The Longer Isoform and Cys^{-1} Disulfide Bridge of Rat Surfactant Protein A Are Not Essential for Phospholipid and Type II Cell Interactions†

Mei Zhang,‡ Mamatha Damodarasamy,§ Baher M. Elhalwagi,§ and Francis X. McCormack*,§

Division of Pulmonary/Critical Care Medicine, Department of Internal Medicine, and Department of Pathobiology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Received July 27, 1998; Revised Manuscript Received September 14, 1998

ABSTRACT: Rat SP-A is a heterooligomer of two closely related isoforms, that requires interchain disulfide linkage for several functions including SP-A-mediated phospholipid vesicle aggregation and modulation of surfactant secretion and uptake by isolated alveolar type II cells. While the Cys^{6} disulfide bond of rat SP-A is known to be critical for function, the importance of the second interchain disulfide linkage within the N-terminal Isoleucine^{-3}-Lysine-Cysteine^{-1} (IKC) sequence of the alternatively processed isoform is not clear. To examine the role of the Cys^{-1}-dependent multimerization in SP-A function, we disrupted the Cys^{-1} disulfide bond by deletion of the IKC sequence (SP-A$^{hyp,\Delta IKC}$) or the substitution Cys^{-1} to Ser (SP-A$^{hyp,C-1S}$) in mutant recombinant proteins produced in insect cells. N-terminal sequence analyses revealed that the mutations influenced signal peptidase cleavage specificity, resulting in an increase in the abundance of the longer isoform of SP-A$^{hyp,C-1S}$ and in N-terminal truncation of a fraction of the SP-A$^{hyp,\Delta IKC}$ polypeptides at Gly^{8}. On nonreducing SDS−PAGE analysis, both mutant proteins migrated as monomers and dimers but not the higher multimers that are characteristic of the wild-type recombinant protein (SP-A^{hyp}). Cross-linking analyses demonstrated that the association between trimeric SP-A subunits remained intact despite disruption of the Cys^{-1} bond. The SP-A$^{hyp,C-1S}$ eluted in the same volume as SP-A^{hyp} from the gel sizing column with an apparent mass of 440 kDa, indicative of association of at least 9−10 monomers. The phospholipid binding and aggregation activities of the SP-A$^{hyp,C-1S}$ were approximately 75% and 55% of the SP-A^{hyp}, respectively, but the SP-A$^{hyp,\Delta IKC}$ was functionally comparable to SP-A^{hyp}. Similarly, both mutant proteins regulated the secretion and uptake of surfactant from isolated type II cells, but the SP-A$^{hyp,\Delta IKC}$ was somewhat more potent than the SP-A$^{hyp,C-1S}$. Competitive binding to the SP-A receptor on type II cells was reduced by both Cys^{-1} mutations. We conclude that neither Cys^{-1}-dependent multimerization nor the longer SP-A isoform is absolutely required for oligomeric association of trimeric SP-A subunits, SP-A/phospholipid interactions, or the regulation of surfactant secretion or uptake from type II cells by rat SP-A.

Pulmonary surfactant is a mixture of phospholipids and proteins that maintains airspace patency by reducing surface pressure at the alveolar air−liquid interface. Rat pulmonary surfactant protein A (SP-A)[1] is a Ca^{2+}-dependent phospholipid binding protein that is complexed with tubular myelin and other surfactant aggregates in the alveolar space (*1*). The contribution of SP-A to the surface tension lowering properties of surfactant appears to be less than the hydrophobic surfactant proteins SP-B or SP-C (*2*), but recent evidence suggests that SP-A is important for the structure and stability of surfactant aggregates (*3*−*5*) and the maintenance of low alveolar surface pressure in the presence of protein inhibitors (*6, 7*). SP-A regulates the secretion and uptake of surfactant phospholipid by isolated alveolar type II cells, the primary source of surfactant in the lung, suggesting a role for SP-A in the modulation of surfactant homeostasis (*8*−*10*). While normal tissue and alveolar surfactant pool sizes in the SP-A gene-targeted mouse did not support a role for SP-A in the regulation of lung surfactant levels in the resting mouse, additional studies will be required to determine if the protein performs a homeostatic function in animals under stress (*11*). As a member of the collectin family of preimmune opsonins, SP-A also has potential host defense properties. Reports that SP-A binds to several species of microbes and activates macrophages, and that absence of SP-A in the SP-A gene-targeted mouse results in a bacterial clearance defect, indicate that SP-A may play a role in innate immunity in the lung (*12, 13*). Mutagenesis studies that have defined domains of SP-A that are essential for surfactant and antimicrobial functions (*14*−*20*) may ultimately be useful to predict the

† This research was supported by a Career Investigator Award from the American Lung Association, the Medical Research Service of the Department of Veteran Affairs, and NIH Grant P30ES-0609606.

* To whom correspondence should be addressed. Telephone: 513-558-0480. Fax: 513-558-0835. E-mail: frank.mccormack@uc.edu.

‡ Department of Pathobiology and Laboratory Medicine.

§ Division of Pulmonary/Critical Care Medicine.

[1] Abbreviations: SP-A, surfactant protein A; CRD, carbohydrate recognition domain; IKC, isoleucine-lysine-cysteine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; DSG, disuccinimidyl glutarate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; PG, phosphatidylglycerol, SP-A^{hyp}, hydroxyproline-deficient recombinant SP-A; SP-A$^{hyp,C-1S}$, recombinant SP-A with Cys^{-1} to Ser substitution; SP-A$^{hyp,\Delta IKC}$, recombinant SP-A containing a deletion of the IKC sequence that defines the longer isoform.

10.1021/bi9817966 CCC: $15.00
Published on Web 11/05/1998

minimal structural elements of the molecule required for SP-A replacement in hosts with surfactant dysfunction or pulmonary infection.

Rat SP-A is an oligomer of 18 polypeptide chains that are derived from a single gene (*21*). The primary structure of the protein is characterized by four sequential domains including a short N-terminal segment, a collagen-like region of Gly x−y repeats, a hydrophobic 'neck' domain, and a globular C-terminal carbohydrate recognition domain (*22*). Alternative N-terminal proteolytic processing gives rise to two isoforms with different N-termini (*16*). Approximately 80% of rat SP-A isolated from bronchoalveolar lavage contains the N-terminus that was originally predicted from the deduced amino acid sequence (Asn^1) (*21*), and 20% contains three additional amino acids, Ile-Lys-Cys, that precede Asn^1. Like other collectins, trimerization of monomeric SP-A subunits results from triple-stranded folding of the collagen-like region (*23*, *24*) and the neck domain (*23*, *25*). The fully assembled protein is composed of six trimers that are laterally associated through the first half of the collagen-like domain and stabilized by inter- and intrachain disulfide bonds. SP-A resembles a bouquet of flowers under the electron microscope, like the structurally related complement protein, C1q (*26*).

