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Alanine Mutagenesis of Surfactant Protein A Reveals That Lipid Binding and pH-Dependent Liposome Aggregation Are Mediated by the Carbohydrate Recognition Domain†

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ABSTRACT: The carbohydrate recognition domain (CRD) of surfactant protein A (SP-A) is critical for the modulation of surfactant secretion from isolated type II cells and for the Ca^{2+}-dependent aggregation of surfactant liposomes, but the domains of SP-A that mediate lipid binding have not been precisely mapped. To determine the role of the CRD in lipid interactions and other functions, the conserved amino acids of the putative Ca^{2+} and carbohydrate binding site (Glu^{195}, Glu^{202}, Asn^{214}, Asp^{215}) were substituted with alanine. The wild-type recombinant protein, SP-A^{hyp}, and mutant SP-As, SP-$A^{hyp,E195A}$, SP-$A^{hyp,E202A}$, SP-$A^{hyp,N214A}$, and SP-$A^{hyp,D215A}$, were expressed in insect cells using baculovirus vectors and compared functionally. The Ca^{2+}-dependent binding and aggregation of liposomes at pH 7.0 by SP-$A^{hyp,N214A}$ were comparable to SP-A^{hyp}, but these activities were blocked in SP-$A^{hyp,E195A}$, SP-$A^{hyp,E202A}$, and SP-$A^{hyp,D215A}$. In contrast, the SP-$A^{hyp,D215A}$ but not the other mutant proteins induced the Ca^{2+}-independent aggregation of phospholipid liposomes at pH 4.0. The mutant recombinant proteins did not compete with ^{125}I-labeled rat SP-A for high-affinity receptor occupancy on isolated type II cells and were much less potent than SP-A^{hyp} as regulators of surfactant secretion and uptake from type II cells. We conclude that (1) lipid binding and pH-dependent liposome aggregation are mediated by the CRD of SP-A, (2) distinct but overlapping domains within the CRD are required for pH- and Ca^{2+}-dependent liposome aggregation, and (3) conserved acidic and polar residues of the carbohydrate binding site of SP-A are essential for interactions with type II cells.

Pulmonary surfactant is a complex mixture of lipids and proteins which reduces surface tension forces at the alveolar air−liquid interface and stabilizes pulmonary alveoli. Surfactant protein A (SP-A)[1] is an oligomeric glycoprotein which is synthesized and secreted into the distal airspaces by alveolar type II cells and Clara cells of the pulmonary epithelium [for review, see Weaver and Whitsett 1991)]. The primary structure of SP-A is characterized by 4 discrete domains including (Sano et al., 1987; White et al., 1985): (1) a short NH_2-terminal segment, (2) a collagen-like region of 24 proline and hydroxyproline-rich Gly-x-y repeats, (3) a hydrophobic "neck" region, and (4) a C-terminal carbohydrate recognition domain (CRD) which bears sequence homology to mannose binding protein (MBP) (Drickamer et al., 1986). Several recent studies have elucidated the role of SP-A in pulmonary homeostasis. The deficiency of the surfactant aggregate tubular myelin and altered surface active properties of surfactant from SP-A gene targeted ("knockout") mice provide direct evidence that SP-A is important for surfactant function and structure in the airspace (Korfhagen et al., 1996). The animals did not have any alterations in resting surfactant pool sizes, however, challenging the physiological relevance of *in vitro* evidence that SP-A modulates surfactant homeostasis by receptor-mediated (Kuroki et al., 1988a; Ryan et al., 1989; Wright et al., 1989) enhancement of the uptake (Wright et al., 1987) and secretion of surfactant from isolated type II cells (Dobbs et al., 1987; Kuroki et al., 1988c; Rice et al., 1987). SP-A binds and aggregates liposomes containing the phospholipid dipalmitoylphosphatidylcholine (DPPC) *in vitro* and may play an important role in the adsorption and stability of surfactant lipids at the alveolar air−liquid interface (Hawgood et al., 1987). In addition, structural homologies identify SP-A as a member of the family of preimmune opsonins called the "collectins", which suggests a potential role in pulmonary host defense (Sastry & Ezekowitz, 1993).

Recent mutagenesis studies have identified the CRD as essential for interactions with lipids and alveolar type II cells (Kuroki et al., 1994; McCormack et al., 1994b; Ogasawara

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[1] Abbreviations: SP-A, surfactant protein A; CRD, carbohydrate recognition domain; SP-D, surfactant protein D; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's media; D_0, DMEM with antibiotics and glutamine; D_{10}, D_0 with 10% fetal calf serum; SP-A^{hyp}, recombinant SP-A produced in insect cells; SP-$A^{hyp,E195A}$, recombinant SP-A with the substitution of alanine for glutamic acid 195; SP-$A^{hyp,E202A}$, recombinant SP-A with the substitution alanine for glutamic acid 202; SP-$A^{hyp,N214A}$, recombinant SP-A with the substitution of alanine for asparagine 214; SP-$A^{hyp,D215A}$, recombinant SP-A with the substitution of alanine for aspartic acid 215.

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et al., 1994). The neck and CRD of SP-A grafted onto the collagen-like domain of the structurally related protein, SP-D, are sufficient to confer DPPC binding and aggregation properties to the chimeric molecule (Ogasawara et al., 1994). Interactions between SP-A and phospholipids are blocked by an epitope-specific monoclonal antibody which recognizes a C-terminal segment of the CRD (Kuroki et al., 1994). Tandem amino acid substitutions, $Glu^{195} \rightarrow Gln$ and $Arg^{197} \rightarrow Asp$, introduced into this same region of the CRD (SP-$A^{hyp,E195Q,R197D}$) dissociate the lipid binding and aggregation functions of SP-A (McCormack et al., 1994b). Specifically, the SP-$A^{hyp,E195Q,R197D}$ binds to lipid vesicles but does not cause them to aggregate. Further, the dual amino acid substitutions in SP-$A^{hyp,E195Q,R197D}$ block competitive binding to the type II cell receptor, SP-A-mediated lipid association with alveolar type II cells, and inhibition of surfactant secretion (McCormack et al., 1994b). Collectively, these studies demonstrate that the regions which are essential for Ca^{2+}-dependent lipid aggregation, receptor binding, and surfactant regulation colocalize to the distal portion of the CRD, but the mutagenesis experiments have not revealed the structural requirements for lipid binding or pH-dependent lipid aggregation.

We have recently reported that the conserved amino acids Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215} of the CRD are required for the binding of SP-A to carbohdyrate-linked beads and to the oligosaccharides attached to the major surface glycoprotein (MSG) of the pulmonary pathogen *Pneumocystis carinii* (McCormack et al., 1997). These charged and polar amino acids of SP-A were predicted to coordinate binding to Ca^{2+} and carbohydrate, based on comparisons with the crystal structure of an MBP−oligosaccharide complex (Weis et al., 1992). Individual substitution of each residue with alanine reduced the affinity of SP-A for both monosaccharides and MSG, and the rank order for binding of the mutant recombinant proteins to the two ligands was identical. These data indicated that Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215} participate in the binding of SP-A to carbohydrate, either by direct interaction with the ligand or by contributing to the binding conformation of the CRD through coordination of Ca^{2+}.

