Surfactant Protein A2 Mutations Associated with Pulmonary Fibrosis Lead to Protein Instability and Endoplasmic Reticulum Stress*

Received for publication, March 8, 2010, and in revised form, May 11, 2010. Published, JBC Papers in Press, May 13, 2010, DOI 10.1074/jbc.M110.121467

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Rare heterozygous mutations in the gene encoding surfactant protein A2 (SP-A2, SFTPA2) are associated with adult-onset pulmonary fibrosis and adenocarcinoma of the lung. We have previously shown that two recombinant SP-A2 mutant proteins (G231V and F198S) remain within the endoplasmic reticulum (ER) of A549 cells and are not secreted into the culture medium. The pathogenic mechanism of the mutant proteins is unknown. Here we analyze all common and rare variants of the surfactant protein A2, SP-A2, in both A549 cells and in primary type II alveolar epithelial cells. We show that, in contrast with all other SP-A2 variants, the mutant proteins are not secreted into the medium with wild-type SP-A isoforms, form fewer intracellular dimer and trimer oligomers, are partially insoluble in 0.5% Nonidet P-40 lysates of transfected A549 cells, and demonstrate greater protein instability in chymotrypsin proteolytic digestions. Both the G231V and F198S mutant SP-A2 proteins are destroyed via the ER-associated degradation pathway. Expression of the mutant proteins increases the transcription of a BiP-reporter construct, expression of BiP protein, and production of an ER stress-induced XBP-1 spliced product. Human bronchoalveolar wash samples from individuals who are heterozygous for the G231V mutation have similar levels of total SP-A as normal family members, which suggests that the mechanism of disease does not involve an overt lack of secreted SP-A but instead involves an increase in ER stress of resident type II alveolar epithelial cells.

Inhaled oxygen and exhaled carbon dioxide gas must diffuse across the distal lung for effective gas exchange. This interface includes alveolar surfactant, which lowers the surface tension of the distal lung, the alveolar epithelial cells, and the supporting interstitium in which the capillary vascular bed resides. The phospholipids and proteins in surfactant are made and secreted by the type II alveolar epithelial cells, which cover about 5% of alveolar surface area and have a distinct morphology with characteristic lamellar bodies and apical microvilli (1).

The pulmonary surfactant protein B and C, SP-B and SP-C, are very hydrophobic proteins that stabilize the surfactant lipids and reduce the surface tension in the alveolus. Mutations in the genes encoding SP-B and SP-C are associated with respiratory failure and a range of interstitial lung diseases in children and adults (2). Mutations in either of these genes result in decreased expression of secreted surfactant protein SP-B and SP-C. A subset of heterozygous mutations in the gene encoding SP-C has a dominant-negative effect on protein biosynthesis and leads to the formation of intracellular protein aggregates (3). Mutations in SP-C that prevent the protein from folding into a stable conformation lead to the activation of the unfolded protein response that increases transcription of ER chaperones, including BiP, and promotes their destruction via the ER-associated degradation pathway (4–6).

Surfactant protein A and D, SP-A and SP-D, are hydrophilic calcium-dependent lectins known as collectins that have a primary role in innate immunity of the lung (7). SP-A knock-out mice demonstrate that SP-A is not essential for lowering the surface tension of surfactant, although it is required for the formation of tubular myelin in the alveolar lumen (8). SP-A knock-out mice are more susceptible to infection by multiple different microorganisms, demonstrating its importance in host defense (9). SP-A can bind, aggregate, opsonize, and permeabilize microorganisms (10). The binding of pathogens and lipids to SP-A is mediated by the carbohydrate binding domain (CRD). This region is very highly conserved; the crystal structure of the rat SP-A helical neck and globular CRD domains demonstrates an extensive hydrophilic binding surface (11).

Idiopathic pulmonary fibrosis (IPF) is a lethal disease affecting older adults that results in progressive scarring of the lung parenchyma. Understanding of the molecular pathogenesis of this disease has come from human genetic studies of the familial form of this disease. Approximately 15% of all kindreds with familial adult-onset pulmonary fibrosis have heterozygous mutations in the gene that encodes the protein component of telomerase, TERT (12, 13). Rare heterozygous mutations in the gene that encodes the RNA component of telomerase, TERC (12, 14), and the gene that encodes surfactant protein C, SFTPC (15), have also been reported.

Recently, a genome-wide linkage scan of a large kindred with familial idiopathic pulmonary fibrosis and lung adenocarcinoma identified a linked region on chromosome 10 that includes two genes encoding the closely related isoforms of surfactant protein A2 and C. 

**This work was supported, in whole or in part, by National Institutes of Health Grants HL093096 (NHLBI) (to C. K. G.) and HL050022 (to C. R. M.). This work was also supported by the Doris Duke Charitable Foundation.

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2 The abbreviations used are: SP, surfactant protein; ER, endoplasmic reticulum; 3-MA, 3-methyladenine; XBP-1, X-box-binding protein-1; CRD, carbohydrate binding domain; IPF, idiopathic pulmonary fibrosis; PBS, phosphate-buffered saline.
facnt protein A, SP-A1 and SP-A2 (16). A rare heterozygous missense mutation in SP-A2 that predicts a valine instead of glycine at position 231 (G231V) was identified in the proband and segregated with the lung disease. An independent mutation encoding for a different missense mutation of SP-A2 (F198S) was found in another family with an identical phenotype. Both mutations affect highly conserved residues in the CRD of the protein. These two variants are very rare; they were not detected in a large (n = 3557) multiethnic population.