The functions of SP-A are dependent on the presence of intact disulfide bonds (*15*, *27*−*29*). There are two interchain disulfide-forming cysteines in rat SP-A, at Cys^{-1} and Cys^6, which link up to six or more polypeptide chains in the octadecameric molecule. The Cys^6 disulfide bond has recently been shown to be required for lipid aggregation by SP-A, and for potent lipid binding and the regulation of surfactant secretion and uptake by type II cells (*15*). The Cys^{-1} disulfide bridge is known to be required for the formation of multimers (*16*), but its role in SP-A function has not been examined. The objectives of this study were to determine the functional consequences of limiting covalent linkage of SP-A subunits to dimerization by selective disruption of the Cys^{-1} bond, and to determine whether both rat SP-A isoforms are required for function.

EXPERIMENTAL METHODS

Production of Mutant Recombinant Proteins. Mutant cDNAs encoding the deletion of amino acids Ile^{-3}-Lys^{-2}−Cys^{-1} (ΔIKC) and the substitution of Cys^{-1} by Ser (C−1S) were produced from a 1.6 kb rat SP-A cDNA template (*21*) by overlapping extension PCR (*30*). The mutagenic oligonucleotides pairs that were used to introduce the ΔIKC mutation were 5′-GTTGTCGCTGGTAATGTGACAGAC-3′ and 5′-GTCTGTCACATTACCAGCGACAAC-3′, and the pairs for the C−1S mutation were 5′-ATCAAGTCCAATGTGACAGACG-3′ and 5′-CGTCTGTCACATTGGACTTGAT-3′. The primers used for amplification were derived from sequences within the polyhedrin gene of the PVL 1392 vector (5′AAATGATAACCATCTCGC-3′) and at nucleotide 1200 of the SP-A cDNA (5′-GCAATGGCCTCGTTCTCCTCA-3′). The mutated cDNAs were ligated into the *Eco*R1 site of PVL 1392 transfer vector (Invitrogen) and the correct orientations were confirmed with *Kpn*1. Sequencing of the entire coding region for both constructs confirmed the intended nucleotide changes and the absence of spurious mutations (*31*). Recombinant baculoviruses containing the mutant cDNAs were produced by homologous recombination in *Spodoptera frugiperda* (Sf-9) cells following contransfection with linear viral DNA and the PVL 1392/mutant SP-A constructs (Baculogold, Pharmingen), as described (*18*, *19*). Fresh monolayers of 10^7 Trichoplusia Ni (T. Ni) cells were infected with plaque-purified recombinant viruses at a multiplicity of infection (MOI) of 10, and then incubated with serum-free Excell 400 media supplemented with antibiotics for 72 h. Recombinant SP-A was purified from the culture media by adsorption to mannose−Sepharose 6B columns in the presence of 1 mM Ca^{2+} and elution with 2 mM EDTA (*32*). The purified recombinant SP-A was dialyzed against 5 mM Tris (pH 7.4) and stored at −20 °C.

Purification of Native SP-A. Surfactant was isolated by bronchoalveolar lavage of silica-pretreated Sprague Dawley rats (*33*), floated on NaBr gradients and sedimented by centrifugation, as described (*27*). SP-A was isolated and purified from the surfactant pellet by delipidation, mannose−Sepharose affinity chromatography, and gel permeation chromatography with Biogel A-5m (*34*). The migration profile of silica rat-derived SP-A on reducing and nonreducing SDS−PAGE gels is indistinguishable from that of SP-A purified from normal rats, and their inhibitory activities on the secretion of surfactant from type II cells are comparable (*27*).

Protein Assays. Recombinant SP-A levels in tissue culture media were determined with a rabbit polyclonal IgG against rat SP-A using a sandwich ELISA (*35*). The lower limit of sensitivity of the assay was 0.20 ng/mL, and the linear range extended from 0.16 to 10.0 ng/mL. Routine protein concentrations were determined with the bicinchoninic protein assay kit (BCA) (Pierce) using bovine serum albumin as a standard.

Analysis of Recombinant SP-A. Protein samples were separated by 8−16% SDS−PAGE and stained with Coomassie blue (*36*). The effects of mutations on the oligomeric structure of the recombinant proteins were assessed by chemical cross-linking. Variant SP-As (10 μg) were incubated with 5 mM disucciniidyl glutarate (DSG)(Pierce) in 20 mM HEPES containing 0.1 mM EGTA, 0.15 M NaCl, and 100 mM KCl at room temperature for 30 min. The reaction was stopped by addition of reducing sample buffer, and the proteins were size-fractionated on 8−16% gradient SDS−PAGE gels.

Protein Microsequencing. The N-terminal amino acid sequence of native and recombinant SP-As was determined using a modification (*37*) of the technique of Edman degradation (*38*) and an automated gas-phase microsequencer (Applied Biosystems 470A). The elution of phenylthiohydantoin (PTH)-derivatized amino acids was monitored with a Model 120A PTH analyzer. Proteins were submitted for analysis in water or bound to PVDF membranes after transfer from SDS−PAGE gels. The lower limit of detection for this method is about 1 pmol of protein. The relative abundance of the SP-A isoforms was estimated by comparison of the yield of representative amino acids from each sequence. Residues in the second or third cycle were usually selected for quantitation, to avoid the higher background in the first cycle and the declining yields due to technical limitations in subsequent cycles.

Primary Culture of Alveolar Type II Cells and Secretion of Phosphatidylcholine. Experiments to assess the effect of

Cys^{-1} mutations on the SP-A-mediated inhibition of surfactant secretion were performed as described previously (*15*). Briefly, alveolar type II cells were isolated from male Sprague−Dawley rats by tissue dissociation with elastase and purification on metrizamide gradients (*39*). The type II cells were seeded into tissue culture flasks and incubated overnight in [^{3}H]choline (0.5 μCi/mL) supplemented Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (D_{10}) at 37 °C in a 10% CO_2 atmosphere. Fresh D_{10} medium was applied, and SP-A variants were tested for their ability to antagonize 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (10^{-7} M) stimulated surfactant secretion, by coincubation with the monolayer for 3 h. In some experiments, 0.125 M α-methylmannoside was added simultaneously with the TPA and SP-A, to determine if the SP-A effect was reversible with excess monosaccharides. 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)].

Liposome Aggregation and Lipid Binding. Liposome binding and aggregation experiments were performed using lipids purchased from Avanti Polar Lipids, as previously described (*19*). 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, 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 5 mM Ca^{2+} (final). For lipid binding, multilamellar liposomes produced by vigorous vortexing of DPPC/PG, 75:15, 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 mixtures were 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 (*10*) with minor modifications (*18*). 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.