The purpose of this study was to map the functional domains of the CRD of SP-A that are required for type II cell and surfactant lipid interactions by alanine mutagenesis. We found that Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215} of the CRD of SP-A are essential for phospholipid binding and aggregation and for regulation of surfactant secretion and uptake by type II cells.

EXPERIMENTAL PROCEDURES

Mutant Recombinant Protein Production. The synthesis and purification of the mutant recombinant proteins used in this study have been reported (McCormack et al., 1997). Briefly, the 1.6 kb cDNA for SP-A was modified using mutagenic oligonucleotides and overlapping extension PCR to encode alanine substitutions for Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215}. The mutant proteins were synthesized in insect cells using baculovirus vectors and purified by affinity chromatography with mannose−Sepharose 6B.

Purification/Modification of Native SP-A. Surfactant was isolated from Sprague-Dawley rats 4 weeks after the intratracheal instillation of 40 mg/kg of silica (Dethloff et al., 1986). SP-A was isolated from sodium bromide gradient purified surfactant by delipidation, mannose−Sepharose affinity chromatography, and gel permeation chromatography on Biogel A-15m (Hawgood et al., 1985). Iodination of SP-A was performed using the Bolton−Hunter reagent, as described (McCormack et al., 1994b).

Protein Assays. The SP-A content of tissue culture media containing recombinant SP-A was determined using a rabbit polyclonal IgG against rat SP-A by a sandwich ELISA (McCormack et al., 1990). Routine protein concentrations were determined with the bicinchoninic protein assay kit (BCA) (Pierce) using bovine serum albumin (BSA) as a standard.

Proteolysis of Recombinant SP-As. Variant SP-As (10 μg) were incubated in EDTA- or Ca^{2+}-containing buffers for 15 min and then digested with trypsin or subtilisin at a protein to enzyme ratio of 20:1 at 37 °C. After 20 min, the reaction was stopped by the addition of hot reducing SDS-sample buffer and boiling for 5 min. The proteins were size-fractionated on SDS−PAGE and either stained with Coomassie blue or transferred to nitrocellulose. For immunoblotting, the membranes were incubated with 10 μg/mL rabbit anti-rat SP-A IgG followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG. Blots were developed by HRP-dependent oxidation of *o*-phenylenediamine.

Intrinsic Fluorescence. The intrinsic fluorescence of SP-A (100 μg) was measured at room temperature in 1 mL of 5 mM Tris containing 0.1 mM EDTA, 1 mM Ca^{2+}, or 5 mM Ca^{2+}. The samples were excited at 280 nm, and emission spectra were recorded from 300 to 400 nm on a SPEX DM3000 fluorescence spectrophotometer (SPEX Industries, Edison, NJ).

Primary Culture of Alveolar Type II Cells and Secretion of Phosphatidylcholine. Alveolar type II cells were isolated from male Sprague-Dawley rats by tissue dissociation with elastase and purification on metrizamide gradients (Dobbs & Mason, 1979). The type II cells were seeded into tissue culture flasks and incubated overnight in [^{3}H]choline (0.5 μCi/mL) containing Dulbecco's modified Eagle's medium (DMEM) and 10% fetal calf serum (D_{10}) at 37 °C in a 10% CO_2 atmosphere. The concentration of Ca^{2+} in the DMEM and D_{10} was 1.7 mM, but the concentration was increased to 10 mM where indicated. Secretion of radiolabeled phosphatidylcholine from type II cells was performed as described (Kuroki et al., 1988c), using SP-A variants as antagonists of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (10^{-7} M) stimulated surfactant secretion. In some experiments, 0.125 M methyl α-mannoside was added simultaneously with the TPA and SP-A. Secretion was measured using [^{3}H]phosphatidylcholine as a marker for surfactant and expressed as percent secretion [radioactivity in the media/(radioactivity in the cells + media)].

Receptor Binding. A whole cell competitive binding assay was performed to determine the ability of various recombinant forms of SP-A to compete with ^{125}I-labeled rat SP-A for receptor occupancy on the surface of isolated type II cells (Kuroki et al., 1988b). Following isolation, 2×10^6 type II cells/35 mm dish were incubated overnight in D_{10} at 37 °C in a 10% CO_2 atmosphere. The following morning, the nonadherent cells were removed by washing the monolayers 3 times at 4 °C with 10 mL of DMEM containing 1 mg/mL BSA. The monolayers were then incubated with 1 μg/mL ^{125}I-labeled rat SP-A and various recombinant SP-As in D_{10}

for 3 h at 37 °C in a 10% CO_2 atmosphere. After being washed 3 times on ice with buffer containing 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM $CaCl_2$, and 1 mg/mL BSA, the cells were solubilized in 0.1 N NaOH, and radioactivity was quantified in a gamma radiation counter.

Liposome Aggregation and Lipid Binding. Liposome binding and aggregation experiments were performed using lipids purchased from Avanti Polar Lipids, as previously described (McCormack et al., 1994a). Unilamellar vesicles were produced by probe sonication of lipid mixtures composed of DPPC/ egg phosphatidylcholine (PC)/phosphatidylglycerol (PG), 9:3:2, and equilibrated with SP-As (lipid:protein ratio 20:1 by weight) in 50 mM Tris (pH 7.4), 150 mM NaCl buffer (buffer A) at 20 °C. Aggregation was determined by measuring light scattering ($A_{400\ nm}$) at 1 min intervals after the addition of 1, 5, 10, or 50 mM Ca^{2+} (final). In some experiments, Ca^{2+}-independent aggregation was induced under low-pH conditions using a buffer composed of 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) and 150 mM NaCl at pH 4.0. For lipid binding, multilamellar liposomes produced by vigorous vortexing of 85:15 DPPC/PG mixtures (1 mg/mL) were mixed with 10 μg/mL SP-A in buffer A containing 2.0% BSA and 5 mM Ca^{2+}. Following incubation for 1 h at room temperature, the mixture was centrifuged at 14000g_{av} for 10 min and washed once, and the SP-A contents of the pellet and pooled supernatant fractions were determined by ELISA. Percent binding was defined as ([SP-A_{pellet}]/[SP-$A_{pellet\ +\ supernatant}$]) × 100. Control experiments in which liposomes or Ca^{2+} were individually deleted were also performed.

Phospholipid Uptake by Type II Cells. Uptake of phospholipid liposomes by type II cells was performed according to the method of Wright (Wright et al., 1987) with minor modifications (McCormack et al., 1994b). Freshly isolated alveolar type II cells (1×10^6/tube) were incubated with unilamellar liposomes (100 μg/mL) composed of [^{3}H]DPPC (1600 cpm/nmol)/egg PC/PG (7:2:1) and SP-A variants in 0.5 mL of DMEM/10 mM HEPES (pH 7.4) for 1 h at 37 °C. The media and cells were separated by centrifugation at 160g for 5 min at 4 °C, and the cells were washed 3 times in ice-cold PBS containing 1 mg/mL BSA. An additional volume of 0.5 mL of phosphate-buffered saline was added to each tube, and the cells and media were transferred to separate liquid scintillation vials and counted. Percent uptake was calculated according to the equation: [[^{3}H]$DPPC_{cells}$/([^{3}H]$DPPC_{cells}$ + [^{3}H]$DPPC_{media}$)] × 100.

