Published in final edited form as:

Int J Biochem Cell Biol. 2012 January ; 44(1): 101–112. doi:10.1016/j.biocel.2011.10.003.

MULTIPLE WAYS TO DIE: DELINEATION OF THE UNFOLDED PROTEIN RESPONSE AND APOPTOSIS INDUCED BY SURFACTANT PROTEIN C BRICHOS MUTANTS

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Abstract

Epithelial cell dysfunction is now recognized as an important mechanism in the pathogenesis of interstitial lung diseases. Surfactant Protein C (SP-C), an alveolar type II cell specific protein, has contributed to this concept with the observation that heterozygous expression of *SFTPC* gene mutations are associated with chronic interstitial lung disease. We have shown that transient expression of aggregation prone mutant SP-C isoforms (SP-C BRICHOS) destabilizes ER quality control mechanisms resulting in the intracellular accumulation of aggregating propeptide, inhibition of the ubiquitin/proteasome system, and activation of apoptosis. The goal of the present study was to define signaling pathways linking the unfolded protein response (UPR) and subsequent ER stress with intrinsic apoptosis events observed following mutant SP-C expression. *In vitro* expression of the SP-C BRICHOS mutant, SP-C^{Δexon4}, was used as a model system. Here we show stimulation of a broad ER stress response in both transfected A549 and HEK293 cells with activation of all 3 canonical sensing pathways, IRE1/XBP-1, ATF6, and PERK/eIF2α. SP-C^{Δexon4} expression also resulted in activation of caspase 3 but failed to stimulate expression of the apoptosis mediating transcription factors ATF4/CHOP. However, inhibition of either caspase 4 or c-jun kinase (JNK) each blocked caspase 3 mediated cell death. Taken together, these results suggest that expression of SP-C BRICHOS mutants induce apoptosis via activation multiple CHOP independent but specific UPR signaling pathways, and provide new therapeutic targets for the amelioration of ER stress induced cytotoxicity observed in fibrotic lung remodeling.

Keywords

BRICHOS Family; Unfolded Protein Response; ER stress; Apoptosis; Interstitial Lung Disease

1. INTRODUCTION

Idiopathic pulmonary fibrosis (IPF), and the associated family of interstitial pneumonias represent a group of devastating interstitial lung diseases (ILD) of unknown etiology. Recently, in addition to cytokine / chemokine network imbalance, effector cell activation,

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and myofibroblast infiltration, the concept of abnormal wound healing has emerged as a cornerstone in the pathophysiology of ILD (Selman et al., 2001; Selman et al., 2004; Thannickal et al., 2004). However, the mechanisms promoting epithelial cell dysfunction and linking it with fibroproliferative lung remodeling are incompletely defined. Surfactant protein C (SP-C), an alveolar type II (AT2) epithelial cell-specific protein, has provided an important clue to ILD pathogenesis, as multiple reports have described over 20 different mutations in the *SFTPC* gene associated with ILD in both adult and pediatric patients (Nogee et al., 2001; Nogee et al., 2002; Thomas et al., 2002).

SP-C, which co-isolates with a phospholipid-rich fraction of bronchoalveolar lavage, imparts important biophysical stability to the extracellular surfactant monolayer (Clements, 1957; Rooney et al., 1994). This protein is synthesized in AT2 cells as a 21 kDa precursor (proSP-C) that undergoes a number of posttranslational processing steps, including palmitoylation and four sequential proteolytic cleavages. Processing is dependent upon 1) successful transit through the regulated secretory pathway, 2) cytosolic targeting motifs within the NH₂ terminus of the primary translation product (Johnson et al., 2001; Russo et al., 1999), and 3) progressive acidification of multivesicular, composite, and lamellar bodies. These events ultimately result in generation of the 3.7 kDa mature product that accumulates in lamellar bodies where it is secreted along with surfactant protein B (SP-B) and phospholipids in a regulated fashion (Beers, 1998; Beers and Lomax, 1995; Vorbroker et al., 1995; Weaver, 1998). Although incompletely processed, when the wild-type isoform is transfected into non-lamellar body containing epithelial cell lines (A549, HEK293, MLE, CHO), the SP-C proprotein localizes to acidic, lysosomal-like organelles. Such models have been used as surrogates to characterize normal and abnormal SP-C biosynthesis.