Receptor Binding. A whole cell binding assay was performed to determine the ability of various recombinant forms of SP-A to compete with ^{125}I-rat SP-A from the SP-A receptor on the surface of isolated alveolar type II cells (*28*). ^{125}I-rat SP-A with specific activities of 179.6, 226.8, and 303.6 cpm/ng was prepared using the Bolton−Hunter reagent as described (*40*). Alveolar type II cells were isolated from Sprague−Dawley rats, and plated at a density of 2×10^6 type II cells/35 mm dish. After overnight incubation in D_{10} at 37 °C in a 10% CO_2 atmosphere, nonadherent cells were removed by washing 3 times at 4 °C with 10 mL of DMEM/1 mg/mL BSA. The monolayers were then incubated with 0.5 or 1 μg/mL ^{125}I-rat SP-A and different concentrations of the mutant recombinant SP-As in D_{10} for 3 h at 37 °C in a 10% CO_2 atmosphere. After washing 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.

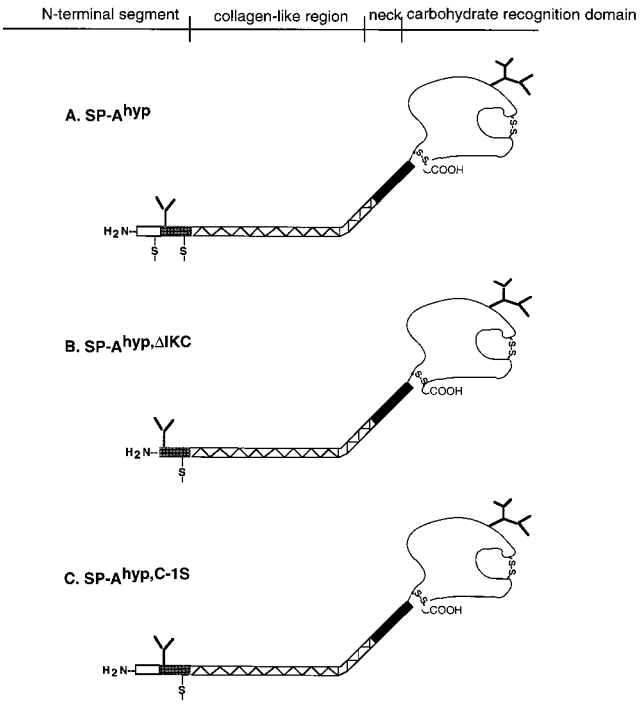

FIGURE 1: Schematic of Cys^{-1} mutant recombinant SP-As. The structures of the longer isoform of wild-type recombinant SP-A, SP-A^{hyp} (panel A), and Cys^{-1} mutant recombinant SP-As bearing a deletion of the IKC sequence, SP-$A^{hyp,\Delta IKC}$ (panel B), or a Cys^{-1} to Ser substitution, SP-$A^{hyp,C-1S}$ (panel C), are shown. The N- and C-terminal branching structures represent protein-associated carbohydrate.

RESULTS

Production and Characterization of SP-$A^{hyp,C-1S}$ and SP-$A^{hyp,\Delta IKC}$. Recombinant proteins used in this study were synthesized in insect cells using baculovirus vectors. The wild-type recombinant SP-A (SP-A^{hyp}) is relatively deficient in hydroxyproline (designated "hyp" for hydroxyproline deficient), but is functionally comparable to rat SP-A isolated from lung lavage (*19*). To evaluate the role of the Cys^{-1} interchain disulfide in SP-A function, two different mutant recombinant forms of SP-A were produced by overlapping extension PCR. The Cys^{-1}-containing N-terminal extension that defines the longer isoform of rat SP-A was deleted (SP-$A^{hyp,\Delta IKC}$), or the Cys^{-1} was substituted by Ser (SP-$A^{hyp,C-1S}$) (Figure 1). Both proteins were secreted from the insect cells and isolated from the culture media by Ca^{2+}-dependent adsorption to the mannose−Sepharose affinity column, indicating that the Cys^{-1} interchain disulfide bond is not required for proper folding and trafficking through the secretory pathway or for competence to bind carbohydrate.

Table 1

	amino acid[a]											
SP-A^{hyp}	−3	−2	−1	1	2	3	4	5	6	7	8	%
sequence 1[b,c]				N_{10}	V_{43}	T	D	V	*C*	A		78
sequence 2[d]	I	K_{12}	−	−	V	T	D					22

	amino acid											
SP-A$^{hyp,\Delta IKC}$	−1	1	2	3	4	5	6	7	8	9	10	%
sequence 1		N_2	V_{10}	T	D	V	*C*	A				34
sequence 2[e]									G	S	P_{19}	66

	amino acid											
SP-A$^{hyp,C-1S}$	−3	−2	−1	1	2	3	4	5	6	7	8	%
sequence 1				N	V_{16}	T	D	V	−	A		12
sequence 2	I_{121}	K	S	−	V	T	D					88

[a] Numbered according to position in the reported native SP-A sequence. [b] Subscripted numbers represent molecular yield (picomoles) of the associated amino acid. [c] Italicized cysteines were disulfide-linked based on elution in the position of diPTH-Cys. [d] (−) = blank cycle. [e] Deletion of IKC exposed a cryptic signal peptide cleavage site resulting in a proteolytic product.

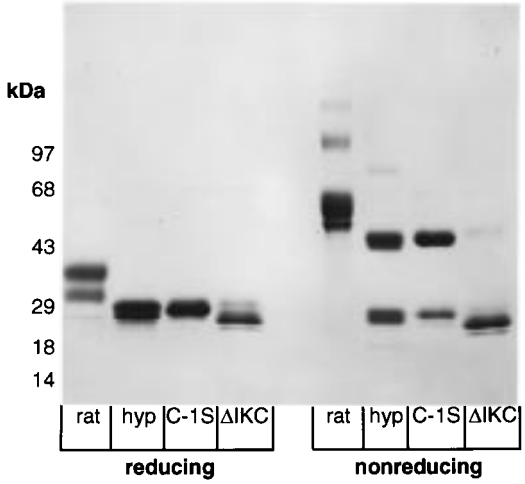

FIGURE 2: Electrophoretic analysis of recombinant SP-As. Rat SP-A, wild-type recombinant SP-A, SP-A$^{hyp,\Delta IKC}$, and SP-A$^{hyp,C-1S}$ were subjected to 8−16% SDS−PAGE under reducing and nonreducing conditions and stained with Coomassie Brilliant Blue.

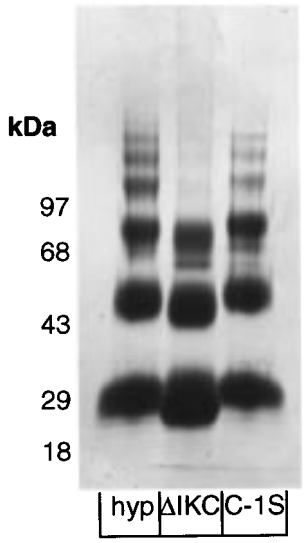

FIGURE 3: Cross-linking analysis of oligomeric association of polypeptide chains in the SP-A$^{hyp,\Delta IKC}$ and SP-A$^{hyp,C-1S}$. The wild-type recombinant SP-A (SP-A^{hyp}), SP-A$^{hyp,\Delta IKC}$, and SP-A$^{hyp,C-1S}$ were incubated with the cross-linking reagent DSG for 30 min at room temperature and then size-fractionated on reducing 8−16% SDS−PAGE gels and stained with Coomassie Brilliant Blue.