Surfactant protein A2 is very polymorphic. A number of different variants have been described in normal populations and patients with idiopathic pulmonary fibrosis. Determining the functional significance of variants will increase our understanding of their impact on lung health and disease. Here we examine the synthesis, secretion, oligomerization, detergent solubility, and protein stability of different SP-A2 variants and determine the mechanism by which substitution of highly conserved residues in the CRD region can affect the function of SP-A2.

EXPERIMENTAL PROCEDURES

Materials—Culture medium and fetal bovine serum were obtained from Invitrogen and Atlanta Biologicals (Lawrenceville, GA), respectively. Protease inhibitor mixture tablets were purchased from Roche Applied Science. The rabbit polyclonal surfactant A antibody was used as previously described (16); anti-V5 mouse monoclonal antibody (R960–25) was obtained from Invitrogen; anti-myc mouse monoclonal antibody (9E10) was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-SP-B rabbit polyclonal antibody (WRAB-SPB) was from Seven Hills Bioreagents (Cincinnati, OH); anti-SP-D mouse monoclonal antibody (sc-25324) was from Santa Cruz; anti-Bip/GRP78 mouse monoclonal antibody (610978) was from BD Transduction Laboratories; horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit were from Jackson ImmunoResearch; bovine serum albumin (9E10) was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-V5 mouse monoclonal antibody (R960–25) was obtained from Invitrogen and Atlanta Biologicals (Lawrenceville, GA) was utilized so that the coding sequence of the wild-type V5 epitope tag (GKPIPNPLLGLDST) or three copies of the c-myc epitope (EQKLISEEDLN) (17) were placed after the glutamic acid at amino acid 21 by primer extension mutagenesis and zipper PCR. The DNA sequence of the V5 tag was 5′-GGTAAGCCTATCCTAAACCTCTCTCGGTCTCGATTCTACG-3′.

A549 Cell Culture, Transfections, and Cell Lysates—A549 cells (ATCC, Manassas, VA) were plated in 6-well plates on day 0 at 350,000 cells per well in 2 ml of complete medium (Ham's F-12 medium with 10% fetal bovine serum, 100 units of penicillin, and 100 µg of streptomycin). On day 1, the cells were transfected with 1–2 µg of DNA using 3 µl of FuGENE HD Transfection Reagent (Roche Applied Science) per µg of DNA in medium without antibiotics according to the manufacturer’s protocol. The cells were fed with complete medium on day 2 and harvested on day 4. One ml of cultured medium was removed from each well on the day of harvest and centrifuged at 13,000 × g for 10 min at 4 °C; the protein concentration of the conditioned medium was determined by the Pierce BCA assay (Thermo Scientific, Rockford, IL). The cells were washed once with 2 ml of ice-cold phosphate buffered saline (PBS), harvested by scraping in 300 µl of radioimmunoprecipitation lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 1 mM EDTA, pH 8, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholate with 1 tablet of protease mixture (Roche Applied Science) per 10 ml of buffer), sonicated 10 s, and centrifuged at 13,000 × g for 10 min at 4 °C. The protein concentration of the cell lysate was determined as above. Aliquots of medium and cell lysates were subjected to SDS-PAGE and immunoblot analysis.

Recombinant Adenovirus and A549 Cell Infections—cDNAs were subcloned into the pACCMVpLpAl-(-)loxP-SSP shuttle vector (18), and recombinant adenovirus was constructed by in vitro cre/loxP-mediated recombination using a cosmid containing the adenovirus type 5 genome with a large deletion of the E3 region (19). Viral clones were propagated in 911 cells; viral supernatants were clarified by centrifugation and titered by plaque assay. Aliquots of virus were stored frozen at –80 °C until use. Adenovirus derived from the empty shuttle vector (AdCMV/pLpAl-(-)loxP) was used as a negative control. A549 cells were plated in 6-well plates at 350,000 cells per well. 24 h later recombinant adenovirus was added in minimal medium (0.5 ml complete medium/well) at a multiplicity of infection of 100. The cells were washed once with PBS, fed with complete medium, and harvested 48 h later.

Alveolar Type II Cells and Adenovirus Infections—Pulmonary alveolar type II cells were isolated and cultured from midgestation human fetal lung tissue obtained from Advanced Bioscience Resources, Inc. (Alameda, CA) as described (20). The fetal lung tissues were acquired in accordance with the Donors Anatomical Gift Act of the State of Texas; protocols were approved by the Human Research Review Committee of the University of Texas Southwestern Medical Center at Dallas. Briefly, the tissue was minced and cultured in Waymouth's MB756/1 media (Life Sciences) in the presence of 1 mM dibutyryl-cAMP (Roche Applied Science). After 3–5 days of culture with daily medium changes, lung explants were dissociated by digestion with collagenase. After digestion, the cell suspension was depleted of fibroblasts, purified on a Percoll gradient, and plated onto 60-mm tissue culture dishes that were coated with extracellular matrix prepared from Madin-Darby canine kidney cells (ATCC) at 4 × 10⁶ cells/60-mm dish in 1.0 ml of
medium containing 10% fetal bovine serum. On the following day the cells were washed and infected with recombinant adenovirus at a multiplicity of infection of 30 pfu/cell. After overnight infection (20 h), the cells were washed once with PBS, fed with serum-free medium containing dibutyryl-cAMP and harvested 48 h later.