Data has shown that *SFTPC* mutations result in two distinct protein expression phenotypes (mistargeted vs. aggregating), each capable of triggering a characteristic subset of cellular responses. The mistargeted proSP-C mutants disrupt the endosome/lysosome system through abnormal accumulation, producing both organellar dysfunction and cytotoxicity (Hawkins et al., 2011; Mulugeta et al., 2010). In contrast, missense and splicing mutations that reside within the *SFTPC* BRICHOS domain, a ~100 amino acid region in the distal COOH-terminal flanking peptide with homology to a number of proteins linked to familial neurodegenerative disease, produce proprotein misfolding and intracellular aggregate formation (Sanchez-Pulido et al., 2002). *In vitro*, transfection of two different SP-C BRICHOS mutants associated with ILD (SP-C^{Δexon4} and SP-C^{L188Q}) has been shown to induce the Unfolded Protein Response (UPR) in an attempt to resolve improper folding or promote protein degradation. Conversely, these mutants have also been shown to inhibit ERAD clearance pathways (specifically the Ubiquitin Proteasomal System) (Bridges et al., 2003; Mulugeta et al., 2007; Mulugeta et al., 2005), and to induce cell death through apoptosis (Meusser et al., 2005). Evidence of UPR activation and apoptosis has been found in the lungs of patients with both idiopathic IPF as well as IPF patients harboring *SFTPC* mutations {1878}.

The UPR is comprised of three component signaling cascades to lessen the protein burden in the ER, and upregulate chaperone production. The upstream sensors of misfolding include the ER localized IRE1 (insulin response element 1), PERK (pancreatic ER kinase), and ATF6 (activating transcription factor 6) proteins, which remain “quiescent” under non-stress conditions through association with the signaling molecule, GRP78/BiP. In the setting of intraluminal accumulation of misfolded protein, BiP dissociates from these sensors, thereby allowing the UPR to activate (Bertolotti et al., 2000). The contribution of each of these pathways to UPR signaling may be cell-type specific and is unknown for SP-C BRICHOS mutants. The present study mechanistically extends our previous observations by undertaking the systematic identification of specific downstream UPR signaling pathways

that are activated by expression of mutant SP-C, and their interface with the intrinsic apoptosis pathways. Using SP-C^{Δexon4} as a model, results presented in this study demonstrate that SP-C BRICHOS expression induces a broad based ER stress response in epithelial cells and that the resultant apoptosis that occurs is independent of the traditionally recognized ATF4/CHOP pathway, but is dependent upon both caspase 4 activation and JNK signaling.

2. MATERIALS AND METHODS

2.1. Reagents

Generation of EGFP tagged human SP-C isoforms was done using a full-length human SP-C cDNA insert of 875 bp corresponding to the published sequence of Warr, et al (Warr et al., 1987) (SP-C^{WT}) subcloned into pEGFP-C1 eukaryotic expression vector (Clontech, Inc. Palo Alto, CA, USA). In addition, the BRICHOS mutants (SP-C^{Δexon4} SP-C^{L188Q}) and a non-BRICHOS mutant (SP-C^{I73T}) generated by PCR from this backbone as previously described (Mulugeta et al., 2007; Wang et al., 2002; Wang et al., 2003) were subcloned into the pEGFP-C1 vector. Human SP-C^{WT}, SP-C^{Δexon4} and SP-C^{L188Q} in DSRed-C1 vector (Clontech, Inc. Palo Alto, CA, USA) were created using the EGFP tagged wild type and mutant proSP-C plasmids as templates and a single PCR reaction with two primers as previously published (Mulugeta et al., 2007).