The N-terminal sequence analyses of the mutant proteins are shown in Table 1. The SP-A$^{hyp,\Delta IKC}$ was composed of two polypeptide sequences, one with the expected N-terminal sequence beginning with Asn^1 and a second fragment which began with Gly^8. The abundance of the latter product, which most likely resulted from the exposure of a cryptic signal peptidase cleavage site by the IKC deletion, ranged from 33% to 66% of the total protein on three separate sequence analyses. The SP-A$^{hyp,C-1S}$ contained a polypeptide sequence with the N-terminus that is characteristic of the shorter isoform, and the longer SP-A variant containing the expected IKS extension. The abundance ratio of the two isoforms was inverted compared to SP-A^{hyp}, however, such that the longer isoform comprised 88% of the mutant protein compared to 22% of SP-A^{hyp}. The reduced yield of Asn^1 compared to Val^2 in the SP-A$^{hyp,C-1S}$ and SP-A$^{hyp,\Delta IKC}$ proteins is consistent with glycosylation of Asn^1 (*16*) and demonstrates that neither the Cys^{-1} disulfide bond nor the IKC sequence is required for carbohydrate modification at the N-terminal position. On reducing SDS−PAGE analysis, the migration profile of the SP-A^{hyp} differs from that of rat SP-A primarily because of differences in the extent and complexity of glycosylation, as described (*19*). Both mutant SP-As migrated similarly to SP-A^{hyp} as a series of bands between 25 and 32 kDa (Figure 2), although the lower molecular mass species near 25 kDa were enriched in SP-A$^{hyp,\Delta IKC}$ and the higher molecular mass bands near 32 kDa were more abundant in SP-A$^{hyp,C-1S}$. Under nonreducing conditions, rat SP-A appeared as a ladder of disulfide-dependent multimers. SP-A^{hyp} migrated as monomers, dimers, trimers, and less abundant higher species as previously reported (*19*), but no bands larger than dimers were observed for the Cys^{-1} mutant proteins. The monomeric species were present for both SP-A$^{hyp,C-1S}$ and SP-A$^{hyp,\Delta IKC}$, but the dimeric species were much more abundant for SP-A$^{hyp,C-1S}$ than SP-A$^{hyp,\Delta IKC}$. This pattern is consistent with the truncation of a portion of the SP-A$^{hyp,\Delta IKC}$ at Gly^8 (33−66%), which removes both cysteines that can form interchain linkages. Cross-linking analyses were used to assess the effect of the Cys^{-1} mutations on the oligomeric structure of the protein, and the results are shown in Figure 3. Cross-linking and electrophoretic separation of SP-A^{hyp} resulted in a ladder of nonreducible bands that migrated with apparent

molecular masses consistent with monomers, dimers, and trimers. There were also least five or six fainter bands at higher molecular masses, indicating that a total of up to eight or nine individual polypeptide chains are associated in the oligomeric protein. Rat SP-A produced a nearly identical pattern, except that no monomeric species were present (not shown). For $SP\text{-}A^{hyp,C-1S}$, there were at least six bands clearly visible on the gel, indicating that disulfide formation at Cys^{-1} is not required for the association of SP-A subunits as trimers or higher oligomers in solution. The electrophoretic profile of cross-linked $SP\text{-}A^{hyp,\Delta IKC}$ was similar to $SP\text{-}A^{hyp,C-1S}$ in that both were at least trimeric but the cross-linked species containing more than three chains were much fainter for the $SP\text{-}A^{hyp,\Delta IKC}$. This result suggests that the $Gly^{8}-Phe^{228}$ truncated fragment which does not contain cysteines available for interchain disulfide formation reduces the association of trimeric subunits in solution for this protein. To examine the effect of disruption of the Cys^{-1} interchain disulfide bond on assembly of SP-A, the $SP\text{-}A^{hyp,C-1S}$ was analyzed using gel permeation chromatography on Bio-Gel A-5m (not shown). Rat SP-A eluted near the position of blue dextran, with an apparent molecular mass that was approximately 1.6 mDa, as previously reported (*27*). The peak for $SP\text{-}A^{hyp}$ was centered around the elution position of ferritin (440 kDa), predicting the association of multiple (>10) SP-A subunits, consistent with our prior report (*15*). The $SP\text{-}A^{hyp,C-1S}$ eluted as a single peak in exactly the same position as $SP\text{-}A^{hyp}$, indicating that the Cys^{-1} interchain disulfide bond is not required for the oligomeric assembly of SP-A.

Direct Binding of $SP\text{-}A^{hyp,C-1S}$ and $SP\text{-}A^{hyp,\Delta IKC}$ to Multilamellar Liposomes. The binding of $SP\text{-}A^{hyp,C-1S}$ and $SP\text{-}A^{hyp,\Delta IKC}$ to DPPC-containing liposomes was compared, and the results are shown in Figure 4A. In the presence of Ca^{2+}, $56.9 \pm 4.3\%$ of $SP\text{-}A^{hyp,\Delta IKC}$ and $49.3 \pm 2.4\%$ of $SP\text{-}A^{hyp,C-1S}$ cosedimented with the lipid vesicles. This value was very similar to the $55.3 \pm 2.2\%$ $SP\text{-}A^{hyp}$ that bound to the liposomes, and to values that had been reported previously for $SP\text{-}A^{hyp}$ (*15*). Higher than background nonspecific binding by the $SP\text{-}A^{hyp,C-1S}$ protein ($8.3 \pm 4.6\%$) resulted in a value for specific binding that was slightly less for $SP\text{-}A^{hyp,C-1S}$ ($41.0 \pm 3.2\%$) than for either $SP\text{-}A^{hyp}$ ($53.8 \pm 2.2\%$) or $SP\text{-}A^{hyp,\Delta IKC}$ ($56.2 \pm 4.0\%$). It is clear, however, that neither the longer isoform of SP-A nor the Cys^{-1} interchain disulfide bond is required for the binding of SP-A to phospholipid liposomes.