RESULTS

Recombinant Proteins. The production and initial characterization of the mutant SP-As used in this study have been described (McCormack et al., 1997). The recombinant proteins were synthesized in insect cells, which only partially hydroxylate prolines within the collagen-like region of SP-A (designated "hyp" for hydroxyproline deficient). The wild-type recombinant protein, SP-A^{hyp}, has been shown to have measurable functional properties which are comparable to rat SP-A (McCormack et al., 1994a,b). We have reported that individual alanine substitutions for Glu^{195} (E195A), Glu^{202} (E202A), Asn^{214} (N214A), and Asp^{215} (D215A) of the CRD reduced the binding of SP-A to mannose−Sepharose beads and to a natural carbohydrate ligand on the surface of *P. carinii* (MSG), but substitution of the less conserved Arg^{197} by Gly within this domain did not affect binding (McCormack et al., 1997). Each of the mutant proteins was purified by adsorption to a mannose−Sepharose affinity matrix, indicating that some degree of Ca^{2+}-dependent binding was preserved despite the neutral amino acid substitutions.

Table 1: Intrinsic Fluorescence Spectra of Recombinant SP-As[a]

	0.1 mM EDTA	1 mM Ca^{2+}	5 mM Ca^{2+}
hyp	344	342 (+6.7)	340 (+3.9)
hyp,E195A	341	344 (−4.8)	346 (−7.7)
hyp,E202A	346	347 (−1.0)	343 (−2.7)
hyp,N214A	342	344 (−10.5)	344 (−13.9)
hyp,D215A	343	343 (−2.6)	346 (−2.5)

[a] The emission maximum (nanometers) and the percent change in maximal emission intensity relative to that in EDTA buffer (parentheses) are given.

Calcium-Induced Conformational Changes in the Mutant SP-As. The binding of Ca^{2+} to SP-A induces a conformational change which causes a small shift in the intrinsic fluorescence emission maximum and a marked increase in resistance to protease digestion (Haagsman et al., 1990). These properties were used to evaluate the integrity of the Ca^{2+} binding site in the alanine mutant SP-As. Rat SP-A contains only two tryptophans, at positions 191 and 213 of the CRD. Upon excitation at 280 nm in EDTA-containing buffer, the emission peak of SP-A^{hyp} occurred at 344 nm (Table 1). After the addition of Ca^{2+} at a concentration of 1 mM, the emission maximum shifted toward the blue end of the spectrum to 342 nm, and the emission intensity increased about 7%, as has been described for native SP-A (Haagsman et al., 1990). When the Ca^{2+} concentration was increased to 5 mM, the wavelength of the SP-A^{hyp} peak shortened further to 340 nm. In the EDTA buffer, the emission maxima of SP-$A^{hyp,E195A}$, SP-$A^{hyp,N214A}$, and SP-$A^{hyp,D215A}$ occurred at a shorter wavelength (341 nm, 343 nm, and 343 nm, respectively) than for SP-A^{hyp}, and that for SP-$A^{hyp,E202A}$ was slightly longer (346 nm). In 1 mM Ca^{2+}, the peak wavelength of the alanine mutant recombinant SP-As did not change or increased, and the emission intensity decreased by 1−10%. However, at 5 mM Ca^{2+}, the peak wavelength of the SP-$A^{hyp,E202A}$ shifted downward by 3−4 nm to 342 nm. Thus, although all of the mutant proteins exhibited alterations in the fluorescent spectra which were consistent with Ca^{2+}-induced changes in the environment for tryptophan, only SP-$A^{hyp,E202A}$ retained the characteristic shortening of the peak emission wavelength at 5 mM Ca^{2+} that was seen with SP-A^{hyp}. The effect of Ca^{2+} on the sensitivity of the variant SP-As to trypsin and subtilisin digestion was next tested. At zero time, before the addition of protease, the wild-type and mutant recombinant proteins migrated similarly on reducing SDS−PAGE with an apparent molecular mass of about 30 kDa, as described previously (Figure 1A, lanes a and b) (McCormack et al., 1997). Following incubation with trypsin for 20 min, the SP-As were cleaved into several fragments which migrated more rapidly than the untreated protein. The major fragment of each recombinant protein had an apparent mass of about 20 kDa, and was demonstrated to contain the CRD by Western blotting using the 1D-6 monoclonal antibody (not shown) (Kuroki et al., 1988c; McCormack et al., 1994b). The 20 kDa fragment of the SP-A^{hyp} was at least partially resistant

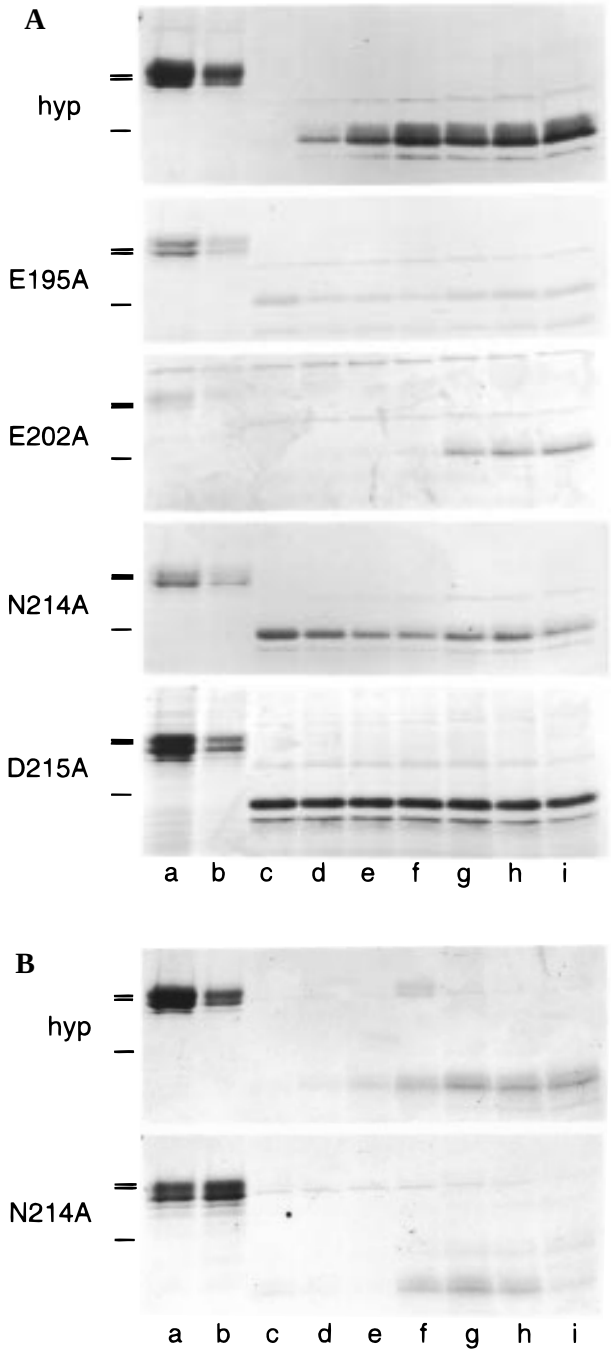

FIGURE 1: Calcium-dependent resistance of recombinant SP-As to proteolysis. Wild-type recombinant SP-A (SP-A^{hyp}) and mutant SP-As with alanine substitutions for amino acids in the CRD were incubated with trypsin (panel A) or subtilisin (panel B) in the presence of EDTA (lane c) or 0.01, 0.1, 1, 5, 10, or 50 mM Ca^{2+} (lanes d−i) for 20 min at 37 °C. The reaction was stopped by boiling in 1% SDS and 2% β-mercaptoethanol. The treated SP-As were size-fractionated on 16% SDS−PAGE gels, and compared to the 10 μg (lane a) or 4 μg (lane b) of the untreated SP-A. The apparent molecular masses of 20 kDa (single line) and 30 kDa (double line) are shown.