The ERA1-ΔDBD (XBP1/ΔDBD-Venus) plasmid for detection of IRE1 activation was the generous gift of Dr. Masayuki Miura of the University of Tokyo (Japan). Tissue culture medium was produced by the Cell Center Facility, University of Pennsylvania. Precast gels and buffers for SDS/PAGE (Novex®) were obtained from Invitrogen, Inc. (San Diego, CA). 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).

2.2. Cell Lines

Human lung epithelial (A549) and embryonic kidney epithelial (HEK293) cell lines were grown as monolayers in DMEM supplemented with 2mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum at 37°C, in humidified air equilibrated with 5% CO₂. Human fetal lung epithelial cells were prepared as described (Gonzales LW et al., 2002), and cultured in 10 nM dexamethasone, 0.1 mM 8-Br-cAMP, and 0.1 mM isobutylmethylxanthine to induce and maintain AT2 cell differentiation.

2.3. Cell Transfection and Pharmacologic Treatments

A549 or HEK293 cells grown to 75% confluence were transiently transfected with the indicated plasmid constructs using either CaPO₄ precipitation or Lipofectamine 2000 reagent (Invitrogen, San Diego, CA) as previously described (Maguire et al., 2011). Human lung epithelial cells were transfected using the Amaxa Mammalian Epithelial Cell Nucleofector solution and Amaxa program M05 as recommended by the manufacturer. Consistent with our previously published results (Mulugeta et al., 2007; Mulugeta et al., 2005; Wang et al., 2002; Wang et al., 2003), using either Western blotting for protein expression or cell counting for GFP positive cells, transfection efficiencies of the wild-type and mutant SP-C isoforms were nearly equivalent in both HEK and A549 cells. Where indicated, positive controls were generated by treatment of non-transfected cells 15 hours prior to harvest with ER stress inducers Thapsigargin (2 μM) or Tunicamycin (20 μg/ml). Pharmacologic treatment with a caspase 4 specific inhibitor (LEVD-CHO, Calbiochem, Gibbstown, NJ), or the JNK inhibitor (SP600125, St. Louis, MO) was carried out for 18 hours at the indicated concentrations.

2.4. Cell harvesting and mRNA isolation

At the indicated time points, cells were washed with ice-cold BSS, scraped, and pelleted by centrifugation at 450×g for 5 minutes at 4° C. The pellet was washed three times with BSS and suspended in cell lysis buffer (1% Triton X-100, 150mM NaCl, 25mM Tris Cl, pH 7.4) containing protease inhibitors (pepstatin (10µg/ml), leupeptin (10µg/ml), aprotonin (1µg/ml), and phenylmethylsulfonylfluoride (1mM)). The nuclei were pelleted by centrifugation at 12,000×g for 5 minutes and the protein content of the resulting supernatant (lysates) was determined by the method of Lowry using bovine serum albumin as a standard (Lowry et al., 1951). Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

2.5. Semiquantitative rtPCR

Total RNA was subjected to reverse transcription (RT) using the Ambion RETROscript Kit. Each RT reaction contained 2µg of RNA, and was carried out under the following conditions: heat denaturation at 80°C for 3 min, amplification for 1 hour at 44°C, then heat inactivation at 92°C for 10 minutes. The resultant cDNA's were subjected to PCR using the Promega PCR Master Mix. Each reaction contained approximately 250 ng of cDNA template. DNA amplification was carried out as follows: denaturation at 94°C for 3 min, followed by the specified number of cycles at 94°C for 30 sec, 62°C for 45 sec, and 72°C for 1 min. The reactions were then incubated at 72°C for 10 min to increase the yield of amplification.

The CHOP cDNA was amplified with the following primers: 5'-GCCAAAATCAGAGCTGGAACCT-3' (forward) and 5'-ACAGTGTCCCGAAGGAGAAAGG-3' (reverse) using 22 cycles. ATF4 cDNA was amplified with the following primers: 5'-TTCCTGAGCAGCGAGGTGTG-3' (forward) and 5'-TCCAATCTGTCCCGAGAAGG-3' (reverse) using 22 cycles. HERPUD1 gene expression was detected with the following primers: 5'-CTACTCCTCCCTGAGCAGATTC-3' (forward) and 5'-GGTTGGGGTCTTCAGTTTCAGG-3' (reverse) using 25 cycles. The β -Actin gene was amplified with the following primers: 5'-TGGGTCAGAAGGATTCCTATGT-3' (forward) and 5'-CAGCCTGGATAGCAACGTACA-3' (reverse) using 22 cycles. The resulting bands were resolved in 1% agarose gels and quantified by densitometry. Data from each gene was normalized to β -Actin.

