Published in final edited form as:

Traffic. 2011 September ; 12(9): 1196–1210. doi:10.1111/j.1600-0854.2011.01223.x.

A non-aggregating Surfactant Protein C mutant is misdirected to early endosomes and disrupts phospholipid recycling

Michael F. Beers¹, Arie Hawkins¹, Jean Ann Maguire¹, Adam Kotorashvili¹, Ming Zhao¹, Jennifer L. Newitt¹, Wenge Ding¹, Scott Russo¹, Susan Guttentag², Linda Gonzales², and Surafel Mulugeta^{1,*}

¹ Pulmonary, Allergy, and Critical Care Division, Department of Medicine, University of Pennsylvania, Philadelphia, PA

² Division of Neonatology, Department of Pediatrics, Children's Hospital of Philadelphia, PA

Abstract

Interstitial lung disease in both children and adults has been linked to mutations in the lung-specific Surfactant protein C gene (*SFTPC*). Among these, the missense mutation (isoleucine to threonine at codon 73 = hSP-C^{I73T}) accounts for ~30% of all described *SFTPC* mutations. We reported previously that unlike the BRICHOS misfolding *SFTPC* mutants, expression of hSP-C^{I73T} induces lung remodeling and alveolar lipoproteinosis without a substantial ER stress response or ER-mediated intrinsic apoptosis. We show here that, in contrast to its wild type counterpart that is directly routed to lysosomal-like organelles for processing, SP-C^{I73T} is misdirected to the plasma membrane and subsequently internalized to the endocytic pathway via early endosomes, leading to the accumulation of abnormally processed proSP-C isoforms. Functionally, cells expressing hSP-C^{I73T} demonstrated both impaired uptake and degradation of surfactant phospholipid, thus providing a molecular mechanism for the observed lipid accumulation in patients expressing hSP-C^{I73T} through the disruption of normal phospholipid recycling. Our data provide evidence for a novel cellular mechanism for conformational protein associated diseases, and suggest a paradigm for mistargeted proteins involved in the disruption of the endosomal/lysosomal sorting machinery.

Keywords

Surfactant Protein C; post-translational processing; sorting; lysosomes; interstitial lung disease

Introduction

The hydrophobic human surfactant protein C (hSP-C) secreted into the pulmonary alveolus is a product of a larger bitopic, type II integral membrane precursor protein (proSP-C) (depicted in Figure 1) that undergoes palmitoylation and a series of proteolytic cleavages to yield the 3.7 kDa mature form (1, 2). Produced exclusively by alveolar type II cells, mature hSP-C is packaged into specialized lysosomal-like organelles (lamellar bodies) and released via regulated exocytosis together with other surfactant proteins and phospholipids. In the alveolus, SP-C along with another surfactant protein, SP-B, play a critical role in the modulation of lung mechanics from their direct effects on alveolar surface tension. Through enhancement of the adsorption and spreading of surfactant phospholipids and by

*Address correspondence to: Surafel Mulugeta, Ph.D., Pulmonary, Allergy, and Critical Care Division, University of Pennsylvania School of Medicine, Vernon & Shirley Hill Pavilion, 380 South University Avenue, Suite H418, Philadelphia, PA 19104. mulugeta@mail.med.upenn.edu.

maintenance of interfacial film at the air-liquid interface, hSP-C promotes the efficient generation of a surfactant monolayer shown to be critical in the prevention of alveolar collapse at end expiration. [For review see (1, 2)].

Interstitial lung disease (ILD) represents a diverse group of disorders of largely unknown etiology characterized by variable types and degree of interstitial and alveolar inflammation, fibrosis, and impaired gas exchange. Mutations in genes critical for surfactant metabolism and function are now recognized as one of the etiological causes of ILD (3). Of these, the gene encoding hSP-C (*SFTPC*) has been seminal in establishing this linkage with multiple reports of both sporadic cases as well as an autosomal dominant inheritance pattern with variable penetrance. Histological phenotypes of mutation-associated *SFTPC* ILD range from chronic pneumonitis of infancy and nonspecific interstitial pneumonitis (NSIP) in children to usual interstitial pneumonia (UIP) and idiopathic pulmonary fibrosis (IPF) in adults (4, 5). The great majority of *SFTPC* mutations are located in the distal C-terminus of proSP-C termed the BRICHOS domain (6), a region of ~100 amino acids with sequence and structure homology of proteins most notably found in association with familial neurodegenerative dementias. Using a variety of *in vitro* models, expression of the BRICHOS mutant hSP-C cDNAs results in intracellular aggregation of misfolded protein, ER stress, and subsequent induction of apoptotic cell death (7–10).

In addition to these aggregation prone hSP-C mutations, a different phenotype is emerging for a second group of missense mutations situated outside the BRICHOS domain, but within the C-terminal region of the propeptide adjacent to the transmembrane domain (6, 11). Among these, a heterozygous substitution mutation of threonine for isoleucine at amino acid 73 (hSP-C^{I73T}) of the proprotein accounts for ~30% (over 40 cases reported) of all hSP-C mutations associated with pulmonary disease (11–17) in both de novo (sporadic) as well as in inherited (autosomal dominant) cases. While symptomatic patients with an I73T mutation show some variability in clinical phenotype and course most likely due to the effects of additional modifier genes and/or environmental interactions (4, 5, 11, 17, 18), the associated histological and biochemical abnormalities most often include a pattern of NSIP with alveolar type II cell hyperplasia, cellular thickening of alveolar septa, and preserved alveolar architecture, often accompanied by evidence of alveolar accumulation of phospholipids and protein (lipoproteinosis). Analyses of the broncho-alveolar lavage of patients with hSP-C^{I73T} also show accumulation of abnormal C-terminal proSP-C products (11, 19, 20) although the consequences of their presence toward surfactant function is unknown. Moreover, in contrast to hSP-C BRICHOS mutations, *in vitro* expression of hSP-C^{I73T} fails to either induce ER stress responses, or activate caspase 4 mediated apoptosis (9) suggestive of a mechanism for cellular dysfunction distinct from those elicited by grossly misfolded BRICHOS proteins.

Given the central role of the alveolar epithelium in surfactant metabolism and the unique consequences for the lung resulting from expression of the hSP-C^{I73T} mutation including disrupted surfactant homeostasis, we hypothesized that the trafficking and processing as well as the functional consequences of hSP-C^{I73T} expression *in vitro* would be distinct from SP-C BRICHOS mutations. In this study, we demonstrate that unlike either its wild type counterpart or SP-C BRICHOS mutants, the hSP-C^{I73T} proprotein is initially mistargeted to the plasma membrane and subsequently internalized to early endosomes accompanied by aberrant post-translational processing. Functional assays using H³-labeled dipalmitoylphosphatidylcholine (DPPC), the major surfactant lipid species, demonstrated significant reduction of both lipid uptake and degradation by cells stably expressing hSP-C^{I73T}. Thus, expression of this non-BRICHOS mutant results in a unique cellular phenotype distinct from previously characterized *SFTPC* mutations.

Results

The human I73T mutant propeptide is abnormally trafficked to early endosome compartments

To characterize the influence of the I73T missense mutation on the biosynthesis of proSP-C, as well as on cellular homeostasis, a previously described human proSP-C cDNA clone was systematically altered where isoleucine was substituted with threonine at residue 73 by site directed mutagenesis and fused to the EGFP reporter (Figure 1) (10, 21–23). Because proSP-C is normally trafficked by homomeric association during its biosynthesis (24), initial studies were performed in transfected cell lines lacking endogenous SP-C to minimize interference from oligomeric association with an endogenous wild type isoform.

When wild type (EGFP•hSP-C^{WT}) and mutant hSP-C (EGFP•hSP-C^{I73T}) isoforms were transiently transfected into A549 cells (steady state expression, as determined by immunofluorescence microscopy), the proteins segregated into two distinct patterns of subcellular localization. The EGFP•hSP-C^{WT} fusion was predominantly localized in cytosolic vesicles which were also positive for CD63 (a marker antigen for type II cell lamellar bodies and lysosomes) and negative for EEA1 (an early endosome marker) (Figure 2A) as previously described (10, 24). In contrast, the EGFP•hSP-C^{I73T} mutant isoform was preferentially trafficked to compartments positive for EEA1 and predominantly negative for CD63 (Figure 2B). In addition, a significant pool of EGFP•hSP-C^{I73T} was also observed on the plasma membrane (Figure 2B, arrows), suggesting discrete routing differences between the two hSP-C isoforms.

The intracellular distribution of the hSP-C isoforms in subcellular fractions was further characterized biochemically using density gradient centrifugation and immunoblotting. Lamp1, another lamellar body/lysosome marker, overlapped with EGFP•hSP-C^{WT} in higher density sucrose fractions (Figure 2C), while EGFP•hSP-C^{I73T} was detected in lower density fractions which contained EEA1 (Figure 2D).