to proteolytic attack by trypsin in the presence of 0.01, 0.1, 1, 5, 10, and 50 mM Ca^{2+}, but was completely degraded in 1 mM EDTA. The protective effect of Ca^{2+} appeared to plateau at 1−5 mM Ca^{2+}. The SP-A^{hyp,E202A} was also partially trypsin resistant in the presence of 1−50 mM Ca^{2+}, but was degraded in 0.01−0.1 mM Ca^{2+}, or 1 mM EDTA. The SP-A^{hyp,E195A}, SP-A^{hyp,N214A}, and SP-A^{hyp,D215A} were partially resistant to protease digestion even in the presence of EDTA, which indicated that the mutations had induced

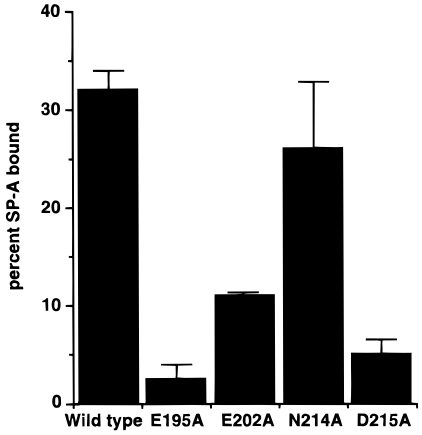

FIGURE 2: Direct binding of recombinant SP-As to phospholipid liposomes. Wild-type and alanine-substituted mutant SP-As were coincubated with multilamellar liposomes composed of DPPC/PG, 85:15, for 1 h at 20 °C in the presence of 5 mM Ca^{2+}. Following centrifugation, the SP-A content of the pellet and supernatant fractions was determined by ELISA. Nonspecific sedimentation of SP-A which occurred in the presence of EDTA was subtracted from total binding to determine specific binding. The data shown are mean ± SE, $n = 3-5$.

trypsin-resistant conformational changes that were not Ca^{2+}-dependent. For the latter proteins, there was no evidence of an increase in protection by Ca^{2+} concentrations as high as 50 mM. Another protease used, subtilisin, also cleaves SP-A^{hyp} into smaller fragments, including a 14 kDa band which reacts with the ID-6 antibody on Western analysis (not shown). As shown in Figure 1B, the SP-A^{hyp} exhibited partial resistance to subtilisin proteolysis in the presence of 0.1, 1, 5, 10, and 50 mM Ca^{2+}. Unlike the result obtained with trypsin, however, the SP-A^{hyp,N214A} was resistant to proteolytic attack by subtilisin at 1, 5, 10, and to a lesser extent 50 mM Ca^{2+}. The subtilisin sensitivity of the other recombinant proteins in the presence of Ca^{2+} was very similar to that obtained with trypsin. We conclude that Ca^{2+}-dependent conformational changes of the CRD that confer protease resistance were at least partially preserved despite the substitution of alanines for Glu^{202} and Asn^{214} but were blocked by alanine substitutions of Glu^{195} and Asp^{215}.

Lipid Binding. The binding of SP-A to DPPC-containing phospholipid vesicles is mediated by the region of the protein which is C-terminal to the collagen-like domain, but it is unclear whether the CRD alone or the combination of the neck + CRD is required (Kuroki et al., 1994; McCormack et al., 1994b; Ogasawara et al., 1994). To more precisely map the domains which are essential for binding to phospholipid, we compared the lipid binding properties of SP-A^{hyp}, and the alanine mutant forms SP-A^{hyp,E195A}, SP-A^{hyp,E202A}, SP-A^{hyp,N214A}, and SP-A^{hyp,D215A}. The variant SP-As were incubated with multilamellar phospholipid liposomes for 1 h, and the fraction of the protein which cosedimented with the 12000*g* pellet was determined (Figure 2). Nonspecific binding which occurred in the presence of EDTA was less than 1.7 ± 0.83% for all proteins and was subtracted from total binding to determine specific binding. Under the conditions of the experiment, 94 ± 0.1% of rat SP-A (not shown) and 32.0 ± 1.9% of SP-A^{hyp} bound specifically to lipid. These values are consistent with what we have reported previously (McCormack et al., 1994a,b). Alanine substitutions for the acidic amino acids Glu^{195} and Asp^{215}