2.6. Western Blotting

Proteins in cell lysates were separated by SDS-PAGE as described by Laemmli (Laemmli, 1970). Samples were run on a 4% stacking and 10% resolving gel, transferred to nitrocellulose (O/N, 20V), and incubated in 5% nonfat dry milk in BSS, 0.1% Tween20 at 4°C overnight. Incubations were carried out in either polyclonal antisera: anti-CHOP, anti-GRP78, anti-ATF-6a (Santa Cruz Biotech, Santa Cruz, CA), anti-Myc (Cell Signaling, Beverly, MA) or monoclonal antibodies: Anti-caspase 4 (Stressgen, Plymouth Meeting, PA), anti-eIF2 α /anti-phospho-eIF2 α (Ser51) (Cell Signaling Technologies), anti-HDJ2 (Neomarkers, Fremont, CA), and anti-GADD-153/CHOP (Santa Cruz). Bands were detected by chemiluminescence using Kodak X-Omat AR film. Specific band intensities were quantitated by densitometry using Kodak1 D software (Version 3.0).

2.7. Luciferase Reporter Gene Assays

Reporter systems were used to detect total UPR (p5×ATF6-GL3, single construct system) and ATF6 specific activation (GAL4-ATF6 and p5×GAL4-E1b-GL3, double construct system) (gift of Dr. Ron Prywes, Columbia University) (Shen and Prywes, 2005). The

plasmids were transiently introduced into A549 or HEK293 cells using the calcium phosphate precipitation transfection method. The pRL-SV40 *Renilla* luciferase vector was used to normalize transfection efficiencies. 48 hours post-transfection, cells were lysed and assayed for firefly and *Renilla* luciferase activities using the dual luciferase kit (Promega, Madison, WI).

2.8. Detection of Caspase-3 Activity

A Myc/FLAG-tagged human caspase 3 ORF construct was purchased from Origene (Rockville, MD) and transiently transfected into HEK293 cells. Lysates were analyzed for cleaved and uncleaved caspase 3 using an anti-Myc antibody (Cell Signaling).

Cells transfected with empty vector, SP-C^{WT} or mutant constructs for 72 hours were harvested by scraping and washed with cold PBS. Cell pellets were resuspended in Lysis Buffer (10mM HEPES, 0.1% CHAPS, 5mM DTT, 2mM EDTA, and 1mM PMSF, pH 7.4). 50µg of cell lysate was used with 25µM of caspase substrate (Ac-DEVD-AFC, caspase 3), and samples were incubated at 37°C for 1 hour in caspase assay buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 10mM DTT, 100µM EDTA, 10% glycerol, pH 7.4). Incubated samples were read at an excitation wavelength of 400nm, and an emission wavelength of 505nm in a multiwavelength excitation dual wavelength-emission fluorimeter (Delta RAM; Photon Technology International).

2.9. Detection of Cell Death

The Cell Death Detection ELISA^{PLUS} photometric enzyme immunoassay (Roche, Branchburg, NJ) was used to quantitate *in vitro* cytoplasmic histone-associated DNA fragments. A549 and HEK293 cells were transiently transfected for 48 hours with the indicated DSRed-tagged SP-C constructs. Negative and positive controls included untransfected cells and overnight treatment of cells with 25 ng/ml TNFα and 25µg/ml cycloheximide, respectively. Cells were harvested and assayed according to the manufacturer's protocol.