**SDS-PAGE and Immunoblot Analysis**—Equivalent amounts of protein from the cell lysates and medium were added to sample loading buffer with or without β-mercaptoethanol. The samples were heated to 95 °C for 5 min, loaded on 10% SDS-polyacrylamide gels, run at 70 – 110 V, and transferred to nitrocellulose Protran membranes (Whatman, Dassel, Germany). Membranes were incubated in blocking buffer (5% dried milk in TBST (150 mM NaCl, 10 mM Tris, pH8, 0.1% Tween 20)) for 60 min at room temperature before the addition of primary antibodies. Primary antibodies were diluted in blocking buffer and incubated with membranes overnight at 4 °C with rocking. Membranes were washed 4 times with TBST for 5 min each at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG was diluted in blocking buffer and incubated with membranes for 30 min. Membranes were washed 4 times in TBST for 5 min each and visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL) according to the manufacturer’s protocol. Quantitative measurement of immunoblots was performed by using the integrated intensity values measured by the Odyssey infrared imaging system from Licor Biosciences.

**Immunoprecipitation**—Myc-tagged SP-A and V5-tagged SP-A2 expression constructs were co-transfected in A549 cells. 48 h after transfection the cells were washed with ice-cold PBS, scraped on ice in 0.5 ml of freshly prepared lysis buffer (100 mM NaCl, 50 mM HEPES, pH 7.4, 1.5 mM MgCl2, 0.5% (v:v) Nonidet P-40 with 1 tablet of protease mixture (Roche Applied Science) per 10 ml) (22). Cellular lysates were incubated at 4 °C on a rotator for 30 min. Samples were centrifuged 16,000 × g at 4 °C for 15 min. The supernatants were transferred to a new tube and incubated with 2 μg of 9E10 monoclonal antibody directed against the c-myc epitope and 200 μg of a protein A-agarose slurry (EMD Biosciences, San Diego, CA) for 16 h at 4 °C. Protein A-bound antibodies and proteins were separated from the supernatant by centrifugation (800 × g, 15 s) and washed twice for 10 min in 800 μl of the lysis buffer at 4 °C. The agarose pellet was resuspended in 2 × Laemmlin buffer and boiled 5 min before SDS-PAGE.

**Nonidet P-40 Fractionation Assay**—A549 or type II alveolar epithelial cells were washed once with 2 ml of ice-cold PBS and scraped in ice-cold lysis buffer (described above) into a 1.5-ml microcentrifuge tube. Cellular lysates were incubated at 4 °C on a rotator for 30 min. Samples were centrifuged 16,000 × g at 4 °C for 10 min, and the supernatants were saved as the Nonidet P-40-soluble fractions. Pellets were washed once with lysis buffer and solubilized with 2 × SDS buffer (125 mM Tris-HCl, pH 6.8, 4% (w:v) SDS, 5% β-mercaptoethanol, 20% glycerol, 0.01% bromphenol blue) and saved as Nonidet P-40-insoluble fractions. SDS-PAGE sample buffer was added to the supernatant fractions for subsequent electrophoresis.

**Chymotrypsin-limited Proteolysis Assay**—A549 or type II alveolar epithelial cells were washed once with 2 ml of ice-cold PBS and scraped in ice-cold lysis buffer not containing protease inhibitors. A 1:50 ratio (w/w) of chymotrypsin (dissolved in 50 mM Tris-HCl, pH 8, 100 mM NaCl) to protein was incubated at room temperature. The reactions were stopped at each time point by the addition of SDS loading buffer and visualized on a 15% SDS-PAGE gel.

**BiP-luciferase Assay**—The BiP-luciferase plasmid (Grp78 promoter-169 luciferase plasmid) was kindly provided by Amy Lee, University of Southern California. 48 h after co-transfection of BiP-luciferase and SP-A plasmids, A549 cells were washed once with 2 ml of ice-cold PBS and scraped in 1× reporter lysis buffer (Promega) into a 1.5-ml microcentrifuge tube. The lysates were vortexed for 10–15 s and centrifuged 12,000 × g at 4 °C for 5 min. The supernatant was transferred to a new tube and used for the luciferase assay using the Promega luciferase assay system.

**XBP-1 Splicing Assay**—Total RNA was harvested from A549 cells infected with adenovirus expressing various V5-tagged SP-A2 constructs and reverse-transcribed using an iScript cDNA synthesis kit (Bio-Rad). PCR using the primers XBP1-F, 5′-GATGCTTGGTCTGAG-3′, and XBP1-R, 5′-GAGTCAATACGCCCCAGATCC-3′ (21), was performed and digested with PstI (22). Cells treated with or without 10 μM MG-132 were harvested in an identical manner and used as controls. The PCR bands were quantitated using Image J (rsb.info.nih.gov).

**Human Subjects**—This study was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center at Dallas. Kindred were previously described (16). Written informed consent was obtained from all subjects. Bronchoscopy was performed under conscious sedation. With the bronchoscope wedged in the right middle bronchus, three successive 60-ml aliquots of sterile 0.9% NaCl was instilled into the lung and retrieved with gentle suction. The bronchoalveolar lavage fluid was centrifuged at 2,000 × g at 4 °C for 10 min, and the supernatant was saved in separate aliquots at −80 °C until use. Total phospholipid content of the fluid was assayed by measuring the organic phosphorus (23).