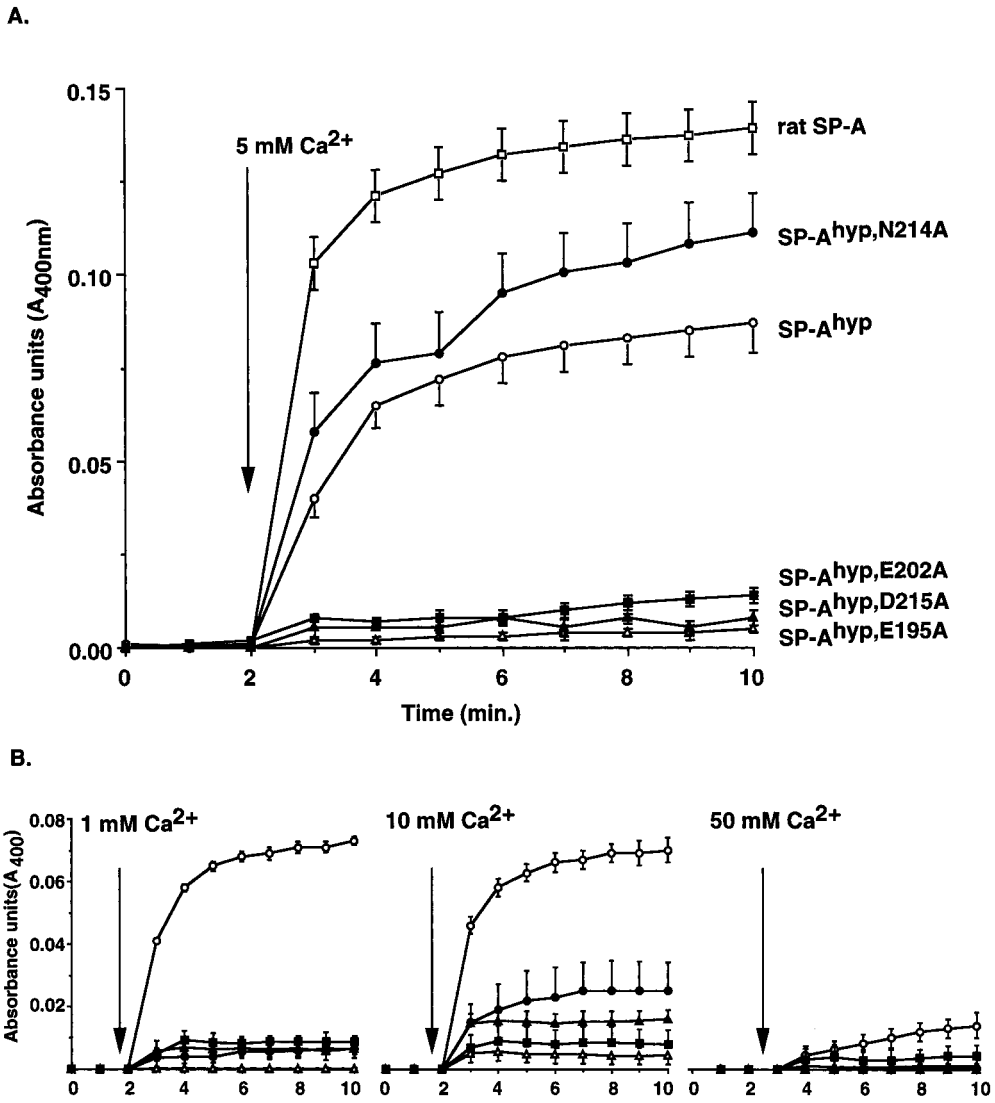

FIGURE 3: Calcium-dependent aggregation of phospholipid liposomes by recombinant SP-As. Unilamellar liposomes composed of DPPC/egg PC/PG, 9:3:2, were incubated with rat SP-A(□), SP-A^{hyp}(○), SP-$A^{hyp,E195A}$ (△), SP-$A^{hyp,E202A}$ (■), SP-$A^{hyp,N214A}$ (●), or SP-$A^{hyp,D215A}$ (▲) in Tris buffer, pH 7.4, in a quartz cuvette. After equilibration for 2 min at 20 °C, 5 mM Ca^{2+} (panel A) or 1, 10, or 50 mM Ca^{2+} (panel B) was added, and light scattering (A_{400}) was measured in a spectrophotometer. The data shown are mean ± SE, $n = 3$.

markedly reduced the binding of SP-A to lipid, such that only 2.5 ± 1.5% of the SP-$A^{hyp,E195A}$ and 5.0 ± 1.5% of the SP-$A^{hyp,D215A}$ added cosedimented with the liposomes. The substitution of Glu^{202} by alanine reduced the lipid binding activity of SP-A to a lesser extent, resulting in the association of 11.0 ± 0.1% of the SP-$A^{hyp,E202A}$ with the lipid pellet. The substitution of alanine for Asp^{214} only slightly inhibited lipid binding to 26.0 ± 6.8% of added SP-$A^{hyp,N214A}$. Collectively, these results indicate that the acidic amino acids Glu^{195}, Glu^{202}, and Asp^{215}, but not the polar residue Asn^{214}, are critical for the Ca^{2+}-dependent binding of SP-A to phospholipid.

Lipid Aggregation. SP-A aggregates unilamellar liposomes containing DPPC at physiologic pH in the presence of Ca^{2+}, at least in part through the interaction of the CRD with the lipid vesicles (Kuroki et al., 1994; McCormack et al., 1994b; Ogasawara et al., 1994). The amino acids of the CRD which are required for aggregation were analyzed by comparing the aggregating properties of the SP-A^{hyp} with each of the alanine-substituted mutant recombinant proteins, and the results are shown in Figure 3. After the addition of 5 mM Ca^{2+}, the rat SP-A and SP-A^{hyp} induced light scattering in DPPC-containing lipid mixtures to values approaching equilibrium end points of 0.145 ± 0.001 and 0.102 ± 0.003 OD A_{400} unit, respectively. The maximum aggregation induced by SP-$A^{hyp,N214A}$ was slightly greater than SP-A^{hyp}, approaching an asymptote at 0.126 ± 0.005 A_{400} unit. The maximum aggregation by SP-$A^{hyp,E195A}$, SP-$A^{hyp,E202A}$, and SP-$A^{hyp,D215A}$ were only 0.007 ± 0.001, 0.016 ± 0.003, and 0.007 ± 0.001 A_{400} unit, respectively, however. The aggregation profile of the wild-type and mutant recombinant proteins was also examined at a range of Ca^{2+} concentrations (Figure 3B), to determine if the activity of the mutant proteins could be restored by excess Ca^{2+}. The light scatterings induced by SP-A^{hyp} and SP-$A^{hyp,E202A}$, SP-$A^{hyp,E195A}$, and SP-$A^{hyp,D215A}$ at 1 and 10 mM Ca^{2+} were similar to those at 5 mM Ca^{2+}, but the SP-$A^{hyp,N214A}$ was less active under both conditions. Very limited aggregation of any of the proteins, including SP-A^{hyp}, occurred at 50 mM Ca^{2+}. Collectively, these results indicate that Glu^{195}, Glu^{202}, and Asp^{215}, but not Asn^{214}, are required for Ca^{2+}-dependent aggregation at pH 7.4. Thus, the amino acid requirements for Ca^{2+}-dependent lipid binding and aggregation are identical, and we conclude that conserved acidic amino acids of the CRD of SP-A are essential for the interaction with phospholipids.

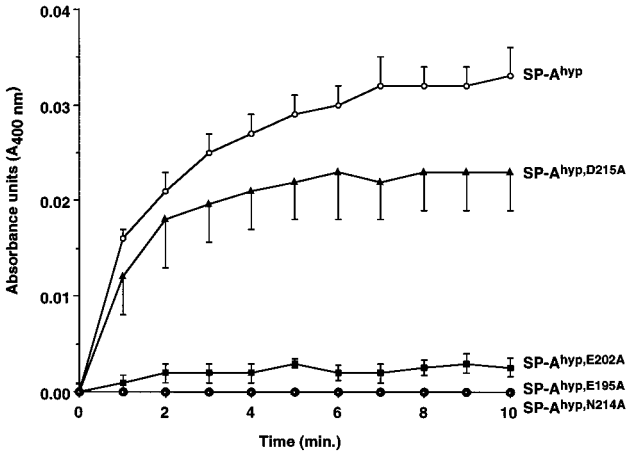

FIGURE 4: pH-dependent aggregation of phospholipid liposomes by recombinant SP-As. Unilamellar liposomes composed of DPPC/egg PC/PG, 9:3:2, were incubated in HEPES buffer, pH 4.0, in a quartz cuvette. After equilibration for 2 min at 20 °C, SP-A^{hyp} (○), SP-A^{hyp,E195A} (△), SP-A^{hyp,E202A} (■), SP-A^{hyp,N214A} (●), or SP-A^{hyp,D215A} (▲) were added, and light scattering (A_{400}) was measured in a spectrophotometer. The data shown are mean ± SE, $n = 3$.