2.10. Statistical Analysis

Parametric data were analyzed with Instat version 3.0 (Graph Pad Software, Inc., La Jolla, CA), and was expressed as mean ± standard deviation ($X \pm SD$), and experimental groups compared utilizing *a*) unpaired 2-tailed Student's *t* test for comparing two samples, or *b*) analysis of variance between sample groups using the Tukey-Kramer multiple comparison post test. Significance was accepted at $p < 0.05$.

3. RESULTS

3.1. Expression of GFP-SPC^{Δexon4} results in a generalized induction of the UPR

The response of the ER to mutant or increased protein load can be sensed by a number of distinct transducers, including GRP78, IRE1, PERK, and ATF6. In order to assess generalized UPR induction, GRP78 protein expression was determined using Western blotting with a GRP specific polyclonal antibody. Treatment with thapsigargin served as a positive control for UPR induction. Expression of the SPC^{Δexon4} mutant resulted in significant elevation of GRP78 compared to SP-C^{WT} (Figure 1A–1B) in both A549 and HEK293 cell lines. Similar results were found for the expression of another general ER stress product, HERPUD1 (data not shown).

General UPR activation was confirmed using a single construct reporter system (p5×ATF6-GL3 (Shen and Prywes, 2005)) in which luciferase gene transcription driven by five tandem repeats of a ATF6/XBP-1 consensus binding sequence within the construct promoter can be

triggered by binding of either one or both of the endogenous UPR transcription factors, ATF6 or IRE1 derived XBP-1 (Mori, 2000; Shen and Prywes, 2005; Yoshida et al., 2001). When A549 or HEK293 cells were transiently transfected with the p5×ATF6-GL3 reporter, each cell line exhibited a 2–3 fold increase in ATF6/XBP-1-driven luciferase activity upon co-transfection with SP-C^{Δexon4} when compared to control transfections with either EGFP-C1 (A549 cells) or SP-C^{WT} (A549 and HEK293) (Figure 1C–1D). Taken together, these results indicate that expression of the SPC^{Δexon4} mutant elicits a wide-ranging UPR *in vitro*.

3.2. The IRE1 signaling pathway is specifically activated upon SPC^{Δexon4} expression

The IRE1 / XBP-1 pathway has been closely linked with ER stress induced inflammatory signaling (Urano et al., 2000), and with apoptosis (Groenendyk and Michalak, 2005). Using splicing and expression of XBP-1 protein as a surrogate, functional IRE-1 activity in living cells was assessed using the ERA1 expression plasmid developed by Miaura (Iwawaki et al., 2004). ERA1 contains an insert encoding for a fusion protein of XBP-1 and *Venus*, a GFP-like reporter protein. Since XBP-1 cDNA contains a stop codon which normally prevents full read-through, IRE-1 dimerization initiated at the onset of the UPR results in splicing by IRE-1 endoribonuclease activity and expression of *Venus*. As shown in Figure 2A–B, when co-transfected with HA-SP-C constructs, only cells expressing HA-SP-C^{Δexon4} exhibited *Venus* fluorescence in a nuclear pattern characteristic of successful transcription, translation and nuclear translocation of XBP-1.

3.3. The ATF6 signaling pathway is specifically activated upon SP-C^{Δexon4} expression

In order to identify a role for ATF6 in SP-C^{Δexon4} UPR induction, a double construct reporter system developed by Prywes (Shen et al., 2002) was utilized to assess specific ATF6-p50 nuclear translocation. A549 and HEK293 cells were transiently co-transfected with the p5×GAL4-E1b-GL3 and GAL4-ATF6 constructs, the indicated SP-C construct, and a *Renilla* luciferase plasmid, pRL-SV40, for normalization of transfection efficiency. The first construct contains the ATF6 sequence Terminally fused to GAL4. The second construct contains 5 tandem repeats of the GAL4 DNA Binding Site (DBS) tagged with firefly luciferase. Upon cleavage of ATF6 and translocation to the nucleus, GAL4 is able to bind to the reporter construct and upregulate luciferase transcription. As illustrated in Figure 3A–B, when compared to either EGFP or EGFP-SP-C^{WT}, expression of EGFP-SP-C^{Δexon4} incurred a significant increase in ATF6-driven luciferase activity in both cell lines.