Temporal expression patterns for hSP-C^{I73T} are distinct from those of hSP-C^{WT}

To further investigate SP-C expression patterns, co-localization studies were performed using EGFP•hSP-C^{WT} or EGFP•hSP-C^{I73T} and a DsRed fusion construct containing the human ATP binding cassette class A3 (hABCA3), a polytopic transmembrane lipid transporter protein that has been shown to be abundant in the limiting membrane of lamellar bodies of alveolar type II cells (25, 26) and lysosomes of transfected cell lines (27). Using lipofectamine to accelerate early expression, EGFP•hSP-C^{WT} or EGFP•hSP-C^{I73T} and DsRed tagged wild type hABCA3 (hABCA3^{WT}•DsRed) were co-expressed in A549 cells. At 12 to 16 hr following transfection, co-expression of EGFP•hSP-C^{I73T} with hABCA3^{WT}•DsRed was virtually undetectable (Figure 3B, top row, and D). However, 24 to 36 hr following transfection, increasing amounts of vesicle-colocalization (~45%) of EGFP•hSP-C^{I73T} and ABCA3 occurred (Figure 3B, bottom row, and 3D) and was similar to CD63 colocalization (Figure 3C). In contrast, co-localization of EGFP•hSP-C^{WT} with hABCA3^{WT}•DsRed was nearly 100% at all time points examined (Figure 3A) with quantitative counting (Figure 3D) suggesting that the biosynthetic pathway taken by the two SFTPC isoforms was divergent.

Mistargeted hSP-C^{I73T} is also aberrantly processed

In alveolar type II epithelial cells, SP-C is synthesized as a 21 kDa bitopic integral membrane precursor that undergoes four proteolytic cleavages as it is trafficked through the biosynthetic pathway to yield a 3.7 kDa mature peptide (2). However, in A549 and other cell lines, only the initial of these four cleavages occurs at or near residue 145 in early post-

Golgi compartments (Fig. 1) (24, 28, 29). Moreover, epithelial cell lines such as A549 or HEK293 secrete neither the mature nor the processed forms of SP-C. However, these cell lines have been proven advantageous for their own benefits over primary alveolar type II cells since the primary cells are phenotypically unstable in culture.

Western blot analyses of EGFP•hSP-C isoforms in transfected A549 cell lysates using antisera directed against the N-terminally placed EGFP tag demonstrated that proSP-C^{I73T} undergoes abnormal post-translational processing. As shown in Figure 4A, a major product of 27 kDa was detected in cells transfected with EGFP alone. When wild type EGFP•hSP-C was introduced, lysates contained two anti-EGFP-positive bands that included the primary translation product of the fusion protein (48 kDa) (Figure 4A, arrowhead) and a smaller processed intermediate consistent with previous reports of partial C-terminal processing of normally routed proSP-C by A549 cells (10, 24). Contrary to wild-type proSP-C, expression of EGFP•hSP-C^{I73T} resulted in multiple mutant proSP-C^{I73T} products composed of the primary translation and processed intermediate forms (similar to those observed in the wild type isoform) as well as two additional forms (Figure 4A, arrows), one migrating at higher M_r (~50 kDa) and the other, with M_r between the primary translation and processed intermediate products. The lower product migrated at a higher molecular weight than the normally processed product suggesting that the cleavage site is in the C-terminus and more distal than the normal cleavage site. Treatment with PNGase F, O-glycosidase, or protein phosphatases did not alter the migration of any of the proSP-C^{I73T} products (data not shown).

hSP-C^{I73T} is preferentially targeted to plasma membrane

In addition to EEA1 vesicles, a significant fraction of EGFP•hSP-C^{I73T} fluorescence appeared to be localized to plasma membrane (Figure 2, arrows). To confirm the appearance of EGFP•hSP-C^{I73T} at the plasma membrane, surface biotinylation of intact transfected cells was performed at 4°C. Compared to EGFP•hSP-C^{WT}, immunofluorescence imaging of EGFP•hSP-C^{I73T} transfected A549 cells showed co-localization of the mutant isoform with biotin-Texas-Red-streptavidin conjugates on the plasma membrane (Figure 4B, arrows). Analysis of biotinylated cell surface protein by streptavidin pull down followed by Western blot, provided biochemical confirmation that significant amounts of the EGFP•hSP-C^{I73T} primary translation product reached the plasma membrane (Figure 4C, arrowhead, and Figure 4D). Interestingly, the two aberrantly processed forms of hSP-C^{I73T} also appeared on the cell surface (Figure 4C, arrows).

hSP-C^{I73T} colocalizes with transferrin both at the plasma membrane and in early endosomal compartments

To link the observation of cell surface expression and localization of EGFP•hSP-C^{I73T} to early endosomes, time dependent analysis of mutant propeptide trafficking following plasma membrane localization was performed using Alexa Fluor-conjugated transferrin as a plasma membrane and endocytosis marker. In the absence of endocytosis (4°C), EGFP•hSP-C^{I73T} co-localized with Alexa Fluor-transferrin at the cell surface (Figure 5B, top row). Increasing the temperature to 37°C for 5 minutes consistently resulted in internalization of transferrin with subsequent co-localization in EGFP•hSP-C^{I73T} containing vesicles primarily in proximity to the plasma membrane (Figure 5B, middle row). Further incubation at 37°C for 1hr resulted in further co-localization in vesicles distributed throughout the cell (Figure 5B, bottom row). In contrast, EGFP•hSP-C^{WT} predominantly remained within cytosolic vesicles and rarely colocalized with transferrin at any of the time points examined (Figure 5A).

hSP-C^{I73T} is initially routed directly to plasma membrane

While the current data demonstrate the presence of SP-C^{I73T} at the cell surface, there are several possible routes that could be utilized for initial delivery of the protein to plasma membrane (30). To investigate whether the mutant protein is trafficked via Golgi to endosome to plasma membrane pathway, or is directly delivered to the cell surface, time-dependent colocalization studies were performed utilizing the mannose-6-phosphate receptor (M6PR) as an endosome marker, or wheat germ agglutinin (WGA) as a plasma membrane marker in A549 cells expressing EGFP-tagged hSP-C^{I73T}. At earlier time points (6 and 12 hr), faint EGFP•hSP-C^{I73T} expression was observed that only sparsely colocalized with M6PR (Fig. 6A and 6B) and did not reach a maximum until 24 hr post-transfection (Figure 6B). In contrast, significant early colocalization of the mutant isoform with WGA was observed starting at 6 hr post transfection and peaking as early as 16 hr post transfection (Figure 6A and 6B). These results suggest that the initial route taken by hSP-C^{I73T} to the plasma membrane is through a direct, anterograde, non-endosomal pathway.

hSP-C^{I73T} is internalized from plasma membrane to cytosolic vesicles

Although intracellular co-localization of transferrin with SP-C^{I73T} was apparent following transferrin internalization, there was no direct evidence demonstrating whether the SP-C^{I73T} found in transferrin-containing organelles was actually from the plasma membrane. To substantiate SP-C^{I73T} endocytosis, an epitope specific antisera raised against the C-terminal region of the propeptide (anti-CTERM) (31) was used. As anticipated, in the absence of endocytosis (4°C), EGFP•hSP-C^{I73T} was localized at the cell surface (Figure 6C, middle row, arrowheads). Increasing the temperature to 37°C for 30 minutes consistently resulted in the internalization of the antibody-labeled EGFP•hSP-C^{I73T} to punctate donut-shaped vesicles (Figure 6C, bottom row, arrows). In contrast, EGFP•hSP-C^{WT} predominantly remained within cytosolic vesicles, did not reach the plasma membrane, and was not labeled by α -CTERM (Figure 6C, top row)

Expression patterns of hSP-C^{I73T} in primary human alveolar type II cells are similar to epithelial cell lines

Primary alveolar type II cells were isolated (from human fetal lung in limiting quantities) and transfected in order to verify SP-C^{I73T} expression patterns observed in A549 and HEK293 (see Figure 8) cell lines. Confocal immunofluorescence imaging of transiently transfected alveolar type II cells showed distinct distribution of the wild type (Figure 7, left panel) versus the mutant (Figure 7, right panel) EGFP•SP-C fusion proteins. As expected, wild type SP-C vesicles were double positive for CD63 and DC-Lamp (another marker antigen for human alveolar type II cell lamellar bodies and lysosomes) and negative for EEA1. In contrast, the EGFP•hSP-C^{I73T} mutant isoform was preferentially expressed in compartments that were positive for EEA1 but also positive for CD63 and DC-Lamp likely secondary to the homomeric/heterotypic association with endogenous SP-C in these cells (see Results, first paragraph). In addition, EGFP•hSP-C^{I73T} but not EGFP•hSP-C^{WT} was found on the plasma membrane (arrowhead) co-localizing with the plasma membrane marker, epithelial cell adhesion molecule (EpCAM) (arrow).