**RESULTS**

In contrast with SP-A2 Wild-type or Common Variants, the G231V and F198S Mutant Proteins Are Not Secreted from Cells—To determine the effects of the SP-A2 variants on expression and secretion of SP-A2, we transiently transfected constructs expressing wild-type and variant forms of V5-epitope-tagged SP-A2 in A549 cells, an immortalized human alveolar epithelial cell line derived from a lung adenocarcinoma. The V5-epitope was inserted after the glutamic acid at amino acid position 21 and did not change the N-terminal cysteines at positions 20 and 26 that are important for SP-A intermolecular interactions (24). Immunoblot analyses were performed using antibodies specific for the V5-epitope. Wild-type SP-A2 was expressed as a 28-kDa protein in the cell lysates. A smear 30 – 36 kDa in size representing post-translationally modified protein was detected in the cell medium. By sequencing of genomic DNA, we have found the following naturally occurring nonsynonymous variants of
FIGURE 1. SP-A2 G231V and F198S mutant proteins are not secreted from A549 cells and type II alveolar epithelial cells. A, shown is a schematic of surfactant protein SP-A2 with the amino acid positions at the junctions of various functional domains within the predicted full-length protein. The domains of the signal sequence, collagen-like region, neck, and carbohydrate recognition domain are indicated. The G231V and F198S mutants are listed above, and the nonsynonymous variants are listed below the schematic.

B, A549 cells were transiently transfected with empty vector (Mock), V5-epitope-tagged SP-A2 wild-type, L12W, F198S, G231V, T9N, V50L, A91P, and Q223K variants. 72 h after transfection, equal amounts of cell lysates (C, 40 μg) or media (M, 80 μg) were subjected to SDS-PAGE and subjected to immunoblot analysis using a monoclonal antibody that recognizes the V5 epitope. The V5 epitope was engineered to follow the signal sequence at amino acid position 22. C, A549 cells were infected with recombinant adenovirus expressing no protein (Mock), V5-epitope-tagged SP-A2 wild-type, G231V, F198S, and Q223K variants. 72 h after infection, both cell lysates (C, 30 μg) and media (M, 30 μg) were harvested and analyzed by immunoblot analysis.

D, human type II alveolar cells were infected with recombinant adenovirus, and equal amounts of cell lysates and media were analyzed by immunoblot analysis for V5-epitope-tagged recombinant SP-A2 variants, total SP-A (endogenous and recombinant expression), or SP-B and SP-D expression (data not shown). Each immunoblot is representative of at least three independent experiments. AEC, alveolar epithelial cells.
FIGURE 3. Effect of SP-A2 variants on oligomerization. A, shown are A549 cells transiently transfected with empty vector (Mock) or plasmid expressing either myc-tagged wild-type SP-A1 or SP-A2. A549 cells were transiently transfected with empty vector (Mock) or plasmid expressing either myc-tagged wild-type SP-A1 or SP-A2 and co-transfected with Mock, V5-epitope-tagged SP-A2 wild-type, G231V, F198S, T9N, L12W, V50L, A91P, or Q223K variants. 72 h after transfection, myc-tagged SP-A and associated proteins were immunoprecipitated (IP) from the cell lysates (C, 50 μg) and media (M, 500 μg), subjected to SDS-PAGE, transferred to membranes, and immunoblotted with antibodies directed against the myc or V5 epitopes (upper panels). The arrow and arrowhead indicate the position of the myc-tagged SP-A and V5-tagged SP-A2, respectively. Aliquots of the input cell lysates (15 μg) and media (15 μg) were subjected to SDS-PAGE, transferred to membranes, and immunoblotted with antibodies directed against the myc and V5 epitopes (lower panels). Each immunoblot is representative of two independent experiments.

B. Type II AECs

All SP-A2 Variants Except the G231V and F198S Mutants Are Able to Form Stable Secreted Complexes with Either Wild-type SP-A1 or SP-A2—The fully mature secreted SP-A protein assembles into 18-subunit oligomers that are composed of six disulfide-linked trimers assembled in a “bouquet of flowers” arrangement. To determine whether the SP-A2 variants can form stable complexes, we co-expressed the V5-epitope-tagged SP-A2 variants with myc-tagged wild-type SP-A1 or SP-A2. The myc-tagged SP-A isomers were constructed in a similar manner as the V5-epitope-tagged variants with the tag following the signal peptide and the glutamic acid at amino acid 21. Immunoprecipitated from the media with a co-expressed myc-tagged wild-type SP-A1 or SP-A2. A549 cells were transiently transfected with empty vector (Mock) or plasmid expressing either myc-tagged wild-type SP-A1 or SP-A2. A549 cells were transiently transfected with empty vector (Mock) or plasmid expressing either myc-tagged wild-type SP-A1 or SP-A2 and co-transfected with Mock, V5-epitope-tagged SP-A2 wild-type, G231V, F198S, T9N, L12W, V50L, A91P, or Q223K variants. 72 h after transfection, myc-tagged SP-A and associated proteins were immunoprecipitated (IP) from the cell lysates (C, 50 μg) and media (M, 500 μg), subjected to SDS-PAGE, transferred to membranes, and immunoblotted with antibodies directed against the myc or V5 epitopes (upper panels). The arrow and arrowhead indicate the position of the myc-tagged SP-A and V5-tagged SP-A2, respectively. Aliquots of the input cell lysates (15 μg) and media (15 μg) were subjected to SDS-PAGE, transferred to membranes, and immunoblotted with antibodies directed against the myc and V5 epitopes (lower panels). Each immunoblot is representative of two independent experiments.

