C cleavage targets located in the domains in which acidic phospholipids are bound. Alternatively, the reduced susceptibility to proteolysis of DPPC/DPPG-bound SP-A over DPPC-bound SP-A can be a consequence of the ability of SP-A to aggregate acidic but not neutral vesicles at acidic pH. SP-A would become more protected from proteolysis in such vesicle aggregates.

Effect of the Lipid on the Secondary Structure of SP-A—We analyzed the effect of DPPC and DPPC/DPPG on the CD spectra of SP-A at pH 4.5 (Fig. 10). SP-A was self-aggregated at this pH in the absence of lipids and had a characteristic CD spectrum (Fig. 1). The presence of DPPC vesicles in the medium had no significant effect on the CD spectra of SP-A. Thus, DPPC bound to SP-A without modifying the secondary structure of H^+-induced aggregated form of SP-A. In contrast, the interaction of DPPC/DPPG vesicles with SP-A resulted in a change in the CD spectra of the protein. The negative ellipticity at both 207 and 222 nm increased and the negative maximum was shifted from 209 to 207 nm. These changes to the CD spectra of SP-A might be interpreted in terms of a decrease of the self-aggregation level of the protein as a consequence of the binding of SP-A to acidic vesicles. The presence of DPPC/DPPG vesicles in the medium would prevent protein self-aggregation provided lipid/protein interactions were favored over protein/protein interactions.

DISCUSSION

In exocrine, endocrine, or neuronal cells, it is well recognized that there is a progressive decrease in pH along the exocytic pathway (14). Several lines of evidence indicated that the acidification of the exocytic pathway could play a role in the regulation of sorting, transport, or proteolytic processing of proteins (16–18, 43, 44). The results presented in this paper show that a progressive decrease in pH influences the secondary structure and lipid binding and aggregation properties of SP-A.

pH-dependent Self-aggregation of SP-A—In this study, we have found that SP-A undergoes self-aggregation as the pH is reduced below 6.0. However, there is a marked aggregation of the protein in the pH range of the TGN (6–6.5) when Ca^{2+} is added. This effect is greater as the pH is reduced. There is a synergy between H^+ and Ca^{2+} on SP-A self-aggregation in the pH range of 5–6.5. Ca^{2+} has no effect on the self-aggregation of the protein when the pH is reduced toward the isoelectric pH of SP-A. Self-aggregation of the protein appears to be due to pH-induced conformational change of the protein. Conformational analysis from CD spectra of SP-A at various pH values indicates that the percentage of α-helix progressively decreases and that of β-sheet increases as the pH is reduced from 7.0 to 4.7. The increase of β-sheet content could be associated with protein/protein interactions that occurred between SP-A molecules at acidic pH.

It is of interest to note that proteins traversing the TGN and immature secretory granules, the site of sorting constitutive from regulated proteins, are exposed to mildly acidic pH and high concentrations of Ca^{2+} ions (14–16). The maintenance of an acidic pH and high Ca^{2+} milieu in the TGN and immature secretory granules is known to be important for secretory granule formation. Several individual granule content proteins from pituitary or adrenal chromaffin granules undergo pH-dependent self-aggregation *in vitro* in a Ca^{2+}-dependent (adrenal granules) (17, 18) or Ca^{2+}-independent (pituitary granules) (18) manner. Moreover, these proteins can drive co-aggregation of other granule proteins that do not have the inherent ability to self-aggregate. However, constitutively secreted proteins do not co-aggregate with pituitary or chromaffin granule content proteins (17, 18). The H^+ and Ca^{2+}-dependent aggregation property seems to be the hallmark of vesicle proteins of the regulated secretory pathway, distinguishing the regulated secretory proteins from others in the TGN. This could be the case for SP-A. We suggest that the H^+- and Ca^{2+}-dependent aggregation property of SP-A, together with its ability to bind to membranes, might be important for the segregation of this protein to secretory granules. Although selective aggregation and interaction of proteins with the membrane of the TGN are important elements in the sorting of proteins, there is evidence that suggests that aggregation alone is not sufficient to ensure the packaging of all proteins in secretory granules (45). To date, no sorting receptor has been conclusively identified. However, a putative receptor for chromogranin B may not recognize the primary sequence of the protein, but a determinant generated by higher order structure of the molecule (45). Interestingly, it has been recently reported that human pro-SP-B contains secretory granule targeting determinants in both the NH_2-terminal propeptide and the mature peptide (46).