Aggregation of Unilamellar Liposomes by $SP\text{-}A^{hyp,C-1S}$ and $SP\text{-}A^{hyp,\Delta IKC}$. The Ca^{2+}-dependent aggregation of phospholipid liposomes by SP-A can be quantified by measurement of light scattering in a spectrophotometer (*19, 34*). As shown in Figure 4B, the maximal end point for light scattering induced by $SP\text{-}A^{hyp}$ was 0.09652 ± 0.0003 A_{400} unit, about half the maximal aggregation induced by rat SP-A [0.1822 ± 0.0006 A_{400} unit (not shown)]. The $SP\text{-}A^{hyp,\Delta IKC}$ also caused liposome aggregation, to a greater extent than did $SP\text{-}A^{hyp}$, approaching a maximal end point of 0.1206 ± 0.0004 A_{400} unit. In contrast, the $SP\text{-}A^{hyp,C-1S}$ protein was less active than $SP\text{-}A^{hyp}$ in the aggregation assay, with an end point of 0.06457 ± 0.0013 A_{400} unit. We conclude that neither the longer isoform of rat SP-A nor the interchain disulfide bond at Cys^{-1} is absolutely required for liposome aggregation.

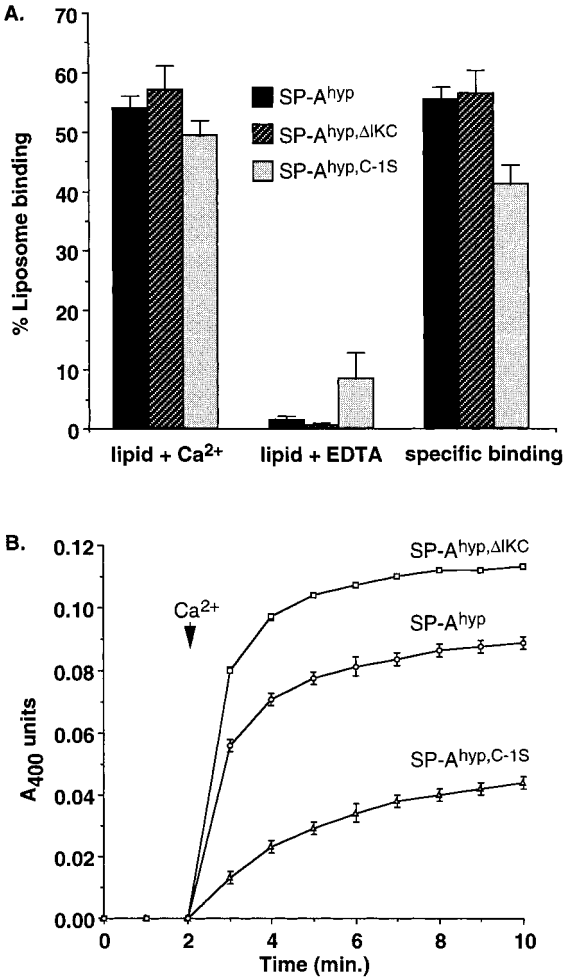

FIGURE 4: Direct binding and aggregation of phospholipid liposomes by recombinant SP-As. Multilamellar liposomes were incubated with $SP\text{-}A^{hyp}$, $SP\text{-}A^{hyp,\Delta IKC}$, or $SP\text{-}A^{hyp,C-1S}$ at room temperature for 1 h. Following centrifugation, SP-As in the pellet and supernatant were quantified by ELISA. Specific binding was determined by subtraction of SP-A sedimentation in the presence of EDTA from total binding (panel A). Unilamellar liposomes were incubated with $SP\text{-}A^{hyp}$, $SP\text{-}A^{hyp,\Delta IKC}$, or $SP\text{-}A^{hyp,C-1S}$ in Tris buffer (pH 7.4) in a quartz cuvette. After equilibration for 3 min at 20 °C, 5 mM Ca^{2+} was added and light scattering ($A_{400\ nm}$) was measured in a spectrophotometer (panel B). The data shown in both panels are mean ± SE, $n = 3$.

Competition of $SP\text{-}A^{hyp,C-1S}$ and $SP\text{-}A^{hyp,\Delta IKC}$ with ^{125}I-Rat SP-A for Receptor Occupancy on Isolated Alveolar Type II Cells. The binding of $SP\text{-}A^{hyp,C-1S}$ and $SP\text{-}A^{hyp,\Delta IKC}$ to the cell surface receptor on isolated alveolar type II cells was assessed in a competitive binding assay (Figure 5). Rat SP-A and $SP\text{-}A^{hyp}$ competed for binding of ^{125}I-rat SP-A to the type II cells in a dose-dependent fashion, with IC_{50}s of 6.0 and 9.2 μg/mL, respectively. The $SP\text{-}A^{hyp,\Delta IKC}$ and the $SP\text{-}A^{hyp,C-1S}$ both competed for receptor occupancy in a dose-dependent manner, but at substantially lower potency than $SP\text{-}A^{hyp}$ with IC_{50}s of approximately 40 and >50 μg/mL. These data indicate that the Cys^{-1} disulfide bond contributes to the affinity of SP-A for the type cell receptor but is not required for receptor binding.

The Inhibition of Surfactant Phospholipid Secretion from Type II Cells by $SP\text{-}A^{hyp,C-1S}$ and $SP\text{-}A^{hyp,\Delta IKC}$. We next studied the effect of disruption of the Cys^{-1} disulfide bridge on the activity of SP-A to inhibit the secretion of surfactant from isolated type II cells. The $SP\text{-}A^{hyp}$ inhibited the TPA-

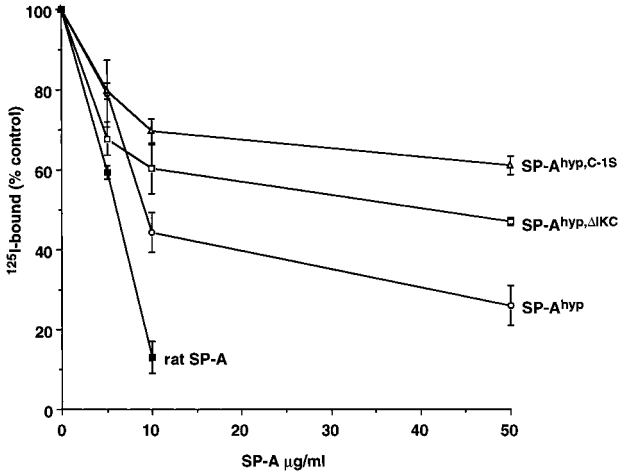

FIGURE 5: Competition of SP-A$^{hyp,\Delta IKC}$ or SP-A$^{hyp,C-1S}$ with ^{125}I-rat SP-A for receptor occupancy on isolated alveolar type II cells. Primary cultures of alveolar type II cells were incubated with 0.5−1 μg/mL ^{125}I-rat SP-A and various concentrations of rat SP-A, SP-A^{hyp}, SP-A$^{hyp,C-1S}$, or SP-A$^{hyp,\Delta IKC}$ for 3 h at 37 °C. The monolayers were washed, dissolved in 0.1 N NaOH, and counted in a gamma radiation counter as described under Experimental Methods.