SP-A2: T9N, L12W, V50L, A91P, and Q223K with allele frequencies of 0.47, 0.01, 0.08, 0.17, and 0.15, respectively (16). The L12W variant, although rare, did not segregate with pulmonary fibrosis in the kindred in which it was found (16). The Q223K variant is located in the CRD region between the positions of the G231V and F198S substitutions (Fig. 1A). All of these variants, both common and rare, were detected in both the cell lysates and medium of transiently transfected A549 cells. In contrast, the two mutant SP-A2 variants, F198S and G231V, were detected in the cell lysate but not in the medium of transfected A549 cells (Fig. 1B). If the same volumes of cell media were loaded on the gels before immunoblot analysis, identical results were obtained (data not shown).

We created replication-defective recombinant human adenovirus expressing the V5-epitope-tagged SP-A2 wild-type, G231V, F198S, and Q223K variants for infection of A549 cells and primary human type II alveolar epithelial cells. Cultured pneumocytes have been shown to express SP-A for up to 2 weeks, to demonstrate regulate-able expression of SP-B and SP-C and to have lamellar bodies by electron microscopy (20). Although the V5-epitope-tagged SP-A2 wild-type and Q223K were found both in the cell lysates and secreted medium, the G231V and F198S mutant proteins were found only in the cell lysates (Fig. 1, C and D). The type II alveolar epithelial cells expressed endogenous surfactant protein A. Immunoblot analysis using an antibody that recognizes both endogenous and recombinant SP-A demonstrated that the mutant G231V and F198S variants were not secreted from these cells even in the presence of endogenous SP-A protein (Fig. 1D). The type II alveolar cells also produce surfactant protein B and D (data not shown).
**SP-A2 Mutations**

**A. A549 Cells**

| Transfected DNA | V5-tagged SP-A |
|-----------------|----------------|
| Mock            | wild-type, G231V, F198S, VS0L, A91P, and Q223K variants. |

72 h after transfection the cells were solubilized with buffer containing 0.5% Nonidet P-40 and partitioned into Nonidet P-40-soluble and -insoluble fractions. Aliquots of Nonidet P-40-soluble and -insoluble fractions (10% of total) were subjected to SDS-PAGE, transferred to nitrocellulose, immunoblotted with antibodies directed against the V5-epitope, and exposed for 2 min. B, A549 cells were transiently transfected with empty vector (Mock) and plasmid encoding V5-epitope-tagged SP-A2 wild-type, G231V, F198S, VS0L, A91P, and Q223K variants. 72 h after transfection the cells were solubilized with buffer containing 0.5% Nonidet P-40 and partitioned into Nonidet P-40-soluble and -insoluble fractions (10% of total) were subjected to SDS-PAGE, transferred to nitrocellulose, immunoblotted with antibody directed against the V5-epitope, and exposed for 2 min.

**B. A549 Cells**

| Transfected DNA | V5-tagged SP-A |
|-----------------|----------------|
| Mock            | wild-type, G231V, F198S, VS0L, A91P, and Q223K variants. |

72 h after transfection the cells were solubilized with buffer containing 0.5% Nonidet P-40 and partitioned into Nonidet P-40-soluble and -insoluble fractions. Aliquots of Nonidet P-40-soluble and -insoluble fractions (10% of total) were subjected to SDS-PAGE, transferred to nitrocellulose, immunoblotted with antibody directed against the V5-epitope, and exposed for 2 min. B, A549 cells were transiently transfected with empty vector (Mock) or plasmid expressing either myc-tagged wild-type SP-A1 or SP-A2 and co-transfected with Mock; V5-epitope-tagged SP-A2 wild-type, G231V, F198S, VS0L, A91P, or Q223K variants. 72 h after transfection the cells were solubilized in buffer containing 0.5% Nonidet P-40 and partitioned into Nonidet P-40-soluble and -insoluble fractions, and immunoblotted (IB) with antibodies directed against the V5 and c-myc epitopes. Myc-tagged wild-type SP-A1 and SP-A2 was present in the Nonidet P-40-insoluble fraction with the V5-tagged SP-A2 G231V mutant protein. C, type II alveolar epithelial cells (AEC) were infected with adenovirus expressing no protein (Mock) and V5-epitope-tagged SP-A2 wild-type, G231V, F198S, and Q223K variants. 72 h after infection, the cells were solubilized with buffer containing 0.5% Nonidet P-40 and partitioned into Nonidet P-40-soluble and -insoluble fractions, and immunoblotted (IB) with antibodies directed against the V5 and c-myc epitopes. Myc-tagged wild-type SP-A1 and SP-A2 was present in the Nonidet P-40-insoluble fraction with the V5-tagged SP-A2 G231V mutant protein. C, type II alveolar epithelial cells (AEC) were infected with adenovirus expressing no protein (Mock) and V5-epitope-tagged SP-A2 wild-type, G231V, F198S, and Q223K variants. 72 h after infection, the cells were solubilized with buffer containing 0.5% Nonidet P-40 and partitioned into Nonidet P-40-soluble and -insoluble fractions, and immunoblotted (IB) with antibodies directed against the V5 and c-myc epitopes.
Under non-reduced conditions, high molecular weight oligomers were present in the cell lysates with an apparent molecular mass of >250 kDa for the wild-type, V50L, A91P, and Q223K variants. Fewer high molecular weight oligomers were detected for the mutant proteins, especially the F198S variant, regardless of whether it was expressed in A549 cells or type II alveolar epithelial cells.