Expression of hSP-C^{I73T} impairs cellular uptake and degradation of lipids

In addition to parenchymal remodeling, patients with hSP-C^{I73T} mutation exhibit alveolar phospholipidosis (11, 32) suggesting a defect in surfactant lipid metabolism. Based on the mistargeting of hSP-C^{I73T}, we hypothesized that cells expressing the mutant isoform would exhibit perturbation of cellular processes that are dependent upon endosomal-lysosomal progression which would include lipid metabolism.

To examine the functional consequences from expression of the mutant hSP-C protein, lipid uptake and degradation were generated from clones of HEK293 cells stably expressing either EGFP tagged wild type or mutant hSP-C isoforms (Figure 8A). As for A549 cells, immunofluorescence analysis of the stable cell lines showed a similarly distinct expression pattern where plasma membrane localization of the mutant isoform with WGA was apparent (Figure 8A, bottom row). By Western blot, the HEK293 clones demonstrated equal expression of EGFP•hSP-C^{WT} or EGFP•hSP-C^{I73T} (Figure 8B).

Cell monolayers were incubated at 37° with H³-choline-labeled dipalmitoylphosphatidylcholine (DPPC)-containing liposomes, and uptake was measured 2 hours following treatment. As demonstrated in Figure 8C, DPPC uptake was significantly reduced in cells expressing EGFP•hSP-C^{I73T} (29% reduction) compared to those expressing EGFP•hSP-C^{WT} or non-transfected cells. Moreover, degradation of DPPC that had been internalized by the cells during the 2 hr incubation period was differentially altered (Figure 8D). In cells expressing EGFP•hSP-C^{WT}, degradation averaged 5.5% of internalized DPPC. In contrast, degradation of DPPC in EGFP•hSP-C^{I73T} expressing cells was reduced by more than a third (36% reduction) or by 25% compared to cells expressing EGFP•hSP-C^{WT} or non-transfected cells, respectively. Taken together, these results suggest that expression of hSP-C^{I73T} functionally impairs lipid uptake and degradation by epithelial cells.

Discussion

The cellular mechanisms underlying the pathogenesis of interstitial lung disease (ILD) associated with *SFTPC* mutations are partly understood. Most of the published reports have focused on a select group of mutations located in the proprotein domain flanking the SP-C C-terminus known as the BRICHOS domain (33) where the unfolded protein response (UPR), ER stress, mitochondrial dysfunction, and apoptosis have been implicated (7, 9, 10). In contrast to these, expression of two different non-BRICHOS mutations (I73T, E66K) of the *SFTPC* gene produces a distinct clinical as well as cellular phenotype. *In vivo*, non-BRICHOS mutants produce both interstitial pneumonia and pulmonary lipoproteinosis accompanied by abnormal localization of the SP-C propeptide in EEA-1 vesicles in alveolar type II cells (11, 34). Since these reports, the hSP-C^{I73T} mutation is now recognized as the most common SP-C mutation to date (13, 14, 16, 17, 35). The present report extends the prior findings by demonstrating that trafficking of the hSP-C^{I73T} mutant is altered by its misrouting to the plasma membrane, subsequent internalization to early endosomes, and delayed appearance within lysosomal-like compartments. This misrouting is accompanied by aberrant post-translational processing of the mutant isoform, and collectively these abnormalities result in functional impairment of lipid uptake and degradation. Thus, the hSP-C^{I73T} non-BRICHOS mutation represents a new class of spatially and functionally distinct mutations of proSP-C that is capable of producing chronic lung disease, but is phenotypically different from those of misfolded BRICHOS mutants.

In general, the diversion of a protein from reaching its targeted destination due to missense, nonsense, deletion, or frame shift mutations is a common phenomenon that occurs in diseases associated with secretory or transmembrane protein mutations. In many cases, protein misfolding is believed to contribute to the pathophysiology. Best known among these are neurodegenerative diseases including Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease. For each, a gain-of-function results from mutations in Huntingtin, beta amyloid, SOD1 and α -synuclein, respectively, and produces ER retention of the misfolded protein leading to the activation of the UPR, ER stress, and protein aggregation (36). These cellular responses in turn lead to the subcellular disruption of essential proteins such as chaperones, interfere with degradation of other proteasome substrates, and compromise the folding and stability of other proteins that are

conformationally challenged to cause the global disruption of cellular homeostasis leading to cell death. We have shown some of these cellular abnormalities also occur *in vitro* in ILDA-associated BRICHOS domain hSP-C mutants, including deletion of the exon 4 transcript of the hSP-C gene (Δ exon 4) and a missense mutation that substitutes leucine for glutamine at residue 188 (L188Q) (6, 10, 37). The absence of mature hSP-C in the bronchoalveolar lavages of patients with the heterozygous expression of these mutations suggests a dominant negative effect by the mutant isoform.

Utilizing multiple experimental techniques, data in the present study demonstrate that the hSP-C^{I73T} mutant propeptide is misrouted to the plasma membrane. In contrast to the BRICHOS mutants, hSP-C^{I73T} fails to induce either a dominant negative effect as some mature SP-C can be detected in patients heterozygous for hSP-C^{I73T} or a classic toxic gain of function as significant amounts of ER stress is lacking (9, 34). In this way, hSP-C^{I73T} induced ILD may be more similar to cytotoxic degenerative diseases such as primary hyperoxaluria type 1 (PH1) disease (38), malonyl-coA decarboxylase (MLYCD) deficiency (39), and hereditary hypophosphatemic rickets with hypercalciuria (HHRH) (40). These diseases all result from misrouting of missense mutants to different subcellular compartments or to the plasma membrane, and in most cases lead to cellular dysfunction. Complementary to this concept, our results suggest expression of the aberrantly targeted mutant hSP-C^{I73T} produces a secondary loss-of-function via disruption of lysosomal degradation processes. Moreover, the functional impairment need not be limited to lipid uptake and degradation, but may also extend to other proteins that share these routing compartments. Aberrant processing intermediates of SP-B have been detected in intra-alveolar surfactant fractions in patients with both hSP-C^{I73T} and hSP-C^{E66K} mutations (11, 19, 34).

In the present study, we have also detected aberrant EGFP hSP-C^{I73T} intermediates which likely result from mistargeting-dependent exposure of the mutant isoform to alternative processing events from different or additional protease(s). As shown in Figures 4 and 6, there are two additional bands that are absent in the expression profile of wild type EGFP hSP-C isoform. These have also been recently identified both in the culture media of cells stably expressing hSP-C^{I73T} and in the lungs of patients with this mutation (19, 28). The first band, migrating faster than the primary translation product, could result from intracellular enzymatic cleavage caused by the propeptide's improper routing to EEA1 vesicles. Alternatively, since the same product was biotinylated (Figure 4C), it is possible that trimming of the C-terminal propeptide at the cell surface by opportunistic extracellular proteases may occur. As shown in Figure 9, since proSP-C is a type II integral transmembrane protein (31, 41), direct delivery to the cell surface would result in a membrane spanning orientation whereby the C-terminal region is exposed to the extracellular milieu making alternative potential protease cleavages a spatial possibility. Biochemical studies of patients with hSP-C^{I73T} as well as the E66K mutation support the findings of abnormal processing. Aberrantly processed 11 kDa and 13 kDa C-terminal segments of hSP-C proprotein were identified by immunoblotting of bronchoalveolar lavage fluid of a patient carrying the SP-C^{I73T} mutation (11, 34). Mass spectrometry has also shown intra-alveolar accumulation of aberrant C-terminal processing products (19).

In addition to abnormal protein processing, other consequences of hSP-C^{I73T} mistargeting include an accompanied ultra structural dysfunction. Alveolar type II cells are actively involved in the clearance of pulmonary surfactant from the intra-alveolar space as well as recycling (via the multivesicular bodies (MVBs)) of internalized surfactant lipids and proteins back to the lamellar body (42, 43). Interestingly, the lamellar body itself exhibits characteristics of a lysosomal-like secretory organelle (44). Consequently, the misrouting of proSP-C^{I73T} to the cell surface and its subsequent internalization not only affects the

lysosomal degradation pathway, but may also disrupt the MVB/lamellar body recycling system including docking and fusion processes of exocytosis. Thus, the observation of abnormal dense core organelles and poorly developed lamellar bodies in alveolar type II cells of patients with hSP-C^{I73T} (11, 17) is likely the product of both disrupted lysosomes and a functionally compromised endosome/MVB/lamellar body recycling system. Moreover, since patients with SP-C^{I73T} have alveolar lipoproteinosis, the increased steady state level of surfactant reflects impaired endocytosis (supported by the present study) and also implies enhanced secretion.