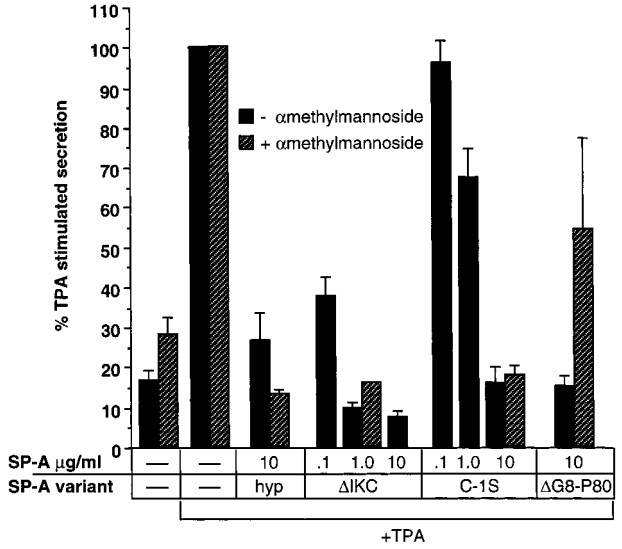

FIGURE 6: Inhibition of phospholipid secretion from alveolar type II cells by recombinant SP-As. Isolated alveolar type II cells in primary culture were incubated overnight with [^{3}H]choline to label cellular phosphatidylcholine. The secretagogue TPA and antagonists SP-A^{hyp}, SP-A$^{hyp,\Delta IKC}$, or SP-A$^{hyp,C-1S}$ were added to the washed cells, and incubated for 3 h at 37 °C. Media and cells were harvested and counted in a scintillation counter. Percent secretion was defined as (counts in the media)/(counts in the media + cells) (solid columns). The specificity of inhibition was assessed by coincubation with 0.125 M α-methylmannoside (hatched columns). The data shown are mean ± SE, $n = 3$.

stimulated secretion of [^{3}H]choline-labeled surfactant phospholipid from isolated type II cells to basal levels at a concentration of 1 μg/mL (Figure 6). The IC_{50} for inhibition by SP-A^{hyp} was approximately 0.6 μg/mL, consistent with previously reported values from this laboratory (*15*). Inhibition by SP-A$^{hyp,C-1S}$ was less potent ($IC_{50} = 4.0$ μg/mL) and inhibition by SP-A$^{hyp,\Delta IKC}$ slightly more potent ($IC_{50} = 0.08$ μg/mL) than the SP-A^{hyp}. The specificity of inhibition of secretion was assessed by coincubation with excess monosaccharides, which are known to block the activity of nonspecific inhibitors such as concanavalin A but not inhibition by

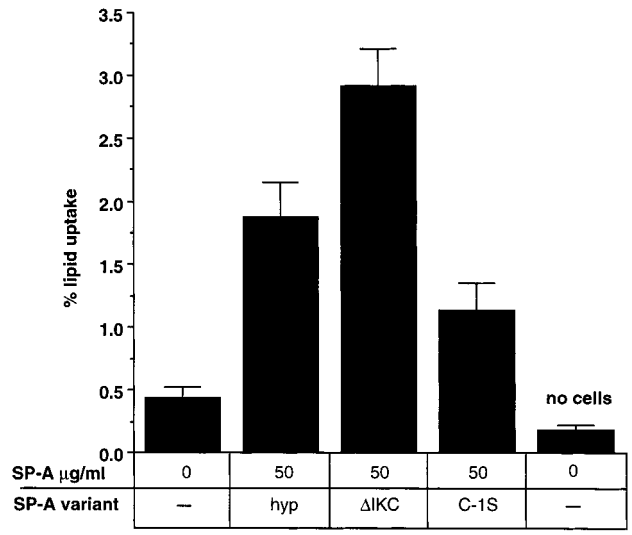

FIGURE 7: Enhanced association of phospholipid liposomes with alveolar type II cells by recombinant SP-As. Unilamellar liposomes labeled with trace [^{3}H]DPPC were incubated with monolayers of isolated alveolar type II cells in the presence of SP-A^{hyp}, SP-A$^{hyp,\Delta IKC}$, or SP-A$^{hyp,C-1S}$. 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 or SP-A^{hyp} (*15*, *27*). The inhibition of secretion produced by the Cys^{-1} mutant proteins was not reversed by the addition of 0.125 M α-methylmannoside, a concentration that partially blocks the nonspecific inhibition of secretion by the previously characterized collagen-region deletion mutant protein, SP-A$^{\Delta G8-P80}$ (Figure 6) (*15*). We conclude that neither the longer isoform of SP-A nor the Cys^{-1} disulfide bond of SP-A is required for specific inhibition of surfactant secretion.

Enhancement of Liposome Association with Type II Cells by SP-A$^{hyp,C-1S}$ and SP-A$^{hyp,\Delta IKC}$. The SP-A-enhanced association (uptake) of phospholipid vesicles by monolayers of isolated alveolar type II cells was assessed using [^{14}C]-DPPC-labeled liposomes, and the results are shown in Figure 7. In the absence of SP-A, approximately 0.43 ± 0.09% of the label was associated with the type II cell monolayer. Control experiments performed in the absence of cells revealed low background levels of radioactivity using this method (0.180 ± 0.05%). SP-A^{hyp} at 50 μg/mL enhanced the monolayer-associated label more than 4-fold to 1.87 ± 0.28%. The SP-A$^{hyp,\Delta IKC}$ and SP-A$^{hyp,C-1S}$ also increased the uptake of the labeled phospholipid, by more than 6-fold (to 2.91 ± 0.3%) and 2.6-fold (to 1.13 ± 0.22%), respectively. These data indicate that neither the longer isoform of SP-A nor the Cys^{-1} intermolecular disulfide bond is required for the SP-A-mediated uptake of liposomes by alveolar type II cells.

DISCUSSION

The purpose of this study was to determine the role of the newly identified Cys^{-1} disulfide bridge and the longer SP-A isoform in the function of rat SP-A (*16*). Two mutant forms of SP-A containing disrupted Cys^{-1} interchain bonds were synthesized in insect cells, and characterized in several in vitro functional assays. We found that while the Cys^{-1} interchain disulfide bond was critical for covalent multimerization of SP-A, unlike the Cys^6 interchain disulfide bond

(*15*), it was not required for the interactions with type II cells and phospholipids that were measured. Collectively, the data indicate that the functions of SP-A are dependent on the disulfide-dependent dimerization of polypeptide chains in the SP-A oligomer, but higher degrees of cross-linking between subunits are not required.