The G231V and F198S Mutant Proteins Form Detergent-insoluble Aggregates in A549 Cells—Because some of the known pathogenic mutations in surfactant protein C form detergent-insoluble aggregates; we analyzed the detergent solubility of expressed SP-A2 variants. Transfected cells were harvested in a 0.5% Nonidet P-40 lysis solution and centrifuged to separate Nonidet P-40-soluble from Nonidet P-40-insoluble proteins (Fig. 4). In transiently transfected A549 cells, all the immunodetectable wild-type SP-A2 was found in the Nonidet P-40-soluble fraction. In contrast with A549 cells, the G231V and F198S mutant proteins were not found in a 0.5% Nonidet P-40-insoluble fraction with the V5-epitope-tagged G231V mutant protein (lanes 3 and 4, Fig. 4B). Whereas the V5-tagged SP-A2 F198S mutant protein was found in both the Nonidet P-40-soluble and -insoluble fractions, we did not find the wild-type myc-tagged isoforms in the Nonidet P-40-insoluble fraction with this mutant protein. Thus, only expression of the G231V mutant protein could affect the solubility of co-expressed wild-type SP-A in A549 cells.

The V5-tagged wild-type, G231V, F198S, and Q223K variants were expressed in primary type II alveolar epithelial cells. In contrast with A549 cells, the G231V and F198S mutant proteins were not found in a 0.5% Nonidet P-40-insoluble fraction in these cell lysates (Fig. 4C).

TABLE 1

| Cell line | SP-A2 variant | t1/2 (min) |
|-----------|---------------|------------|
| A549      | Wild type     | 27.1 ± 0.0 |
|           | G231V         | 7.7 ± 0.6  |
|           | F198S         | 5.8 ± 0.6  |
|           | T9N           | 23.1 ± 11.4|
|           | L12W          | 24.6 ± 8.5 |
|           | V50L          | 25.7 ± 10.3|
|           | A91P          | 22.1 ± 4.1 |
|           | Q223K         | 23.4 ± 3.6 |
| Type II AEC | Wild type    | 53.2 ± 6.5 |
|           | G231V         | 14.5 ± 3.6 |
|           | F198S         | 13.8 ± 5.9 |
|           | Q223K         | 69.5 ± 4.9 |

To further understand how the variants in SP-A2 affect protein stability, we used limited proteolysis to estimate the relative stability of the proteins in comparison with wild-type SP-A2. In these experiments, the N-terminal V5-epitope-tagged protein was expressed in cells, the entire cell lysate was subjected to proteolysis with chymotrypsin for different incubation times, and then the V5-tagged proteins were quantitated using the Odyssey infrared imaging system (Fig. 5). The intensity of the V5-tagged proteins increased linearly in cell lysates of 0–50 μg of protein (data not shown). The amount of immunodetectable wild-type V5-epitope-tagged wild-type SP-A2 at ~28 kDa decreased upon incubation with chymotrypsin. One minor band at ~22 kDa increased concomitantly with proteolysis. The calculated t1/2 of the wild-type SP-A2 expressed in A549 cells was 27.1 ± 9.0 min. The half-life of the T9N, L12W, V50L, A91P, and Q223K variants were very similar (Table 1).

In contrast, the half-life of the G231V and the F198S mutants in A549 cells was much shorter, 7.7 ± 0.6 and 5.8 ± 0.6 min, respectively. The calculated t1/2 of the adenovirus-expressed V5-epitope-tagged SP-A2 wild-type protein in type II alveolar epithelial cells was 53.2 ± 6.5 min. Again, the half-life of the G231V
and F198S mutants was much shorter in this cell system, 14.5 ± 3.6 and 13.8 ± 5.9, respectively, than the wild-type or Q223K variants (Table 1).

**The G231V and F198S Mutant Protein Expression Increases in A549 Cells When Cultured in the Presence of MG-132**—To determine the mechanism of degradation of the mutant SP-A2 proteins in the A549 cells, we cultured them in the presence of MG-132, a nonspecific proteasome inhibitor, and 3-MA, an inhibitor of autophagy (26). There was no increase in immunodetectable V5-epitope-tagged wild-type SP-A2 protein in A549 cells that were pretreated for 17 h with increasing concentrations (1–10 μM) of MG-132. In contrast, increased amounts of V5-tagged G231V mutant protein were detected when cultured in the presence of increasing concentrations of MG-132 (Fig. 6A). The amount of total immunodetectable V5-tagged SP-A2 wildtype, G231V, F198S, and Q223K proteins was quantitated in lysates of A549 cells cultured in the presence or absence of 10 μM MG-132. There was a 2.0- and 1.7-fold increase for the G231V and F198S SP-A2 variants, respectively, that was not seen for the wild-type or Q223K variant (Fig. 6C). The amount of wild-type and Q223K SP-A2 protein secreted into the tissue culture medium also did not change when cultured in the presence of MG-132 (Fig. 6C). No significant change in amount of SP-A2 protein was seen for any of the variants in the cell lysates or media when the cells were cultured in the presence or absence of 3-MA (Fig. 6, B and D).

**Markers of ER Stress Are Increased with the Expression of G231V and F198S Mutant Proteins in A549 Cells and Type II Alveolar Cells**—Because it was apparent that the G231V and F198S SP-A2 variants were being degraded through the proteasome pathway, we investigated whether their expression led to increased ER stress and expression of ER chaperone proteins such
as BiP. A549 cells were transfected with a plasmid expressing firefly luciferase under the control of the BiP promoter (BiP-luciferase). The light intensity units of cell lysates were measured 48 h after transfection. The amount of luciferase activity was measured when the different V5-epitope-tagged SP-A2 variant proteins were co-expressed (Fig. 7A). There was a small increase in luciferase activity when the SP-A2 wild-type, N9T, L12W, V50L, A91P, and Q223K variants were expressed. However, there was a 5.6- and 8.4-fold increase in luciferase activity over wild-type when the G231V and F198S mutants, respectively, were co-expressed.