SP-A/Lipid Interaction—The site where SP-A and surfactant phospholipids are first assembled is unknown, but it is thought to be in the lamellar body. The postulated scheme of formation and enlargement of lamellar bodies involves bulk transfer of phospholipids through budding of vesicles because synexin and other annexins promote fusion of lipid vesicles with lung lamellar bodies (47). This study was focused on the lipid binding and lipid aggregation activity of SP-A at acidic pH in the presence and absence of SP-B and SP-C.

At pH 4.5, SP-A induces massive aggregation of acidic vesicles either with or without SP-B or SP-C or both proteins. The extent of aggregation is higher at acidic than at neutral pH. Efrati *et al.* (27) previously found that SP-A aggregated a lipid

extract from surfactant at pH 4.4 in the absence of Ca^{2+}. One striking result we have found is that SP-A poorly aggregates DPPC or DPPC/PC vesicles at pH 4.5 in the presence of Ca^{2+}. Interestingly, aggregation of DPPC vesicles containing SP-B, SP-C, or both proteins is abrogated at this pH. At the pH of lamellar bodies (approximately 5.5) SP-A-induced aggregation of DPPC containing SP-B or a mixture of both proteins is very low and was Ca^{2+}-dependent. A shift of pH from 5.0 to 7.0 results in a 7-fold increase of DPPC aggregation when liposomes contain surfactant hydrophobic proteins and a 3-fold increase for DPPC vesicles alone. These results indicate that 1) aggregation of DPPC vesicles induced by SP-A strongly depends on pH, 2) Ca^{2+} does not have the same effect on DPPC aggregation at acidic and at neutral pH, and 3) the effect of pH on vesicle aggregation is reversible.

A possible interpretation of these results would be that the binding of SP-A to DPPC is also pH-dependent, being much lower at acidic than at neutral pH and completely abrogated at pH 4.5, when vesicles contain the positively charged hydrophobic proteins. However, we show here that SP-A binds to neutral vesicles at pH 4.5 as well as it does to acidic vesicles. SP-A also binds to DPPC liposomes, which contain SP-B or SP-C at that pH. These results suggest that DPPC binding to SP-A and DPPC aggregation induced by the protein are distinct processes, which probably have different requirements. Binding and aggregation as distinct phenomena have also been described in McCormack *et al.* (22). It is possible that the binding of acidic but not neutral vesicles to SP-A modifies the protein structure or the state of protein self-aggregation. We analyzed the effect of DPPC and DPPC/DPPG vesicles on the secondary structure of SP-A at pH 4.5. At this pH, all of the protein is in the self-aggregated form in the absence of lipids. The interaction of DPPC vesicles with SP-A under acidic conditions does not have any effect on the CD spectra of the protein, suggesting that DPPC does not modify the secondary structure of the self-aggregated form of SP-A. In contrast, the binding of DPPC/DPPG vesicles to SP-A results in a striking change of CD spectra of SP-A toward the typical CD spectra of the nonaggregated form of the protein. These experiments suggest that the presence of DPPC/DPPG in the medium prevents the rapid aggregation of the protein induced by H^+. It is possible that lipid/protein interactions are favored over protein/protein interactions at acidic pH provided that vesicles contain acidic phospholipids. We show here that SP-A markedly interacts with DPPG vesicles at acidic pH and that the interaction is ionogenic. On the other hand, SP-A massively aggregates acidic vesicles at acidic pH. We suggest here that SP-A-induced vesicle aggregation requires the protein to be in a nonaggregated form, or at least without forming large protein aggregates. This hypothesis may explain why SP-A binds to neutral vesicles but poorly aggregates them, because the amount of SP-A molecules available to aggregate liposomes is low as the protein is forming large aggregates. The idea that binding of SP-A to DPPC/DPPG but not to DPPC vesicles, at acidic pH, might lead to a decrease of protein aggregation is supported by epifluorescence microscopy studies of fluorescent Texas Red-labeled SP-A adsorbed to monolayers of either DPPC or DPPC/DPPG at pH 4.5.[2] In these experiments, SP-A was first injected in the subphase at pH 4.5, and then the monolayer was formed. Therefore, SP-A was self-aggregated before interacting with the monolayer of phospholipids. With DPPC monolayers, large aggregates of fluorescent SP-A appear at liquid condensed/liquid expanded boundary regions, indicating that SP-A aggregates are segregated to the lipid packing defects in the monolayer. In contrast, with DPPC/DPPG monolayers, all fluorescent SP-A appears exclusively into the liquid expanded phase, and no aggregates of fluorescent SP-A are observed.