Inter- and intratrimeric disulfide bonds at Cys^{-1} and Cys^{6} rat SP-A link up to six or more polypeptide chains in the fully assembled molecule, and are thought to stabilize the functional configuration of the SP-A oligomer. Kuroki et al. demonstrated that complete reduction and alkylation of SP-A blocked SP-A-mediated aggregation of lipid vesicles and regulation of surfactant secretion and uptake from isolated type II cells, but the protein remained associated as a high molecular weight oligomer (*27, 28*). Similarly, disruption of the Cys^{6} interchain disulfide bond by substitution with Ser also blocked most SP-A functions, without eliminating the oligomeric association of up to nine or more subunits (*15*). These results indicate that intermolecular forces between polypeptide sequences in the neck and collagen-like region are the primary determinants of assembly into higher oligomers, and that interchain cross-links may have functional roles in addition to those related to structural stability.

We have previously reported that the disruption of the disulfide bridge at Cys^{6} blocked lipid aggregation by SP-A and markedly reduced but did not eliminate lipid binding and regulation of phospholipid uptake and secretion by the protein (*15*). The loss of function caused by the disruption of the Cys^{6} disulfide bond in that study was clearly greater than that caused by disruption of the Cys^{-1} linkage described here. One plausible explanation for these findings is that all polypeptide chains in the SP-A oligomer contain Cys^{6} but only 20% contain Cys^{-1}; therefore, extensive dimerization is possible for SP-$A^{hyp,C-1S}$ but not SP-$A^{hyp,C6S}$. Thus, the disruption of Cys^{-1} permitted the dissection of the role of dimerization from higher covalent multimerization, and the deletion of the IKC sequence was designed to analyze the function of the heterooligomeric structure of SP-A by converting all SP-A polypeptides to the shorter isoform.

Both Cys^{-1} mutant proteins were glycosylated and secreted from insect cells, indicating that neither the IKC sequence nor disulfide bond formation at Cys^{-1} is required for eukaryotic glycosylation, processing, or secretion. To our knowledge, rat SP-A is the only reported secreted protein that is known to be glycosylated at the N-terminal residue. Multiple attempts to eliminate all interchain linkages by tandem substitutions at Cys^{-1} and Cys^{6} were unsuccessful, due to rapid intracellular degradation of the double mutant protein in the insect cells (data not shown). This result suggests that at least one disulfide linkage at the N-terminus of SP-A is required for proper folding and trafficking. Both the C−1S and the ΔIKC mutations altered the N-terminal processing of the protein. The C−1S mutation caused an inversion in the ratio of the short to long SP-A isoforms such that the long isoform was predominant. Although Ser is a common amino acid at the −1 position of signal sequence cleavage sites (*41, 42*), it is likely that the Cys^{-1} to Ser mutation enhanced the preference of signal peptidase for the Gly^{-4}−Ile^{-3} bond over the Ser^{-1}−Asn^{1} bond. The ΔIKC mutation exposed a cryptic signal peptidase cleavage site which produced a variably abundant truncated SP-A polypeptide that began at Gly^{8}. We had not predicted that the IKC deletion would result in aberrant cleavage, since the lengths of the signal peptide preceding the Gly^{-4}−Ile^{-3} cleavage site of the SP-A^{hyp} and the Gly^{-4}−Asn^{1} cleavage site of the SP-$A^{hyp,\Delta IKC}$ were identical and Gly is also a preferred amino acid at the −1 position (*41, 42*). In either case, we cannot exclude the possibility that disulfide bond formation at Cys^{-1} of the nascent SP-A^{hyp} has an influence on signal peptidase cleavage that is lost in the Cys^{-1} mutant proteins.

The oligomeric structure of the Cys^{-1} mutant SP-As was investigated by gel permeation chromatography and cross-linking analyses. We have previously reported that gel permeation chromatography predicts that SP-A^{hyp} is at least nonameric in solution, most likely reflecting the oligomeric association of three trimers (*15*). The cross-linking data for SP-A^{hyp} presented in this study are consistent with that estimate, in that the presence of eight or nine distinct bands suggests an equal number of closely approximated individual polypeptide strands in the fully assembled molecule. The deletion or substitution of Cys^{-1} eliminated the disulfide-dependent association of more than two polypeptide chains in SP-A, but both gel permeation chromatography and cross-linking analyses predicted oligomers composed of at least six and perhaps as many as nine polypeptide chains. Noncovalent intermolecular forces in the collagen-like region and the neck are thought to play a major role in the assembly of SP-A and other collectins (*23, 25*). These data indicate that the Cys^{-1} disulfide bond is not required for the formation of trimers, or for association between trimeric subunits in solution.

Both Cys^{-1} mutant proteins retained the functional properties of SP-A, including phospholipid binding and aggregation, SP-A receptor binding, and modulation of surfactant secretion and uptake. These results clearly indicate that Cys^{-1}, and therefore multimerization greater than dimer formation, is not essential for the SP-A functions measured. In most assays, the SP-$A^{hyp,C-1S}$ was slightly less active than the SP-A^{hyp}, suggesting that Cys^{-1} may be required for full activity of the protein. However, the SP-$A^{hyp,\Delta IKC}$ had activity that was equal to or greater than SP-A^{hyp} in all assays except for competitive binding to the SP-A receptor. This result indicates that Cys^{-1} is not required for full functional activity and that the longer (IKC-containing) isoform of rat SP-A is not essential for function. The observation that the SP-$A^{hyp,\Delta IKC}$ was functionally comparable to SP-A^{hyp} despite the presence of an abundant truncated fragment that would be predicted to have little activity invites speculation that removal of the N-terminal IKC extension might enhance the function of the protein. The reduced potency of the SP-$A^{hyp,C-1S}$ may be due to the disrupted folding of N-terminal domains in heterooligomers composed almost exclusively of the longer isoforms, but remains unclear.

The cDNAs for SP-A isolated from human (*22*), rabbit (*43*), guinea pig (*44*), rat (*21*), and mouse (*45*) but not dog (*46*) predict two Cys at the N-termini of the proteins. Of these, both N-terminal Cys from human and rat SP-A have been shown to participate in disulfide bond formation by sequence analysis (*16*), but the migration patterns of rabbit and mouse SP-A on nonreducing SDS−PAGE gels also indicate that at least two intermolecular disulfide bonds are present. Human SP-A contains two additional Cys within the collagen-like region that may also form interchain

disulfide bridges. The data presented here suggest that a single interchain disulfide at the N-terminus is sufficient for the function of rat SP-A, but the results may not necessarily be applicable to SP-A from other species.

In summary, we find that Cys^{6} but not Cys^{-1} is critical for rat SP-A function. The data indicate SP-A composed solely of the shorter isoform is as active as the heterooligomeric protein. We also found that dimerization of SP-A polypeptide chains within the SP-A oligomer is sufficient for protein assembly and for interactions of SP-A with type II cells and phospholipids.