To address this functionally, we modeled the effect of hSP-C^{I73T} expression on clearance of lipid components using stably transfected cell lines. The reduced phospholipid uptake and degradation demonstrated in Figure 8 conforms to disorders associated with defects in clearance and recycling of surfactants from lung. Under normal conditions, both type II cells and alveolar macrophages contribute to surfactant clearance from the distal airways. Previously, in both humans and transgenic mice, defects in granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling either by ligand- or receptor-inhibition (by neutralizing antibodies or mutations) have been associated with disruption of surfactant homeostasis where the bronchoalveolar lavages of patients lacking GM-CSF activity have revealed pulmonary alveolar lipoproteinosis (45–47). Likewise, targeted deletion in mice of the acid independent phospholipase A₂ (aiPLA₂), a lysosomal enzyme that degrades endocytosed surfactant DPPC in alveolar type II cells, results in the time dependent accumulation of both total surfactant phospholipid (mainly disaturated phosphatidylcholine in bronchoalveolar lavage, lung homogenate, and lung lamellar bodies (48). Therefore, the intra-alveolar accumulation of lipids and protein in patients with hSP-C^{I73T} mutation could directly be attributed to a malfunction in type II cell dependent surfactant clearance and recycling and suggests both cell types are important for surfactant homeostasis.

In summary, we report phenotypic and biochemical characterization of a non-BRICHOS mutation of the *SFTPC* gene associated with interstitial pneumonia and alveolar lipoproteinosis. We have determined aberrant protein trafficking and subcellular localization (Figure 9) of this propeptide leading to abnormal protein processing. The nature of the mutation appears to dictate an alternative biosynthetic route taken by the mutant propeptide, which is distinctly different in both spatial and temporal phases from those observed within BRICHOS folding mutants. The acquired impairment of lipid uptake and degradation by cells expressing the mutant propeptide support data from earlier reports of patients with abnormal intra-alveolar contents of lipids and proteins, including increased surfactant protein A content and aberrantly processed surfactant proteins B and C. In addition, this functional impairment points to a novel concept where a protein that is not functionally associated with lysosomes can compromise the lysosomal dependent degradation process. Understanding molecular mechanisms underlying the etiology and evolution of these abnormalities will provide a pertinent perspective to disease progression, and may offer insights into the development of new therapeutic strategies tailored to the specific subcellular deficits and pathways induced by such mistargeted proteins.

Materials and Methods

Reagents

The pEGFP-C1 and DsRed-N1 monomer plasmids were purchased from Clontech, Inc. (Palo Alto, CA). Tissue culture medium was produced by the Cell Center Facility, University of Pennsylvania. Except where noted, all other reagents were electrophoresis, tissue culture, or analytical grade and were purchased from Sigma Chemical, Inc. (St. Louis, MO) or BioRad, Inc. (Melville, NY).

Generation of plasmid constructs

Constructs used for this study are illustrated in Figure 1. Human wild-type proSP-C (amino acids 1–197) inserted into the pEGFP-C1 vector has been previously described, as has EGFP•hSP-C^{I73T} (24, 37). A full length ABCA3^{WT}•DsRed was generated by substitution of the EGFP fluorescence encoding region of the ABCA3^{WT}•EGFP construct (26) with non-dimerizing DsRed encoding region using restriction enzymes XhoI and EcoRI.

Cell lines and transfection

Human A549 and HEK293 epithelial cell lines were originally obtained through the American Type Culture Collection (ATTC, Manassas, VA). A549 cells grown to 85–90% confluency on glass coverslips in 35-mm plastic dishes were transiently transfected with various fusion wild type or mutant hSP-C constructs (4 µg/dish) or co-transfected with post-Golgi distal vesicle marker construct, hABCA3^{WT}•DsRed (2 µg/dish per construct), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). EGFP•hSP-C^{WT} and EGFP•hSP-C^{I73T} stable cell lines in HEK293 cells were generated following a 48 hr transient expression using CaPO₄ transfection method (31), and with subsequent steps taken for clonal selection with 1mg/ml G418 as described previously (49–51). The cells were maintained in Dulbecco's Modified Eagles medium (DMEM) containing 500 µg/ml G418, 10%FBS, and 1% penicillin/streptomycin.

Human fetal lung alveolar type II cell culture and transfection

Isolation and culture of human fetal alveolar type II cells was performed as previously described (52). Briefly, enriched populations of epithelial cells were isolated from second-trimester (13–20 wk gestation) human fetal lung tissue obtained under a protocol approved by the Children's Hospital of Philadelphia Institutional Review Board. After overnight culture as explants without hormones, the tissue was digested with trypsin, collagenase, and DNAase and removed fibroblasts by differential adherence. Nonadherent cells were first transfected with various wild type or mutant SP-C fusion constructs using electroporation with the Amaxa transfection system prior to plating on glass cover slips in 30-mm plastic culture dishes in Weymouth's medium containing 10% fetal calf serum. After overnight culture (*day 1*), attached cells were cultured for an additional 3–9 days in 1 ml of serum-free Weymouth's medium containing 10 nM dexamethasone, 0.1 mM 8-bromo-cAMP, and 0.1 mM isobutylmethylxanthine together (referred to as DCI), previously shown to maximally induce surfactant components consistent with a morphological alveolar type II cell phenotype.

Sucrose gradient fractionation

Subcellular fractionation using discontinuous sucrose gradient was performed using the method of Stockinger *et al.* with some modifications (53). A549 cells in T75 flasks transiently expressing EGFP-tagged hSP-C isoforms for 24 hr were harvested by scraping and were washed twice with phosphate buffered saline (PBS) (137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄, pH 7.4). Cells were subsequently resuspended with 750 µl of 0.2 M sucrose containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], leupeptin [1 µg/ml], pepstatin A [1 µg/ml], and aprotinin [1.5 µg/ml]) and disrupted by 10 strokes using a glass tissue homogenizer and Teflon probe. Nuclei were removed by 5 min centrifugation at 1,000 × g and the supernatant was adjusted to 750 µl with 0.2 M sucrose. The supernatant was loaded on top of 5.25 ml containing 0.4, 0.6, 0.8, 0.9, and 1.0 M discontinuous sucrose gradient (750 µl each) and spun for 1 hr in a Sorvall ultracentrifuge (swinging bucket rotor, TH-641) at 105,000 × g. Individual fractions (750 µl) were collected from the bottom up using Pasteur pipettes and sample proteins were concentrated using trichloroacetic acid (TCA) precipitation method comprised of incubating

samples on ice in equal volume of 20% trichloroacetic acid (TCA) for 30 min; centrifugation at 13,200 g for 15 min; washing precipitates twice with 500 μ l ice cold acetone; and resuspending samples in SDS/PAGE loading buffer.

Cell surface biotinylation

Cell surface protein biotinylation was performed using the Sulfo-NHS-SS-Biotin reagent with Streptavidin Agarose Resins according to the manufacturer's protocol (Thermo Scientific, Rockford, IL) with some modifications. A549 cells transiently expressing isoforms EGFP•hSP-C in T75 flask grown to 85–90% confluency were washed twice with 8 ml ice-cold PBS, pH 7.4. 10 ml of 10 mM of Sulfo-NHS—SS-Biotin was added to the flask and gently agitated for 30 m at 4°C. The biotinylation reaction was stopped by adding 500 μ l of quenching solution (Thermo Scientific, Rockford, IL). Cells were harvested by scraping and washed 3 time by centrifugation in Tris buffered saline (TBS) (20 mM Tris, 500 mM NaCl, pH 7.5) at 500 $\times$ g for 3 min. The resulting pellet was resuspended in 400 μ l HEN buffer (25 mM HEPES, 0.1 mM EDTA, 10 μ M Neocuporine) containing protease inhibitors and 1% SDS (added fresh). Cells were sonicated using a probe sonicator (60 Sonic Dismembrator, Fisher Scientific, Pittsburg, PA) utilizing three burst of 10 sec with 15 sec interval and at 40% maximum power output. Streptavidine binding was accomplished by adding 200 μ l neutralization buffer (20 μ M HEPES, 1 mM EDTA, 10 μ M NaCl, 0.5% Triton X-100) and 500 μ l of streptavidin-agarose beads (Thermo Scientific, Rockford, IL) to the sonicated solution and incubating at room temperature with end to end rotation overnight. The bound biotin-streptavidin-agarose complex was washed 5 times using 500 μ l neutralization buffer containing 600 mM NaCl. The agarose beads were released by adding 20 μ l 2X lithium dodecyl sulfate (LDS) sample buffer (Novex, Carlsbad, CA), 2 μ l 10X sample reducing agent (Novex), and by heating at 95°C for 10 min.