To determine whether there was an increase in expressed BiP protein, lysates of infected A549 cells or primary type II cells were immunoblotted using an antibody that recognizes BiP and quantitated using the Odyssey infrared imaging system. There was only a modest increase in BiP protein in A549 cells expressing the V5-tagged SP-A2 G231V or F198S mutant proteins in comparison with the level of BiP protein in cells expressing the SP-A2 wild-type or Q223K variant (Fig. 7B). There was a 1.5–1.8-fold increase in BiP protein expression when the G231V and F198S mutant proteins were expressed in A549 or type II alveolar epithelial cells in comparison with wild-type SP-A2 (Figs. 7, B and C).

To determine whether the unfolded protein response-activated splicing of XBP-1 was up-regulated, we measured the production of the spliced XBP-1 mRNA as a percent of the total spliced and unspliced mRNA. There was a 2.9-fold increase in spliced XBP-1 in A549 cells when the V5-tagged SP-A2 G231V mutant was expressed in comparison with wild-type protein. Similarly, there was a 1.5- and 1.3-fold increase in spliced XBP-1 when the F198S or Q223K variants were expressed, respectively (Fig. 7D).

The Total Amount of Secreted SP-A in Bronchoalveolar Fluid Is Similar for Patients with and without the G231V Mutation—To determine whether the SP-A2 G231V mutation in the human patients led to a decrease in the amount of secreted surfactant protein A in bronchoalveolar fluid, we collected samples from a number of individuals in family F27. Three of the subjects heterozygous for the G231V mutation have pulmonary fibrosis; one mutation carrier does not express this phenotype. Equal aliquots of bronchoalveolar fluid (each containing 2 nmol of phospholipid) were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies that recognize mature human surfactant protein A, B, and D.

DISCUSSION
The major finding of this study is that both amino acid substitutions in SP-A2 (G231V and F198S), which confer susceptibility to pulmonary fibrosis and lung adenocarcinoma, lead to reduced protein stability and ER stress. Structural modeling of these substitutions had predicted that both would disrupt the tertiary structure of the CRD domain (16). We had previously shown that there is reduced secretion of the untagged mutant proteins when expressed in A549 cells (16). Consistent with the structural predictions, the V5-epitope-tagged proteins demonstrate decreased formation of higher ordered oligomers, evidence of detergent insolubility in A549 cells and increased sensitivity to chymotrypsin digestion. The net result is reduced in

FIGURE 7. Expression of V5-epitope-tagged SP-A2 G231V and F198S variants increase cellular markers of ER stress. A, A549 cells were transiently co-transfected with a plasmid expressing no protein (Mock) or V5-epitope-tagged SP-A2 wild-type (WT), G231V, F198S, N9T, L12W, V50L, A91P, or Q223K variants and a plasmid that expresses luciferase under the control of the BiP promoter. 48 h after transfection the cells were harvested in reporter lysis buffer and directly assayed for luciferase activity. The mean absolute light intensity units of three different transfections are shown. B and C, A549 cells (B) and type II alveolar epithelial cells (C) were infected with adenovirus expressing no protein (Mock) or V5-epitope-tagged SP-A2 wild-type, G231V, F198S, and Q223K variants. 72 h after infection, aliquots of cellular lysates (30 μg) were subjected to immunoblot analysis using antibodies that recognize the V5 epitope (upper panels) and BiP (lower panels). Quantification of BiP protein was performed using the Odyssey infrared imaging system; the fold change of BiP expression over the amount detected in cells expressing wild-type SP-A2 protein is reported. Data are shown as the mean ± S.D. Two to three independent experiments were used for the type II and A549 cells. D, A549 cells were infected with adenovirus expressing no protein (Mock) and V5-epitope-tagged SP-A2 wild-type, G231V, F198S, and Q223K variants. 72 h after infection, total RNA was harvested and reverse-transcribed into cDNA. Control cells were treated with 10 μM MG-132 or vehicle for 17 h before harvest. The XBP-1 gene was PCR amplified and digested with PstI. Background noise was subtracted from each quantitated band. The asterisk indicates a hybrid spliced (S)/unspliced (U) amplicon. The ratio of spliced over (spliced + unspliced) amplicons is reported as the “% spliced XBP1” in the histogram. Data are shown as mean ± S.D. of four independent experiments. rt, nucleotide.
***SP-A2 Mutations***

* in vitro secretion of these mutant proteins even when co-expressed with endogenous or recombinant wild-type SP-A1 or -A2 isoforms.

However, we show that subjects heterozygous for the G231V SP-A2 mutation have the same amount of total secreted SP-A in bronchoalveolar lavage fluid as family member controls without the mutation. This result could be explained by *in vivo* secretion of the mutant proteins, an inability to accurately detect differences in SP-A isoform protein levels, or by up-regulation of wild-type SP-A1 or -A2 expression in the setting of decreased SP-A2 secretion. We are currently unable to quantitate SP-A1 and SP-A2 isoforms in human bronchoalveolar lavage fluid. The four invariant amino acids that distinguish SP-A1 and SP-A2 are all located in the collagen-like domain proximal to the neck region. Our antibody for SP-A recognizes both recombinant SP-A1 and SP-A2 (data not shown). Subjects that are heterozygous for one of these rare mutations have three wild-type SP-A alleles; that is, two wild-type SP-A1 alleles and one wild-type SP-A2 allele. Expression of one of the three wild-type alleles may be up-regulated in the setting of mutant SP-A2 production.