[2] M. L. F. Ruano, K. Nag, C. Casals, J. Pérez-Gil, and K. M. W. Keough, unpublished results.

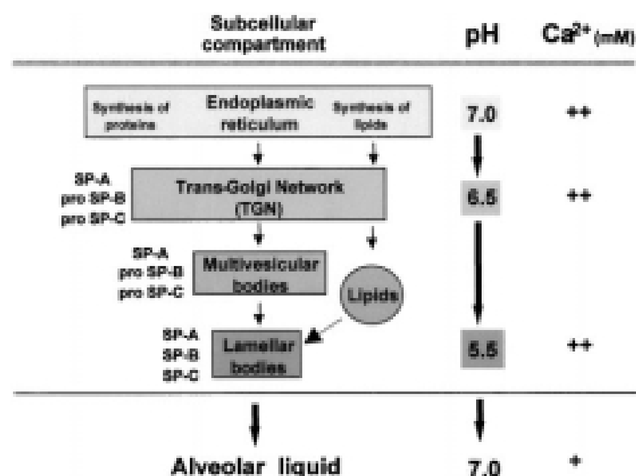

FIG. 11. **Diagrammatic representation of the exocytic pathway of pulmonary surfactant in type II cells.** *Center*, the intracellular compartments involved in posttranslational processing and secretion of surfactant apolipoproteins based upon previous data (7, 44, 48). *Right*, the internal pH of the subcellular compartments of the exocytic pathway (12, 14) and pH of alveolar fluid. These subcellular compartments are exposed to high concentrations of calcium (13, 14, 16). The Ca^{2+} concentration in the alveolar liquid is in the 1-2 mM range (25).

We also report that SP-A bound to DPPC/DPPG vesicles is more protected from endoproteinase Glu-C degradation than SP-A bound to DPPC vesicles or lipid-free SP-A. This result can be explained by the ability of SP-A to induce massive aggregation of acidic but not neutral vesicles. SP-A molecules might form bridges between vesicles and become more protected from degradation. In contrast, large aggregates of protein bind to DPPC vesicles at acidic pH but are not able to aggregate the lipid and remain more susceptible to proteolysis.

Physiological Implications—As in other exocrine cells, the exocytic pathway of type II cells shows a progressive acidification from the endoplasmic reticulum to the secretory lamellar bodies (12, 14, 44). Based upon previous published studies the exocytic pathway of surfactant components is schematically illustrated in Fig. 11. SP-A travels together with the precursors of the hydrophobic surfactant proteins SP-B and SP-C through the same pathway from the Golgi complex to lamellar bodies (7), the site where surfactant phospholipids and surfactant apolipoproteins might be first assembled. The intracellular pH gradient influences the posttranslational processing of pro-SP-C (44) and pro-SP-B (48), which involves intracellular proteolysis of these proteins in acidic subcellular compartments, and the packaging of new synthesized phosphatidylcholine in lamellar bodies (49). In contrast to SP-B and SP-C, no proteolytic events occur in the formation of SP-A except for removal of the signal peptide. However, we found that the secondary structure of the protein, its aggregation state, and its interaction with lipids change with the progressive acidification of the medium. Our results suggest that H^+- and Ca^{2+}-dependent aggregation property of SP-A, together with its ability to bind to membranes, might be important for the sorting of SP-A to secretory granules. Once in lamellar bodies, the presence of acidic phospholipids in the composition of these organelles might be needed for aggregation of membranes induced by SP-A because SP-A hardly aggregates neutral vesicles at acidic pH. This aggregation process together with the synergistic SP-A/SP-B fusion activity at acidic pH (50) could be involved in

the formation of closely packed sheets of lipids in these secretory granules. In addition, the changes of SP-A/lipid interactions with pH shift suggest that the change from the acidic lamellar body to the neutral alveolar subphase could be a factor involved in the reorganization of surfactant material after secretion.

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