SDS/PAGE

Cell pellets collected from 35 mm culture dish by scraping and centrifugation at 300 $\times$ g were solubilized with 40 μ l of lysis buffer containing protease inhibitors. Following centrifugation at 6,000 $\times$ g for 30 seconds to remove nuclei, proteins were separated by electrophoresis on a 12% polyacrylamide gel and transferred to nitrocellulose membrane.

Immunoblotting

Immunoblotting of transferred samples was performed using successive incubations with primary anti-EGFP (Clontech, Mountain View, CA), 1:5000; anti-Lamp 1 (Abcam Inc., Cambridge, MA, USA), 1:1000; anti-EEA1 (BD Biosciences, San Jose, CA), 1:3000; anti- β -actin (Sigma, St. Louis, MO), 1:2000; and horseradish peroxidase-conjugated secondary antibodies as previously described (24). The resulting fluorescence images visualized by ECL were captured either by exposure to film or by direct acquisition using the Kodak 440 Imaging System.

Immunocytochemistry

Colocalization studies were performed by immunostaining cells plated on coverslips and fixed by coverslip immersion in 4% paraformaldehyde as previously described (9). Following permeabilization, cells were immunolabeled with primary antibodies for 1 hr at room temperature at the following dilutions: anti-CD63 (Immunotech, Marseilles, France), 1:500; anti-EEA1 (BD Biosciences, San Jose, CA), 1:3000; anti-Streptavidin 1:1000, (Abcam Inc., Cambridge, MA), DC-Lamp (Beckman Coulter, Brea, CA) 1:100; Ep-CAM (Santa Cruz Biotech, Santa Cruz, CA) 1:200; anti-CTERM (Beers Laboratory, Philadelphia, PA), 1:250; anti-M6PR (Abcam Inc., Cambridge, MA), 1:150. Texas red-conjugated secondary goat anti-mouse IgG monoclonal or secondary goat anti-rabbit IgG polyclonal

antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), were used for visualization at a dilution of 1:2000.

Plasma membrane labeling with wheat germ agglutinin (WGA)

Cells plated on coverslips were fixed by coverslip immersion in 4% paraformaldehyde and without permeabilization. Cells were washed 3×5 min with PBS and 3.3 μ l/ml of 1 mg/ml Alexa Fluor 594 WGA (Invitrogen Molecular Probes, Carlsbad, CA) stock solution was added to cells and incubated with gentle agitation for 30 min. Cells were subsequently washed 3×5 min with PBS and cover slips mounted on slides for fluorescence visualization.

Fluorescence microscopy

EGFP images of permeabilized/nonpermeabilized cells fixed in 4% paraformaldehyde were viewed on an Olympus I-70 inverted fluorescence microscope using a High Q FITC filter packages (Excitation 480 nm; Emission 535/550 nm) (Chroma Technology, Brattleboro, VT). Similarly, DsRed images and Texas Red tagged immunostained images were visualized with High Q TR filter (Excitation 560/555 nm; Emission 645/675 nm) as described (54, 55). Image acquisition, processing, and overlay analysis were performed using the Metamorph 7.5 software (Molecular Devices, Inc., Downingtown, PA) by utilizing identical imaging criteria within experimental groups including equal exposure time, image scaling, and image gamma. For confocal microscopy, cells were examined using the TE300 Nikon coupled Radiance 2000 imaging system, Carl Zeiss (Beltsmd, MD) by using identical imaging criteria within experimental groups including equal exposure time, image scaling and image gamma. All Images were subsequently transformed from 24 bit program files to 8 bit TIFF files and transferred to a Power Point program for assembly and labeling. When necessary, enhancement of images was performed during the Power Point assembly by adjusting the "brightness" and "contrast" of each image within the same figure equally.

Dynamic trafficking of EGFP•hSP-C isoforms

Following 24 hr expression of EGFP•hSP-C isoforms in A549 cells grown on coverslips in 35 mm culture dishes, cell medium was replaced by fresh, ice cold culture medium containing 20 μ g/ml Alexa Fluor 594-labeled transferrin (Invitrogen, Carlsbad, CA) or epitope specific C-terminal polyclonal antibody (anti-CTERM) (31). Cells were incubated with gentle agitation for 60 min at 4°C. Cells were washed with ice-cold culture medium to remove excess transferrin or antibody from the cell surface and either washed with ice cold PBS and fixed directly, or incubated at 37°C for 5 min, 30 min, or 60 min, washed in cold PBS, and then fixed. For fluorescence visualization, the cover slips from the transferrin experiment were directly mounted on slides and the cover slips from anti-CTERM binding were subsequently incubated with Texas Red-conjugated secondary antibody prior to slide mount.

Preparation of liposomes

Unilamellar liposomes were prepared by constituting 0.5 M dipalmitoylphosphatidylcholine (DPPC), 0.25 M egg phosphatidylcholine (PC), 0.15 M cholesterol, and 0.1 phosphatidylglycerol to reflect the approximate composition of lung surfactant (48, 56). Radiolabeled DPPC containing [3 H-methyl] choline was added to a final concentration of 0.052 μ ci/ μ l. Unilamellar liposomes with mean diameter of 108 nm were prepared by evaporating the mixture of lipids under N_2 to dryness, resuspending the evaporated film in PBS with vigorous mixing, freezing and thawing three times by alternating exposure to liquid N_2 and a 50°C water bath, followed by extrusion of the sample at 50°C for 10 cycles through a 100 μ m pore size filter.

Uptake and degradation of DPPC

Uptake of ^3H -DPPC in liposomes by HEK293 cells or by HEK293 cells stably expressing either wild type or mutant isoforms of hSP-C was measured based on methods described previously (48, 57, 58) with some modifications. Growth medium was removed from HEK293 cells grown to confluency in 35 mm dishes and was replaced with DMEM (1 ml/dish) containing 20 nM radiolabeled unilamellar liposomes. Following incubation at 37°C for 2 hr in a 5% CO_2 incubator, cells were sequentially washed twice with DMEM, once with PBS and removed from dishes by trypsinization. Three dishes per sample were pooled for analyses. Cells were pelleted by centrifugation and were resuspended in 0.5 ml PBS followed by cell counts using a hemocytometer. Samples were normalized for equal number of cells per sample by dilution with PBS. Each sample was then equally divided and one half was directly analyzed for radioactivity measured as disintegration per minute (dpm) to determine total DPPC uptake. Uptake of DPPC was calculated from total DPM measured per 0.5 million cells. The remaining half of the sample was fractionated using the Bligh and Dyer method (59) and the amount of DPPC degradation was calculated as the percentage of radioactivity in the aqueous fraction relative to the total radioactivity present in the lipid plus aqueous fractions.

Data analysis

Experimental data were analyzed by one-way analysis of variance with the Tukey-Kramer post hoc test using GraphPad InStat software, version 3.0 for Windows (GraphPad Software, San Diego, CA). All values are means $\pm$ SE. Significance was accepted at $p < 0.05$.

Acknowledgments

We thank Dr. Aron B. Fisher, Chandra Dodia, and Jain-Qin Tao of the University of Pennsylvania, Institute for Environmental Medicine, for their suggestions and technical assistance with lipid assays.

Grants

This work was supported by National Heart, Lung, and Blood Institute Grants HL09073 (SM), HL059959 (SG), HL19737 (MFB), and HL087177 (MFB).