REFERENCES

1. Haller, E. M., Shelly, S. A., Montgomery, M. R., and Balis, J. U. (1992) *J. Histochem. Cytochem. 40*, 1491−1500.
2. Hawgood, S., Benson, B. J., Schilling, J., Damm, D., Clements, J. A., and White, R. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 66−70.
3. Veldhuizen, R. A., Hearn, S. A., Lewis, J. F., and Possmayer, F. (1994) *Biochem. J. 300*, 519−524.
4. Veldhuizen, R. A., Yao, L. J., Hearn, S. A., Possmayer, F., and Lewis, J. F. (1996) *Biochem. J. 313*, 835−840.
5. Suzuki, Y., Fujita, Y., and Kogishi, K. (1989) *Am. Rev. Respir. Dis. 140*, 75−81.
6. Cockshutt, A. M., Weitz, J., and Possmayer, F. (1990) *Biochemistry 29*, 8424−8429.
7. Yukitake, K., Brown, C. L., Schlueter, M. A., Clements, J. A., and Hawgood, S. (1994) *Pediatr. Res. 37*, 21−25.
8. Dobbs, L. G., Wright, J. R., Hawgood, S., Gonzalez, R., Venstrom, K., and Nellenbogen, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1010−1014.
9. Rice, W. R., Ross, G. F., Singleton, F. M., Dingle, S., and Whitsett, J. A. (1987) *J. Appl. Physiol. 63*, 692−698.
10. Wright, J. R., Wager, R. E., Hawgood, S., Dobbs, L., and Clements, J. A. (1987) *J. Biol. Chem. 262*, 2888−2894.
11. Korfhagen, T. R., Bruno, M. D., Ross, G. F., Huelsman, K. M., Ikegami, M., Jobe, A. H., Wert, S. E., Stripp, B. R., Morris, R. E., Glasser, S. W., Bachurski, C. J., Iwamoto, H. S., and Whitsett, J. A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 9594−9599.
12. LeVine, A. M., Bruno, M. D., Huelsman, K. M., Ross, G. F., Whitsett, J. A., and Korfhagen, T. R. (1997) *J. Immunol. 158*, 4336−4340.
13. McCormack, F. X., and Whitsett, J. A. (1996) in *Collectins and innate immunity* (Ezekowitz, A., Reid, K. B. M., and Sastry, K., Eds.) pp 9−50, Landes, Georgetown, TX.
14. McCormack, F. X., Stewart, J. J., Voelker, D. R., and Damodarasamy, M. D. (1997) *Biochemistry 36*, 13963−13971.
15. McCormack, F. X., Pattanajitvilai, S., Stewart, J. J., Possmayer, F., Inchley, K., and Voelker, D. R. (1997) *J. Biol. Chem. 272*, 27971−27979.
16. Elhalwagi, B. M., Damodarasamy, M., and McCormack, F. X. (1997) *Biochemistry 36*, 7018−7025.
17. Honma, T., Kuroki, Y., Tsunezawa, W., Ogasawara, Y., Sohma, H., Voelker, D. R., and Akino, T. (1997) *Biochemistry 36*, 7176−7184.
18. McCormack, F. X., Kuroki, Y., Stewart, J. J., Mason, R. J., and Voelker, D. R. (1994) *J. Biol. Chem. 269*, 29801−29807.
19. McCormack, F. X., Calvert, H. M., Watson, P., Smith, D. L., Mason, R. J., and Voelker, D. R. (1994) *J. Biol. Chem. 269*, 5833−5841.
20. Kuroki, Y., McCormack, F. X., Ogasawara, Y., Mason, R. J., and Voelker, D. R. (1994) *J. Biol. Chem. 269*, 29793−29800.
21. Sano, K., Fisher, J., Mason, R. J., Kuroki, Y., Schilling, J., Benson, B., and Voelker, D. (1987) *Biochem. Biophys. Res. Commun. 144*, 367−374.
22. White, R. T., Damm, D., Miller, J., Spratt, K., Schilling, J., Hawgood, S., Benson, B., and Cordell, B. (1985) *Nature 317*, 361−363.
23. Haagsman, H. P., White, R. T., Schilling, J., Lau, K., Benson, B. J., Golden, J., Hawgood, S., and Clements, J. A. (1989) *Am. J. Physiol. 257*, L42-L429.
24. King, R. J., Simon, D., and Horowitz, P. M. (1989) *Biochim. Biophys. Acta 1001*, 294−301.
25. Hoppe, H. J., Barlow, P. N., and Reid, K. B. (1994) *FEBS Lett. 344*, 191−195.
26. Voss, T., Eistetter, H., Schafer, K. P., and Engel, J. (1988) *J. Mol. Biol. 201*, 219−227.
27. Kuroki, Y., Mason, R. J., and Voelker, D. R. (1988) *J. Biol. Chem. 263*, 3388−3394.
28. Kuroki, Y., Mason, R. J., and Voelker, D. R. (1988) *J. Biol. Chem. 263*, 17596−17602.
29. Ross, G. F., Sawyer, J., O'Connor, T., and Whitsett, J. A. (1991) *Biochemistry 30*, 858−865.
30. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 61−68.
31. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.
32. Fornstedt, N., and Porath, J. (1975) *FEBS Lett. 57*, 187−191.
33. Dethloff, L. A., Gilmore, L. B., Brody, A. R., and Hook, G. E. R. (1986) *Biochem. J. 233*, 111−118.
34. Hawgood, S., Benson, B., and Hamilton, R. J. (1985) *Biochemistry 24*, 184−190.
35. McCormack, F. X., Fisher, J. H., Suwabe, A., Smith, D. L., Shannon, J. M., and Voelker, D. R. (1990) *Biochim. Biophys. Acta 1087*, 190−198.
36. Laemmli, U. K. (1970) *Nature 227*, 680−685.
37. Hewick, R. M., Hunkapiller, M. W., Hood, L. E., and Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990−7997.
38. Edman, P. (1956) *Acta Chem. Scand. 10*, 761−768.
39. Dobbs, L. G., and Mason, R. J. (1979) *J. Clin. Invest. 63*, 378−387.
40. Bolton, A. E., and Hunter, W. M. (1973) *Biochem. J. 133*, 529.
41. von Heijne, G. (1984) *J. Mol. Biol. 173*, 243−251.
42. Izard, J. W., and Kendall, D. A. (1994) *Mol. Microbiol. 13*, 765−773.
43. Boggaram, V., Qing, K., and Mendelson, C. R. (1988) *J. Biol. Chem. 263*, 2939−2947.
44. Yuan, H. T., Gowan, S., Kelly, F. J., and Bingle, C. D. (1997) *Am. J. Physiol. 273*, L900−L906.
45. Korfhagen, T. R., Bruno, M. D., Glasser, S. W., Ciraolo, P. J., Whitsett, J. A., Lattier, D. L., Wikenheiser, K. A., and Clark, J. C. (1992) *Am. J. Physiol. 263(5 Pt. 1)*, L546−L554.
46. Benson, B., Hawgood, S., Schilling, J., Clements, J., Damm, D., Cordell, B., and White, R. T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6379−6383.

BI9817966