SP-A gene expression is essentially lung specific. Both SP-A1 and SP-A2 are expressed in alveolar type II cells, but SP-A2 is also expressed in human airway submucosal gland cells (27, 28). Previous studies have investigated the various structural and functional differences among recombinant SP-A2 variants. The secreted proteins have been purified from medium of expressing hamster CHO-K1 or insect cells (29, 30). These cells, like A549 cells, do not have lamellar bodies, demonstrating that SP-A can be secreted in a non-lamellar body-dependent manner. The variants of SP-A2 have been previously studied based upon their population allele frequencies. The most common allele, 1A', which is found in ~60% of the population, corresponds to the wild-type SP-A2 protein described in this report (31). We find little difference in protein stability among the wild-type, T9N, A91P, and Q223K SP-A2 variants, which are represented in the most common human SP-A2 alleles.

The type II alveolar epithelial cell is specialized to produce large quantities of secreted proteins. High demands to synthesize and fold secreted proteins can overwhelm the ER capabilities and lead to “ER stress.” The cellular homeostatic pathway of the unfolded protein response is triggered by ER stress and can be cytoprotective by decreasing protein translation and increasing ER chaperone expression and protein folding. However, chronic ER stress can lead to cellular apoptosis. Evaluation of surgical lung biopsies from subjects with sporadic idiopathic pulmonary fibrosis has found evidence of severe ER stress and apoptosis of type II alveolar epithelial cells (21). The mutations we focus upon in this report are individually rare and cannot explain the severe ER stress found in sporadic idiopathic pulmonary fibrosis, but they do indicate that the pathway of inherent ER stress resulting from rare inherited surfactant mutations can be an inciting event that leads to pulmonary fibrosis.

Rare mutations in the gene encoding SP-C have been associated with chronic ER stress, cell apoptosis, and pulmonary fibrosis (32). One mutation, the ΔExon 4 mutation, is insoluble in 1% Triton X-100 and 0.05% sodium deoxycholate (6) and acts in a dominant-negative manner by sequestering wild-type SP-C in aggresomes in A549 cells (3). We have shown in this report that the G231V and F198S mutations are insoluble in 0.5% Nonidet P-40. However, only the G231V has a dominant-negative effect of interacting with and trapping the wild-type SP-A isoforms in a detergent-insoluble fraction in A549 cells (Fig. 4B). These mutant proteins were not found in a detergent-insoluble fraction in human type II alveolar epithelial cells. The particular subset of ER chaperones or the kinetics of the secretory machinery of different cell types may influence solubility of the SP-A2 mutant proteins.

Like the ΔExon 4 SP-C mutation, SP-A2 G231V and F198S mutant proteins are also degraded via the ER-associated degradation pathway and are associated with increased expression of BiP and a BiP promoter-driven reporter construct (33). Expression of the ΔExon 4 SP-C mutant also induces apoptosis and activates caspase 3 and NF-κB-dependent pathways (4, 5). So far, we have not been able to detect evidence of increased apoptosis in A549 cells transiently expressing the G231V and F198S variants in comparison with wild-type SP-A2 protein.

Whereas SP-C mutations can cause interstitial lung diseases in children or adults, the SP-A2 mutations have been found associated only with adult-onset disease. What accounts for the delayed presentation of the SP-A2 mutations? The effect of the SP-A2 mutations may be milder because the two human SP-A genes are differentially regulated during development. The SP-A2 gene appears to be more highly expressed than SP-A1 in the adult lung and SP-A1 more highly expressed than SP-A2 in fetal lung tissue. Additionally, SP-A2 is more highly regulated by cAMP than SP-A1 in human fetal lung tissue (34). The phenotypic differences may be related to the toxicity of the mutations in inducing ER stress in the alveolar epithelial cells. Many of the SP-C mutants overwhelm the cellular ER-associated degradation pathway and lead to protein aggregation and reduced SP-C protein secretion into the alveolar space. Like some of the SP-C mutations, we find that one of the SP-A2 mutations (G231V) is able to trap wild-type SP-A1 or SP-A2 isoforms in a detergent-insoluble phase in A549 cells. Unlike the SP-C mutations, we have no evidence that the mutant SP-A2 proteins accumulate in non-native intracellular compartments by immunohistochemistry (data not shown) or affect total SP-A expression in human bronchoalveolar lavage fluid.

Many different epidemiology studies have noted an association between pulmonary fibrosis and lung cancer (35). Lung adenocarcinoma is a phenotype that often segregates with pulmonary fibrosis in SP-A2 mutation carriers (16). Many ER resident proteins display altered expression in cancers and ER proteins have been implicated in cell death signals (36). It is intriguing to speculate that the pathogenesis of both processes may be due to modulation and activation of ER stress in the type II alveolar epithelial cells of the lung.

**Acknowledgments**—We gratefully acknowledge the excellent technical help of Melissa Nolasco and Lauren Miller, Jessica Mullens for the preparation of the recombinant adenovirus, and Meg Smith and Betsy Thomas for the preparation of the human type II alveolar epithelial cells. We want to thank Timothy Weaver, Kevin Gardner, Jonathan Cohen, and Helen Hobbs for helpful discussions.
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