References

1. Whitsett JA, Weaver TE. Hydrophobic surfactant proteins in lung function and disease. *N Engl J Med*. 2002; 347:2141–2148. [PubMed: 12501227]
2. Beers MF, Mulugeta S. Surfactant Proteins B and C (SP-B and SP-C). *Encyclopedia of Respiratory Medicine*. Laurent, GL., Shapiro, SD., editors. Elsevier; Oxford: 2006. p. 148-151.
3. Wert SE, Whitsett JA, Noguee LM. Genetic disorders of surfactant dysfunction. *Pediatr Dev Pathol*. 2009; 12:253–274. [PubMed: 19220077]
4. Loyd JE. Pulmonary fibrosis in families. *Am J Respir Cell Mol Biol*. 2003; 29:S47–S50. [PubMed: 14503554]
5. Percopo S, Cameron HS, Noguee LM, Pettinato G, Montella S, Santamaria F. Variable phenotype associated with SP-C gene mutations: fatal case with the I73T mutation. *Eur Respir J*. 2004; 24:1072–1073. [PubMed: 15572558]
6. Beers MF, Mulugeta S. Surfactant protein C biosynthesis and its emerging role in conformational lung disease. *Annu Rev Physiol*. 2005; 67:663–696. [PubMed: 15709974]
7. Bridges JP, Wert SE, Noguee LM, Weaver TE. Expression of a human surfactant protein C mutation associated with interstitial lung disease disrupts lung development in transgenic mice. *J Biol Chem*. 2003; 278:52739–52746. [PubMed: 14525980]
8. Lawson WE, Polosukhin VV, Stathopoulos GT, Zoia O, Han W, Lane KB, Li B, Donnelly EF, Holburn GE, Lewis KG, Collins RD, Hull WM, Glasser SW, Whitsett JA, Blackwell TS. Increased

- and prolonged pulmonary fibrosis in surfactant protein C-deficient mice following intratracheal bleomycin. *Am J Pathol.* 2005; 167:1267–1277. [PubMed: 16251411]
9. Mulugeta S, Nguyen V, Russo SJ, Muniswamy M, Beers MF. A surfactant protein C precursor protein BRICHOS domain mutation causes endoplasmic reticulum stress, proteasome dysfunction, and caspase 3 activation. *Am J Respir Cell Mol Biol.* 2005; 32:521–530. [PubMed: 15778495]
 10. Mulugeta S, Maguire JA, Newitt JL, Russo SJ, Kotorashvili A, Beers MF. Misfolded BRICHOS SP-C mutant proteins induce apoptosis via caspase-4-and cytochrome c-related mechanisms. *Am J Physiol Lung Cell Mol Physiol.* 2007; 293:L720–L729. [PubMed: 17586700]
 11. Brasch F, Griesse M, Tredano M, Johnen G, Ochs M, Rieger C, Mulugeta S, Muller KM, Bahuau M, Beers MF. Interstitial lung disease in a baby with a de novo mutation in the SFTPC gene. *Eur Respir J.* 2004; 24:30–39. [PubMed: 15293602]
 12. van Moersel CH, van Oosterhout MF, Barlo NP, de Jong PA, van der Vis JJ, Ruven HJ, van Es HW, van den Bosch JM, Grutters JC. SFTPC Mutations are the Basis of a Significant Portion of Adult Familial Pulmonary Fibrosis in a Dutch Cohort. *Am J Respir Crit Care Med.* 2010
 13. Cameron HS, Somaschini M, Carrera P, Hamvas A, Whitsett JA, Wert SE, Deutsch G, Nogee LM. A common mutation in the surfactant protein C gene associated with lung disease. *J Pediatr.* 2005; 146:370–375. [PubMed: 15756222]
 14. Garmany TH, Wambach JA, Heins HB, Watkins-Torrey JM, Wegner DJ, Bennet K, An P, Land G, Saugstad OD, Henderson H, Nogee LM, Cole FS, Hamvas A. Population and disease-based prevalence of the common mutations associated with surfactant deficiency. *Pediatr Res.* 2008; 63:645–649. [PubMed: 18317237]
 15. Abou TR, Jaubert F, Emond S, Le Bourgeois M, Epaud R, Karila C, Feldmann D, Scheinmann P, de Blic J. Familial interstitial disease with I73T mutation: A mid- and long-term study. *Pediatr Pulmonol.* 2009; 44:167–175. [PubMed: 19148933]
 16. Guillot L, Epaud R, Thouvenin G, Jonard L, Mohsni A, Couderc R, Counil F, de Blic J, Taam RA, Le Bourgeois M, Reix P, Flamein F, Clement A, Feldmann D. New surfactant protein C gene mutations associated with diffuse lung disease. *J Med Genet.* 2009; 46:490–494. [PubMed: 19443464]
 17. Crossno PF, Polosukhin VV, Blackwell TS, Johnson JE, Markin C, Moore PE, Worrell JA, Stahlman MT, Phillips JA III, Loyd JE, Cogan JD, Lawson WE. Identification of early interstitial lung disease in an individual with genetic variations in ABCA3 and SFTPC. *Chest.* 2010; 137:969–973. [PubMed: 20371530]
 18. Tredano M, Griesse M, Brasch F, Schumacher S, de Blic J, Marque S, Houdayer C, Elion J, Couderc R, Bahuau M. Mutation of SFTPC in infantile pulmonary alveolar proteinosis with or without fibrosing lung disease. *Am J Med Genet A.* 2004; 126A:18–26. [PubMed: 15039969]
 19. Galetskiy D, Woischnik M, Ripper J, Griesse M, Przybylski M. Aberrant processing forms of lung surfactant proteins SP-B and SP-C revealed by high-resolution mass spectrometry. *Eur J Mass Spectrom (Chichester, Eng).* 2008; 14:379–390.
 20. Abou TR, Jaubert F, Emond S, Le Bourgeois M, Epaud R, Karila C, Feldmann D, Scheinmann P, de Blic J. Familial interstitial disease with I73T mutation: A mid- and long-term study. *Pediatr Pulmonol.* 2009; 44:167–175. [PubMed: 19148933]
 21. Lin Y, Zhang M, Barnes PF. Chemokine production by a human alveolar epithelial cell line in response to *Mycobacterium tuberculosis*. *Infect Immun.* 1998; 66:1121–1126. [PubMed: 9488404]
 22. Heerlein K, Schulze A, Hotz L, Bartsch P, Mairbaurl H. Hypoxia decreases cellular ATP demand and inhibits mitochondrial respiration of A549 cells. *Am J Respir Cell Mol Biol.* 2005; 32:44–51. [PubMed: 15388515]
 23. Beers MF, Lomax CA, Russo SJ. Synthetic processing of surfactant protein C by alveolar epithelial cells. The COOH terminus of proSP-C is required for post-translational targeting and proteolysis. *J Biol Chem.* 1998; 273:15287–15293. [PubMed: 9614145]
 24. Wang WJ, Russo SJ, Mulugeta S, Beers MF. Biosynthesis of Surfactant Protein C (SP-C). Sorting of SP-C proprotein involves homomeric association via a signal anchor domain. *J Biol Chem.* 2002; 277:19929–19937. [PubMed: 11907042]

25. Yamano G, Funahashi H, Kawanami O, Zhao LX, Ban N, Uchida Y, Morohoshi T, Ogawa J, Shioda S, Inagaki N. ABCA3 is a lamellar body membrane protein in human lung alveolar type II cells. *FEBS Lett.* 2001; 508:221–225. [PubMed: 11718719]
26. Mulugeta S, Gray JM, Notarfrancesco KL, Gonzales LW, Koval M, Feinstein SI, Ballard PL, Fisher AB, Shuman H. Identification of LBM180, a lamellar body limiting membrane protein of alveolar type II cells, as the ABC transporter protein ABCA3. *J Biol Chem.* 2002; 277:22147–22155. [PubMed: 11940594]
27. Cheong N, Madesh M, Gonzales LW, Zhao M, Yu K, Ballard PL, Shuman H. Functional and trafficking defects in ATP binding cassette A3 mutants associated with respiratory distress syndrome. *J Biol Chem.* 2006; 281:9791–9800. [PubMed: 16415354]
28. Woischnik M, Sparr C, Kern S, Thurm T, Hector A, Hartl D, Liebisch G, Mulugeta S, Beers MF, Schmitz G, Griesse M. A non-BRICHOS surfactant protein c mutation disrupts epithelial cell function and intercellular signaling. *BMC Cell Biol.* 2010; 11:88. [PubMed: 21092132]
29. Beers MF, Lomax CA, Russo SJ. Synthetic processing of surfactant protein C by alveolar epithelial cells. The COOH terminus of proSP-C is required for post-translational targeting and proteolysis. *J Biol Chem.* 1998; 273:15287–15293. [PubMed: 9614145]
30. Pavelka, M.; Luini, A.; Mironov, AA. Golgi apparatus and epithelial cell polarity. In: Mironov, AA.; Pavelka, M.; Luini, A., editors. *The Golgi Apparatus: State of the Art 110 Years After Camillo Golgi's Discovery*. Springer; New York: 2008. p. 563-575.
31. Mulugeta S, Beers MF. Processing of surfactant protein C requires a type II transmembrane topology directed by juxtamembrane positively charged residues. *J Biol Chem.* 2003
32. Noguee LM, Dunbar AE III, Wert SE, Askin F, Hamvas A, Whitsett JA. A mutation in the surfactant protein C gene associated with familial interstitial lung disease. *N Engl J Med.* 2001; 344:573–579. [PubMed: 11207353]
33. Sanchez-Pulido L, Devos D, Valencia A. BRICHOS: a conserved domain in proteins associated with dementia, respiratory distress and cancer. *Trends Biochem Sci.* 2002; 27:329–332. [PubMed: 12114016]
34. Stevens PA, Pettenazzo A, Brasch F, Mulugeta S, Baritussio A, Ochs M, Morrison L, Russo SJ, Beers MF. Nonspecific interstitial pneumonia, alveolar proteinosis, and abnormal proprotein trafficking resulting from a spontaneous mutation in the surfactant protein C gene. *Pediatr Res.* 2005; 57:89–98. [PubMed: 15557112]
35. Abou TR, Jaubert F, Emond S, Le Bourgeois M, Epaul R, Karila C, Feldmann D, Scheinmann P, de Blic J. Familial interstitial disease with I73T mutation: A mid- and long-term study. *Pediatr Pulmonol.* 2009; 44:167–175. [PubMed: 19148933]
36. Gadsby DC, Vergani P, Csanady L. The ABC protein turned chloride channel whose failure causes cystic fibrosis. *Nature.* 2006; 440:477–483. [PubMed: 16554808]
37. Wang WJ, Mulugeta S, Russo SJ, Beers MF. Deletion of exon 4 from human surfactant protein C results in aggresome formation and generation of a dominant negative. *J Cell Sci.* 2003; 116:683–692. [PubMed: 12538769]
38. Purdue PE, Takada Y, Danpure CJ. Identification of mutations associated with peroxisome-to-mitochondrion mistargeting of alanine/glyoxylate aminotransferase in primary hyperoxaluria type 1. *J Cell Biol.* 1990; 111:2341–2351. [PubMed: 1703535]
39. Wightman PJ, Santer R, Ribes A, Dougherty F, McGill N, Thorburn DR, FitzPatrick DR. MLYCD mutation analysis: evidence for protein mistargeting as a cause of MLYCD deficiency. *Hum Mutat.* 2003; 22:288–300. [PubMed: 12955715]
40. Levi M. Novel NaPi-2c mutations that cause mistargeting of NaPi-2c protein and uncoupling of Na-Pi cotransport cause HHRH. *Am J Physiol Renal Physiol.* 2008; 295:F369–F370. [PubMed: 18524854]
41. Konkright JJ, Bridges JP, Na CL, Voorhout WF, Trapnell B, Glasser SW, Weaver TE. Secretion of surfactant protein C, an integral membrane protein, requires the N-terminal propeptide. *J Biol Chem.* 2001; 276:14658–14664. [PubMed: 11278984]
42. Ban N, Matsumura Y, Sakai H, Takanezawa Y, Sasaki M, Arai H, Inagaki N. ABCA3 as a lipid transporter in pulmonary surfactant biogenesis. *J Biol Chem.* 2007