SP-A has also been reported to aggregate lipid vesicles in the absence of Ca^{2+} under acidic conditions (Efrati et al., 1987). The aggregation of the mutant recombinant SP-A proteins was analyzed at low pH to determine if the domains which participate in Ca^{2+}-dependent and Ca^{2+}-independent cross-linking of lipid vesicles are interrelated. First, the stability of the mutant proteins under acidic conditions was evaluated by mannose−Sepharose affinity chromatography at pH 4.0. All of the recombinant proteins bound to the column in the presence of 1 mM Ca^{2+} and were eluted with EDTA, appearing as a peak in approximately 1−2 bed volumes (not shown). These data demonstrate that an important function of the CRD, Ca^{2+}-dependent carbohydrate binding, was preserved at low pH. Rat SP-A caused aggregation of the liposomes in the absence of Ca^{2+} at pH 4.0, approaching a maximal OD of 0.114 ± 0.012 A_{400} unit (not shown). As shown in Figure 4, the SP-A^{hyp} also induced light scattering at low pH, albeit at a reduced level compared to rat SP-A, approaching 0.038 ± 0.001 A_{400} unit. SP-A^{hyp,D215A} aggregated the liposomes about 70% as effectively as SP-A^{hyp} (max OD 0.026 ± 0.001 A_{400} unit) at pH 4.0, but the SP-A^{hyp,E195A}, SP-A^{hyp,E202A}, and SP-A^{hyp,N214A} did not induce appreciable light scattering. We conclude that Glu^{195}, Glu^{202}, and Asn^{214}, but not Asp^{215}, are essential for Ca^{2+}-independent lipid aggregation at pH 4.0. Thus, the CRD mediates pH-dependent aggregation of liposomes, but the amino acid requirements for the function are not identical to those for Ca^{2+}-dependent aggregation.

Type II Cell Receptor Binding. The activities of SP-A to regulate the uptake and secretion of surfactant lipids by type II cells are mediated by binding to a high-affinity cell surface receptor (Kuroki et al., 1988a; Ryan et al., 1989; Wright et al., 1989). We analyzed the effect of alanine mutations of the CRD of SP-A on the activity of the mutant recombinant proteins to compete with ^{125}I-labeled rat SP-A for receptor occupancy on alveolar type II cells (Figure 5). Monolayers of day 1 alveolar type II cells were incubated with 1 μg/mL ^{125}I-labeled rat SP-A and 0−50 μg/mL recombinant SP-As. At a concentration of 20 μg/mL, rat SP-A reduced the binding of ^{125}I-SP-A to below 30% of that seen in the absence

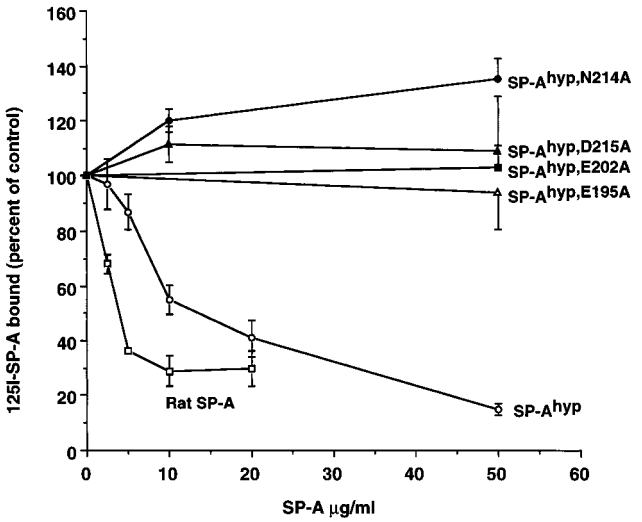

FIGURE 5: Competition of recombinant SP-As with ^{125}I-labeled rat SP-A for receptor occupancy on isolated alveolar type II cells. Primary cultures of alveolar type II cells were incubated with ^{125}I-labeled rat SP-A (1 μg/mL) and various concentrations of rat SP-A (□), SP-A^{hyp} (○), SP-A^{hyp,E195A} (△), SP-A^{hyp,E202A} (■), SP-A^{hyp,N214A} (●), or SP-A^{hyp,D215A} (▲) for 3 h at 37 °C. The monolayers were then washed, dissolved in 0.1 N NaOH, and counted in a γ-counter as outlined under Experimental Procedures.

of antagonist (IC_{50} = 4 μg/mL). The SP-A^{hyp} also competed for the binding of ^{125}I-labeled rat SP-A to the receptor, albeit at slightly lower potency than unlabeled rat SP-A (IC_{50} = 12 μg/mL). The SP-A^{hyp,E195A}, SP-A^{hyp,E202A}, SP-A^{hyp,N214A}, and SP-A^{hyp,D215A} did not compete for receptor occupancy, even at concentrations in 50-fold excess (by weight) of the ^{125}I-labeled rat SP-A ligand. The SP-A^{hyp,N214A} appeared to enhance the binding of ^{125}I-labeled rat SP-A to the monolayer, for unclear reasons. These results indicate that amino acids Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215} are required for SP-A to compete for receptor occupancy on isolated alveolar type II cells.

Inhibition of Surfactant Secretion from Type II Cells. Several studies have suggested that the effect of SP-A to inhibit the secretion of surfactant from type II cells is mediated by the CRD (Kuroki et al., 1994; McCormack et al., 1994b; Ogasawara et al., 1994). We compared the inhibitory activity of mutant forms of SP-A with alanine substitutions for critical amino acids within the CRD, and the results are shown in Figure 6. Rat SP-A inhibited the TPA-stimulated secretion of surfactant from type II cells in a dose-dependent manner, to half-maximal levels (IC_{50}) at an average concentration of 0.08 μg/mL and to below basal levels (36 ± 3.9 %) at 1.0 μg/mL. The IC_{50} for SP-A^{hyp} was also 0.08 μg/mL, and inhibition to below basal levels occurred at a concentration of 10 μg/mL. The SP-A^{hyp,E195A} and SP-A^{hyp,D215A} were much less active than SP-A^{hyp} as inhibitors of surfactant secretion, with IC_{50}s of 68.1 and 133.0 μg/mL, respectively. The SP-A^{hyp,E202A} and SP-A^{hyp,N214A} had intermediate levels of activity, with IC_{50}s of 21.1 and 19.9 μg/mL, respectively. The inhibitory function of these proteins was not increased by supplementing the media with Ca^{2+}. At protein concentrations of 10 μg/mL, the SP-A^{hyp,E202A} inhibited the TPA-stimulated secretion of surfactant to 58.0 ± 1.2% of maximal in 1.7 mM Ca^{2+} (measured concentration in D_{10}) and 63.0 ± 5.1% of maximal in 10 mM Ca^{2+}, and the SP-A^{hyp,N214A} values in 1.7 and 10 mM Ca^{2+} were 51.5 ± 5.1% and 61.5 ± 10.8%, respectively (not

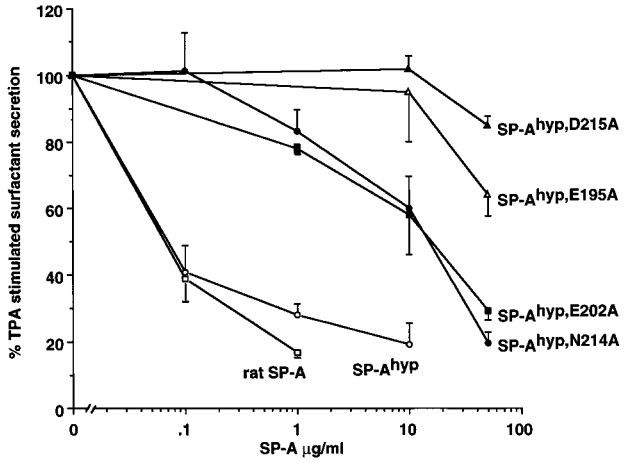

FIGURE 6: Inhibition of secretion of surfactant from type II cells by recombinant SP-As. Isolated alveolar type II cells in primary culture were incubated overnight with [^{3}H]choline to radiolabel phosphatidylcholine. The secretagogue TPA and the antagonists rat SP-A (□), SP-A^{hyp} (○), SP-A^{hyp,E195A} (△), SP-A^{hyp,E202A} (■), SP-A^{hyp,N214A} (●), or SP-A^{hyp,D215A} (▲) were incubated with the monolayers for 3 h. Lipids in the media and cells were organically extracted and counted in a scintillation counter. Percent secretion was defined as label in the media/(media + cells). The data shown are mean ± SE, $n = 3$.