3.4. The PERK-eIF2α pathway is upregulated by expression of SP-C^{Δexon4}

Cell survival during times of UPR and subsequent ER stress is promoted through the protein kinase PERK (Ron, 2002). Upon its homodimerization and autophosphorylation, an active PERK phosphorylates eukaryotic translation initiation factor 2 (eIF2α), inhibiting its ability to bind to the translation machinery, and thereby attenuating protein synthesis. To evaluate the participation of the PERK-eIF2α pathway in SP-C^{Δexon4} induced UPR, cell lysates were probed with a phospho-specific eIF2α antibody. 48 hours after transient expression of the SP-C^{Δexon4} mutant, a significant increase in eIF2α phosphorylation was observed in both A549 and HEK293 cells (Figure 4A–B).

3.5. SP-C^{Δexon4} Induces Apoptosis

Taken together, expression of the SP-C^{Δexon4} folding mutant results in sustained activation of all three arms of the UPR stress response, IRE1, PERK, and ATF6, for at least 48 hours. Prolonged upregulation of the aforementioned UPR components can also trigger apoptosis. In order to identify this transition, Annexin V labeling of the plasma membrane (indicative of inner to outer leaflet translocation of plasma membrane phosphatidylserine) was assessed in cells expressing SP-C wild-type and BRICHOS mutants. As Figure 5A demonstrates,

A549 cells expressing the EGFP-SP-C^{Δexon4} mutant exhibited positive Annexin V labeling suggesting apoptotic induction, which was not observed when EGFP-SP-C^{WT} was substituted. All cells were negative for propidium iodide staining (data not shown).

As further confirmation of programmed cell death, caspase 3 activation was determined in both A549 and HEK cells expressing the SP-C^{Δexon4} mutant. A549 cells show significantly elevated amounts of caspase 3 activity, determined through a caspase specific colorimetric assay, when compared to empty vector and SP-C^{WT} expressing cells (Figure 5B). HEK293 cells transiently transfected with a myc-tagged caspase 3 ORF construct and the mutant SP-C BRICHOS constructs (SP-C^{Δexon4} and SP-C^{L188Q}) showed significantly greater caspase 3 cleavage than the SP-C^{WT} control (Figure 5C).

3.6. A TF4 and CHOP are not involved in SP-C^{Δexon4} mediated apoptosis induction

In some model systems, activation of PERK and phosphorylation of eIF2 α attenuates translation of most genes, but leads to preferential translation of the transcription factor ATF4 (Harding et al., 2000). Although important as a mediator of cell recovery from mutant protein expression, ATF4 in conjunction with ATF6, can upregulate the proapoptotic transcription factor, CHOP (Ma et al., 2002). To determine if the previously documented SP-C^{Δexon4} induced caspase 3-dependent cell death (Mulugeta et al., 2005) occurs through a CHOP-dependent pathway, activation of both ATF4 and CHOP transcription factors were evaluated. Semiquantitative PCR using human specific ATF4 primers failed to demonstrate a significant change in SP-C^{Δexon4} ATF4 mRNA levels compared to nontransfected or SP-C^{WT} A549 and HEK293 controls (Figure 6A–B). CHOP mRNA and protein levels were measured in SP-C mutant and wild-type expressing A549 and HEK293 cells. Accordingly, no increases in CHOP message or protein levels were seen in SP-C^{Δexon4} expressing cells when compared to EGFP or SP-C^{WT} controls (Figure 7A–B), suggesting that SP-C^{Δexon4}-mediated cell death occurs through a CHOP independent pathway. This data was corroborated in human primary AT2 cells, which also fail to upregulate CHOP upon SP-C mutant expression (Figure 7C).