43. Bates SR, Tao JQ, Schaller S, Fisher AB, Shuman H. Lamellar body membrane turnover is stimulated by secretagogues. *Am J Physiol Lung Cell Mol Physiol.* 2000; 278:L443–L452. [PubMed: 10710515]
44. Weaver TE, Na CL, Stahlman M. Biogenesis of lamellar bodies, lysosome-related organelles involved in storage and secretion of pulmonary surfactant. *Semin Cell Dev Biol.* 2002; 13:263–270. [PubMed: 12243725]
45. Kitamura T, Uchida K, Tanaka N, Tsuchiya T, Watanabe J, Yamada Y, Hanaoka K, Seymour JF, Schoch OD, Doyle I, Inoue Y, Sakatani M, Kudoh S, Azuma A, Nukiwa T, Tomita T, Katagiri M, Fujita A, Kurashima A, Kanegasaki S, Nakata K. Serological diagnosis of idiopathic pulmonary alveolar proteinosis. *Am J Respir Crit Care Med.* 2000; 162:658–662. [PubMed: 10934102]
46. Trapnell BC, Whitsett JA. Gm-CSF regulates pulmonary surfactant homeostasis and alveolar macrophage-mediated innate host defense. *Annu Rev Physiol.* 2002; 64:775–802. [PubMed: 11826288]
47. Kitamura T, Tanaka N, Watanabe J, Uchida, Kanegasaki S, Yamada Y, Nakata K. Idiopathic pulmonary alveolar proteinosis as an autoimmune disease with neutralizing antibody against granulocyte/macrophage colony-stimulating factor. *J Exp Med.* 1999; 190:875–880. [PubMed: 10499925]
48. Fisher AB, Dodia C, Feinstein SI, Ho YS. Altered lung phospholipid metabolism in mice with targeted deletion of lysosomal-type phospholipase A2. *J Lipid Res.* 2005; 46:1248–1256. [PubMed: 15772425]
49. Bridges JP, Xu Y, Na CL, Wong HR, Weaver TE. Adaptation and increased susceptibility to infection associated with constitutive expression of misfolded SP-C. *J Cell Biol.* 2006; 172:395–407. [PubMed: 16449190]
50. Dong M, Bridges JP, Apsley K, Xu Y, Weaver TE. ERdj4 and ERdj5 are required for endoplasmic reticulum-associated protein degradation of misfolded surfactant protein C. *Mol Biol Cell.* 2008; 19:2620–2630. [PubMed: 18400946]
51. Wang M, Bridges JP, Na CL, Xu Y, Weaver TE. Meckel-Gruber syndrome protein MKS3 is required for endoplasmic reticulum-associated degradation of surfactant protein C. *J Biol Chem.* 2009; 284:33377–33383. [PubMed: 19815549]
52. Gonzales LW, Guttentag SH, Wade KC, Postle AD, Ballard PL. Differentiation of human pulmonary type II cells in vitro by glucocorticoid plus cAMP. *Am J Physiol Lung Cell Mol Physiol.* 2002; 283:L940–L951. [PubMed: 12376347]
53. Stockinger W, Sailer B, Strasser V, Recheis B, Fasching D, Kahr L, Schneider WJ, Nimpf J. The PX-domain protein SNX17 interacts with members of the LDL receptor family and modulates endocytosis of the LDL receptor. *EMBO J.* 2002; 21:4259–4267. [PubMed: 12169628]
54. Johnson AL, Braidotti P, Pietra GG, Russo SJ, Kabore A, Wang WJ, Beers MF. Post-translational processing of surfactant protein-C proprotein: targeting motifs in the NH(2)-terminal flanking domain are cleaved in late compartments. *Am J Respir Cell Mol Biol.* 2001; 24:253–263. [PubMed: 11245624]
55. Kabore AF, Wang WJ, Russo SJ, Beers MF. Biosynthesis of surfactant protein C: characterization of aggresome formation by EGFP chimeras containing propeptide mutants lacking conserved cysteine residues. *J Cell Sci.* 2001; 114:293–302. [PubMed: 11148131]
56. Manevich Y, Al Mehdi A, Muzykantov V, Fisher AB. Oxidative burst and NO generation as initial response to ischemia in flow-adapted endothelial cells. *Am J Physiol Heart Circ Physiol.* 2001; 280:H2126–H2135. [PubMed: 11299214]
57. Fisher AB, Dodia C. Role of acidic Ca²⁺-independent phospholipase A2 in synthesis of lung dipalmitoyl phosphatidylcholine. *Am J Physiol.* 1997; 272:L238–L243. [PubMed: 9124374]
58. Wissel H, Lehfeldt A, Klein P, Muller T, Stevens PA. Endocytosed SP-A and surfactant lipids are sorted to different organelles in rat type II pneumocytes. *Am J Physiol Lung Cell Mol Physiol.* 2001; 281:L345–L360. [PubMed: 11435209]
59. BLIGH EG, DYER WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* 1959; 37:911–917. [PubMed: 13671378]
60. Kruger NJ. The Bradford method for protein quantitation. *Methods Mol Biol.* 1994; 32:9–15. [PubMed: 7951753]

Figure 1.

Schematic representation of the hSP-C expression constructs used in this study is shown. Additional constructs including EGFP-C1 vector and ABCA3^{WT}•DsRed were used for negative and positive controls. Four proteolytic cleavages of SP-C propeptide at sites “a to d” occur in alveolar type II cells. In contrast, A549 cells cleave the propeptide only once at site “a”. The cytosolic (NH₂) and luminal (COOH) domains are indicated.