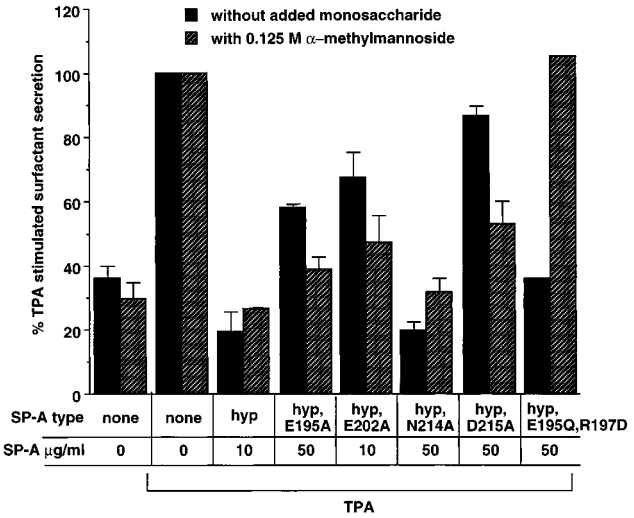

FIGURE 7: Effect of excess saccharides on the inhibition of surfactant secretion by SP-A. The assay for inhibition of secretion was performed as outlined in Figure 4 in the presence of 0.125 M methyl α-mannoside (hatched bars) or no added monosaccharide (black bars). The data shown are mean ± SE, $n = 3$, except SP-A^{hyp,E195Q,R197D} where $n = 2$.

shown). The 10 mM Ca^{2+} conditions decreased the absolute secretion of surfactant from the cells by one-third, but did not affect basal secretion. At the highest protein concentrations tested in the 1.7 mM Ca^{2+} media (50 μg/mL), SP-A^{hyp,E202A} or SP-A^{hyp,N214A} but not SP-A^{hyp,E195A} or SP-A^{hyp,D215A} inhibited secretion to below basal levels. The specificity of inhibition by the mutant SP-As was assessed using a competition experiment with excess free monosaccharides, as described (Kuroki et al., 1988c). Lectins such as concanavalin A have been shown to inhibit surfactant secretion by a nonspecific mechanism that, unlike SP-A, is blocked by methyl α-mannoside. As shown in Figure 7, 0.125 M methyl α-mannoside reversed the inhibitory effect of the previously reported SP-A^{hyp,E195Q,R197D} (McCormack et al., 1994b) but not rat SP-A, SP-A^{hyp}, or the alanine mutant proteins. In the case of SP-A^{hyp,D215A}, and to a lesser extent

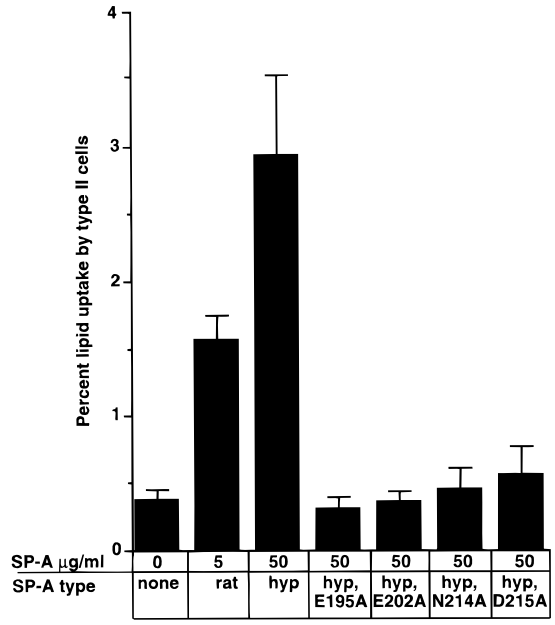

FIGURE 8: Enhanced association of phospholipid liposomes with alveolar type II cells by recombinant SP-As. Unilamellar phospholipid liposomes labeled with trace [^{3}H]DPPC were incubated with freshly isolated alveolar type II cells in the presence of rat SP-A, SP-A^{hyp}, or the mutant recombinant SP-As SP-A^{hyp,E195A}, SP-A^{hyp,E202A}, SP-A^{hyp,N214A}, or SP-A^{hyp,D215A}. Following incubation for 1 h, the cells were washed, dissolved in 0.1 N NaOH, and counted in a scintillation counter. The data shown are mean ± SE, $n = 3$.

SP-A^{hyp,E195A}, coincubation with methyl α-mannoside even appeared to enhance the inhibitory effect of the proteins. The addition of the monosaccharide did not appreciably alter the basal levels of surfactant secretion (29.8 ± 5.15%) or the maximal TPA-stimulated secretion in the absence of inhibitors. Collectively, these data indicate that Glu^{195}, Glu^{202}, Asn^{214} and Asp^{215} are important for the specific inhibition of surfactant secretion by SP-A.

Association of Phospholipids with Type II Cells. Several investigators have shown that SP-A enhances the association of phospholipid vesicles with isolated alveolar type II cells (Horowitz et al., 1996). We tested the activity of the alanine mutant recombinant SP-As to mediate association of liposomes and type II cells, in order to determine amino acids within the CRD that are required for this function. In the absence of added protein, 0.37 ± 0.09% of ^{3}H-labeled liposomes become associated with the freshly isolated type II cells (Figure 8). Rat SP-A at 5 μg/mL enhanced the association of the liposomes with the cells approximately 4.2-fold to 1.56 ± 0.19%. In the presence of 50 μg/mL SP-A^{hyp}, there was an 8-fold increase in type II cell associated label, to 2.94 ± 0.59% of added [^{3}H]DPPC. The SP-A^{hyp,E195A}, SP-A^{hyp,E202A}, SP-A^{hyp,N214A}, and SP-A^{hyp,D215A} all failed to significantly augment the association of the liposomes to monolayers of type II cells, with percent associations of 0.31 ± 0.10, 0.36 ± 0.08, 0.45 ± 0.17, and 0.56 ± 0.22, respectively. These results indicate that Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215} are all required for the property of SP-A to enhance the association of phospholipid vesicles with isolated alveolar type II cells.