Figure 2. EGFP-hSP-C^{I73T} is trafficked to early endosome vesicles

(A and B) Representative epifluorescent images of A549 cells 24 hr following plasmid introduction of EGFP-hSP-C^{WT} (A) and EGFP-hSP-C^{I73T} (B) immunostained with Texas Red-conjugated CD63 and EEA1 antibodies, respectively. Solid boxes from the merged images are magnified to illustrate colocalization or non-colocalization (right panels). Bar, 5 μ m. (C and D) Analysis of the subcellular localization of EGFP-hSP-C isoforms by density gradient fractionation. 24 hr following EGFP-hSP-C^{WT} and EGFP-hSP-C^{I73T} transfection, A549 cells were harvested and normalized for equal protein concentration per sample. Post-nuclear supernatants were subsequently prepared and loaded on top of discontinuous sucrose gradient as described in Materials and Methods. Representative immunoblot for EGFP (C and D, top panels), LAMP 1 and EEA1 (C and D, bottom two panels) of fractionated samples and corresponding histograms of quantified bands are from at least 3 separate experiments. * $p < 0.005$

Figure 3. Time-dependent trafficking of EGFP•hSP-C^{I73T}

Representative epifluorescent images of A549 cells at different time intervals following concomitant expression of ABCA3^{WT}•DsRed with EGFP•hSP-C^{WT} (A) or EGFP•hSP-C^{I73T} (B) in cells transiently transfected using Lipofectamine transfection method. Times shown are 12 to 16 hr and 24 to 36 hr post-transfection. Bar, 5 μm. (C) Partial co-localization of EGFP•hSP-C^{I73T} in CD63+ vesicles following 36 hr expression. (D) Counts of vesicle co-expressing ABCA3^{WT}•DsRed and EGFP•hSP-C isoforms. Data were obtained from three separate experiments, each with counts of 50 -100 vesicles per cell in at least 100 transfected cells. *p < 0.001, **p < 0.01, ***p < 0.05

Figure 4. EGFP•hSP-C^{I73T} is trafficked to the plasma membrane

(A) Representative immunoblot (using EGFP antibody) of whole cell lysates of non-transfected (Blank), vector transfected (EGFP-C1), or transfected with EGFP•hSP-C^{WT} or EGFP•hSP-C^{I73T}. The primary translation products (arrowhead) and processed products (bottom bands) of the hSP-C isoforms are shown. Two aberrantly processed products in EGFP•hSP-C^{I73T} cell lysates (arrows) are apparent. β -actin bands were used to assess equal loading. (B) Representative epifluorescent images of A549 cells following an overnight expression of either EGFP•hSP-C^{WT} (top panel) or EGFP•hSP-C^{I73T} (bottom panel) and cell surface labeling of plasma membrane proteins with biotin/streptavidin complex at 4°C according to protocols described in Materials and Methods. The complex was visualized using Texas Red-conjugated streptavidine antibody. Bar, 5 μ m. (C and D) A549 cells grown in T75 flask were transiently transfected and cell surface labeled with biotin/streptavidin as in (B). (C) Representative immunoblot (using anti-EGFP) of plasma membrane-bound proteins following immunoprecipitation using anti-streptavidin. Unlike EGFP•hSP-C^{WT}, the aberrantly processed products (arrows) in addition to the primary translational product (arrowhead) of the EGFP•hSP-C^{I73T} protein were preferentially localized at the plasma membrane. Biotinylated proteins were normalized by loading equal concentration of each protein sample (Bradford method (60)). (D) Band intensities of plasma membrane expressed EGFP-tagged total SP-C products were quantified and results from at least three separate experiments are shown. * $p < 0.001$

Figure 5. Time-dependent colocalization of EGFP•hSP-C^{I73T} and transferrin at the plasma membrane and early endosomal vesicles

Representative epifluorescent images of live A549 cells following 24 hr expression of wild type and mutant EGFP-tagged hSP-C^{WT} (A) and hSP-C^{I73T} (B) and incubation with Alexa Fluor 594-labeled transferrin at 4°C for 1 hr and fixed (top rows) or transferred and incubated at 37°C for 5 min (middle rows) and 1 hr (bottom rows). Plasma membrane co-localizations of EGFP•hSP-C^{I73T} with transferrin (arrows) followed by co-localization of

the two proteins initially confined in vesicles proximal to the plasma membrane (arrowheads) after a 5 min incubation at 37°C (B, merged middle image), and their subsequent distribution throughout the cell (B, merged bottom image) after one hour incubation at 37°C are shown.

Figure 6. EGFP•hSP-C^{I73T} is initially trafficked to the plasma membrane via a non-endosomal pathway and subsequently internalized

(A) Representative confocal fluorescent images of permeabilized and labeled with anti M6PR (left panel), or non-permeabilized and labeled with WGA (right panel) A549 cells expressing EGFP•hSP-C^{I73T} 6, 12, 16, 20, and 24 hr following transfection. Bar, 5 μ m. Due to low level of fluorescent expression, visualization at 6 and 12 hr post transfection was accomplished by significantly increasing confocal device settings (“iris size”, “gain”, and “offset” was increased up to 4, 4, and 3 times above normal, respectively). (B) Cell count of expressed EGFP•hSP-C^{I73T} colocalized with markers. For M6PR, quantitation was performed by counting cells that showed colocalization of M6PR in at least 3 EGFP•hSP-C^{I73T} containing vesicles. At least 50 cells per marker from 2 separate experiments were counted. * $p < 0.05$, ** $p < 0.01$. (C) Representative confocal fluorescent images of live A549 cells following 24 hr expression of wild type and mutant EGFP-tagged hSP-C^{WT} (top row) and hSP-C^{I73T} (bottom two rows) and incubation with epitope specific C-terminal proSP-C antibody at 4°C for 1 hr and fixed (middle row) or transferred and incubated at 37°C for 30 min (bottom row). Solid boxes from the merged images are magnified to illustrate localization or non-localization (right columns). Membrane localizations of EGFP•hSP-C^{I73T} (arrowheads) followed by internalization in punctate vesicles (arrows) after 30 min incubation at 37°C are shown. Images are representative of at list three separate experiments. Bars, 5 μ m.

Figure 7. Sorting of hSP-C^{I73T} in primary human alveolar type II cells is similar to epithelial cell lines

Representative confocal images, from at least three separate experiments, of primary alveolar type II cells isolated from human fetal lung tissues transiently expressing EGFP-tagged wild type (left panel) or mutant I73T (right panel) SP-C isoforms immunostained with Texas Red-tagged subcellular markers. Both isoforms were found in compartments containing the lysosome/lamellar body markers DC-lamp (top row) and CD63 (second row) (depicted in yellow merged images). Bottom two rows, co-localization of mutant I73T isoform with early endosome and plasma membrane markers EEA1 (third row) and Ep-CAM (bottom row), but not with wild type SP-C. Solid boxes from the merged images are magnified to illustrate colocalization or non-colocalization (right columns). Distinct plasma membrane localization of the mutant isoform is apparent (second row, arrowhead and bottom row, arrow). Bar, 5 μ m.

Figure 8. Lipid uptake and degradation are impaired by hSP-C^{I73T} expression

(A) Representative confocal fluorescent images of non-permeabilized HEK293 cells stably expressing EGFP-tagged wild type (top row) or I73T (bottom row) isoforms of SP-C labeled with the plasma membrane marker, WGA. Plasma membrane colocalization of the SP-C^{I73T} with WGA is apparent with merged (yellow) images (bottom right). (B) Representative anti-GFP immunoblot bands (top panel) and histogram of quantified bands (bottom panel) of HEK 293 whole cell lysate samples stably expressing either EGFP•hSP-C^{WT} (middle lane) or EGFP•hSP-C^{I73T} (right lane) are displayed to demonstrate equal expression of SP-C isoforms. Non-transfected HEK cells were used as control (left lanes). β-Actin was used to normalize for equal loading. (C and D) Histograms of radioactivity measured as disintegration per minute (DPM) of DPPC uptake per 0.5 million cells (C) and % DPPC degradation (D) in HEK293 cells treated with H³-DPPC-containing liposomes stably

expressing EGFP•hSP-C^{WT} (middle lane) or EGFP•hSP-C^{I73T} (right lane). Data were obtained from at least three separate experiments for (A) and (B), and at least seven separate experiments for (C) and (D) each consisting of at least 2 culture dishes per construct. * $p < 0.01$; ** $p < 0.05$.

Figure 9. Model for aberrant hSP-C^{I73T} trafficking

Schematic representation depicting normal and abnormal sorting of wild type and mutant isoforms of SP-C. The hSP-C^{WT} gene/cDNA is transcribed into a 0.9 kb mRNA product which is translated to a 197 amino acid (21 kDa) proprotein. Proteolytic cleavages occur during transit through the secretory pathway that include small vesicles, multivesicular bodies, and lysosome-like vesicles (LLV). In contrast, hSP-C^{I73T} is misdirected to the plasma membrane via a non-endosomal and likely through the constitutive pathway where it is subsequently internalized and sorted to early endosomes and LLV. N and C depict the N- and C-terminus of the proprotein. Hatched arrows imply pathways that may require multiple steps to reach target organelles. (Inset) Topological representation of the bitopic proSP-C with type II orientation. Mature SP-C (blue), cleavable segments of proSP-C (green), Juxtamembrane palmitoylation sites (arrow), and a C-terminal disulfide-bonding site (arrowhead) are shown.