DISCUSSION

The intimate association of SP-A with surfactant lipids in the alveolar space contributes to the formation of tubular

myelin (Korfhagen et al., 1996; Suzuki et al., 1989), the lowering of alveolar surface tension (Hawgood et al., 1987), and protection of the surfactant monolayer from disruption by foreign proteins (Cockshutt et al., 1990). The molecular interactions between SP-A and lipid which are responsible for these activities are not fully understood. Phospholipid binding by SP-A probably involves both hydrophobic and charged interactions, since structural studies using a variety of phospholipid species have shown that binding is sensitive to the number and saturation state of the fatty acid side chains as well as the type of head group (Kuroki & Akino, 1991). Lipid binding analyses performed with naturally occurring and proteolytic SP-A fragments initially suggested that the lipid interaction domain of the protein was located within a hydrophobic stretch of amino acids in the neck region of SP-A (Ross et al., 1986). Subsequent studies have provided evidence that the predominant lipid interaction sites of SP-A may be in the CRD (Kuroki et al., 1994; McCormack et al., 1994b; Ogasawara et al., 1994). Recently, analysis of a mutant form of SP-A with tandem substitutions in the CRD demonstrated that the lipid aggregation domain overlaps with the carbohydrate binding domain of SP-A in the CRD, but the same mutations had no effect on phospholipid binding (McCormack et al., 1994b). Our objective in the current study was to further define structural elements which are required for lipid binding and other activities of SP-A.

The binding of SP-A to lipid ligands has been reported to be Ca^{2+}-dependent by some groups (Kuroki & Akino, 1991) and Ca^{2+}-independent by others (King et al., 1983). The targets for mutagenesis of SP-A corresponded to the Ca^{2+} and carbohydrate binding residues of the homologous lectin, MBP. That model predicts that one oxygen atom from each of the side chains of Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215}, and from the main chain of Asp^{215} of SP-A, may form a coordination set for the binding of Ca^{2+}. Our strategy was to determine the functional consequences of substitution of each of these residues with the neutral amino acid, alanine. In the absence of data to implicate specific amino acids in direct interactions with ligand, we reasoned that point mutations in the Ca^{2+} binding region had the potential to broadly assign lipid binding and other incompletely mapped functions to the CRD. This strategy cannot precisely define binding regions within the CRD, however, since weakened Ca^{2+} binding may interfere with conformational changes in SP-A that expose local or remote ligand interaction sites (Sohma et al., 1992).

Three independent experiments were used to assess the integrity of the Ca^{2+} binding site in the alanine mutant proteins. The recombinant SP-As were purified by Ca^{2+}-dependent adsorption to mannose−Sepharose beads, which demonstrated that the Ca^{2+} binding site was not destroyed in any of the proteins. Second, we demonstrated that Ca^{2+} affected the intrinsic fluorescent spectra of all of the proteins, but only the $SP\text{-}A^{hyp}$ and $SP\text{-}A^{hyp,E202A}$ retained the downward shift of the peak emission wavelength that is observed with native SP-A and $SP\text{-}A^{hyp}$. Finally, we found that ligation of Ca^{2+} rendered $SP\text{-}A^{hyp}$, $SP\text{-}A^{hyp,E202A}$, and $SP\text{-}A^{hyp,N214A}$ at least partially resistant to digestion by either trypsin or subtilisin (Sohma et al., 1992). The Ca^{2+} binding site of $SP\text{-}A^{hyp,E195A}$ and $SP\text{-}A^{hyp,D215A}$ was clearly weakened, however, since trypsin resistance was nonresponsive to Ca^{2+}. These data were consistent with a Ca^{2+} binding role for Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215}, and indicate that Ca^{2+} binding was probably best preserved in $SP\text{-}A^{hyp,E202A}$ and $SP\text{-}A^{hyp,N214A}$.

The data presented here indicate that the CRD mediates lipid binding by SP-A (Kuroki et al., 1994; McCormack et al., 1994b; Ogasawara et al., 1994). The rank order for binding to lipid was $SP\text{-}A^{hyp} > SP\text{-}A^{hyp,N214A} > SP\text{-}A^{hyp,E202A} > SP\text{-}A^{hyp,E195A} > SP\text{-}A^{hyp,D215A}$, which exactly paralleled the rank order for binding to carbohydrate-linked beads (McCormack et al., 1997). The observation that point mutations of conserved acidic amino acids in the putative Ca^{2+} coordination site interfere with the association of SP-A with the liposomes supports a direct role for the ligation of Ca^{2+} in lipid binding. That point mutations in the CRD of $SP\text{-}A^{hyp,E195A}$ and $SP\text{-}A^{hyp,D215A}$ effectively block lipid binding suggests that the neck region, which has also been implicated in the interaction with the ligand, probably does not play a major role. These conserved acidic amino acids of the CRD were also required for Ca^{2+}-dependent lipid aggregation. Taken together, these results demonstrate that the CRD contains the lipid binding site and the conserved acidic amino acids of the Ca^{2+} binding site are required for lipid interactions. Further, the domains of SP-A which are required for carbohydrate binding and phospholipid binding overlap.

SP-A has also been shown to aggregate phospholipid vesicles under acidic conditions in the absence of Ca^{2+} (Efrati et al., 1987), but the domains of SP-A which mediate this process have not been clarified. The data presented here clearly indicate that, like Ca^{2+}-dependent aggregation, the CRD of SP-A also mediates pH-dependent phospholipid aggregation. However, the amino acid requirements for the two modes of aggregation are different. Phospholipid vesicle cross-linking at low pH required Asn^{214} but not Asp^{215}, whereas the opposite was true for Ca^{2+}-dependent aggregation. Both Glu^{195} and Glu^{202} were required for the pH-dependent and Ca^{2+}-dependent aggregation. The role of Ca^{2+} and pH in defining SP-A/lipid interactions may be important in the structure of surfactant aggregates in the alveolar space and the association of SP-A with lipids in the endosomal pathway.

The structural requirements for binding to the high-affinity SP-A receptor on alveolar type II cells were also examined. We have previously reported that $SP\text{-}A^{hyp,E195Q,R197D}$ does not compete for receptor occupancy on isolated type II cells, indicating that Glu^{195} and/or Arg^{197} play a role in ligation of the receptor (McCormack et al., 1994b). In this study, we showed that Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215} all participate in binding to the SP-A receptor. These results, in conjunction with previous studies, provide strong evidence that the CRD of SP-A mediates binding to at least one receptor population on alveolar type II cells.

The property of SP-A to regulate the secretion and uptake of surfactant from isolated alveolar type II cells is mediated by the CRD (Kuroki et al., 1994; McCormack et al., 1994b; Ogasawara et al., 1994). We have previously reported that the amino acids Glu^{195} and/or Arg^{197} were important for these processes, since $SP\text{-}A^{hyp,E195Q,R197D}$ produced only weak, monosaccharide reversible inhibition and did not augment the association of liposomes with the type II cells (McCormack et al., 1994b). The data presented indicate that Glu^{195}, Glu^{202}, Asn^{214}, and Asp^{215} are all important for the regulation of surfactant secretion and uptake by SP-A.

In conclusion, we have mapped critical residues of the CRD of SP-A by alanine mutagenesis. Characterization of these mutants demonstrated that lipid binding and pH-dependent lipid aggregation are mediated by the CRD of SP-A. Further, the data indicate that Ca^{2+}-dependent and pH-dependent lipid aggregation have overlapping but distinct amino acid requirements. The results presented also support a critical role for the CRD in the interaction of SP-A with the receptor on alveolar type II cells and the regulation of surfactant secretion and uptake.

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