3.7. SP-C^{Δexon4} Induces apoptosis through a Caspase 4 and JNK-dependent Mechanism

Apart from ATF4 / CHOP signaling, activation of ER stress-induced apoptotic cell death could involve a number of other signaling pathways, including recruitment of TRAF2 by IRE1, resulting in release and activation of caspase 4/12. We have previously shown that caspase 4 is cleaved upon expression of the SP-C^{Δexon4} mutation (Mulugeta et al., 2007). Binding of TRAF2 to IRE1 is also known to induce downstream activation of the JNK (Leppa and Bohmann, 1999). JNK pathway activation by SP-C BRICHOS mutants was assessed in both A549 and HEK293 cells. As Figure 8A–B illustrates, when compared to SP-C^{WT}, SP-C^{Δexon4} expression significantly increased phosphorylation of p46 JNK in A549 cells, and both the p46 and p54 isoforms in HEK293 cells.

The role of JNK and caspase 4 signaling in apoptosis induced by SP-C BRICHOS mutants was assessed using caspase 3 activation as a readout for the execution-phase of cell apoptosis. In order to assess cleavage and activation of caspase 3, the previously described Myc-tagged human ORF construct was transiently transfected into HEK293 cells, along with the indicated SP-C construct. Transfected cells were then treated with a caspase 4 inhibitor (LEVD-CHO), a JNK inhibitor (SP600125), or a combination of both. Western blotting with an anti-Myc antibody revealed that both SP-C BRICHOS mutants (Δ exon4 and L188Q) induce caspase 3 cleavage, and treatment with caspase 4 and JNK inhibitors significantly diminish this activation (Figure 9). Cell lysates harvested from transiently transfected A549 and HEK293 cells, and treated with the caspase 4 and JNK inhibitors were also assayed for DNA fragmentation using a Cell Death ELISA kit (Suppl. Fig. 1). While

the SP-C^{Δexon4} mutant induced DNA fragmentation (an additional surrogate of apoptotic cell death), inhibition of either caspase 4 or JNK pathways was cytoprotective.

4. DISCUSSION

Mutant protein accumulation in the ER is contested by a complex counter-regulatory mechanism aimed at maintaining cellular homeostasis. Initially, the unfolded protein response attempts to adapt the cell to its increased load of ER client protein via translation attenuation and concomitant upregulation of ER folding machinery (Groenendyk and Michalak, 2005; Zhang and Kaufman, 2006), but if this ultimately fails, cell death via intrinsic apoptosis ensues (Groenendyk and Michalak, 2005). We have previously shown that expression of SP-C BRICHOS mutants triggers both protein aggregation and a UPR response, and have linked its prolonged activation to the induction of apoptosis. This response pattern has overlapping similarities to other “conformational diseases” produced by aggregation prone proteins including the polyglutamine repeat expansion of huntingtin associated with Huntington Disease (Rochet, 2007), accumulation of beta-amyloid and tau protein associated with Alzheimer’s Disease (Carrell and Lomas, 1997; Cohen and Kelly, 2003; Salminen et al., 2009), and hepatic toxicity from the α -1 antitrypsin Z mutants (Lomas, 2007). To precisely define underlying pathways responsible for lung epithelial cell dysfunction and death induced by SP-C mutants, the present study undertook a systematic identification of specific signaling events activated during the UPR, and assessed their role in mediating apoptosis in response to expression of a model SP-C BRICHOS protein, SPC^{Δexon4}. Our data reveal a role for both MAPK signaling as well as ER- based caspase 4 activation in this process.

Misfolded and aggregated proteins upregulate a number of signaling pathways that comprise the UPR, creating a quality control mechanism to prevent cytotoxicity. Although several UPR/ER stress related genes have been shown to be induced by mutant SP-C expression (Bridges et al., 2006; Lawson et al., 2008; Mulugeta et al., 2007; Mulugeta et al., 2005), our current data has further defined these events through the demonstration of a generalized UPR activation (Figure 1), mediated by all three canonical UPR stress pathways (Figures 2, 3, and 4). The ER sensing molecules PERK, ATF6, and IRE1 play essential roles in these pathways, acting to attenuate protein translation, stimulate the production of chaperones that aid in protein folding and trafficking, and to activate proteasomal components for protein degradation, respectively. In this study, the IRE1 pathway was identified through its effects on XBP-1 mRNA splicing (Calfon et al., 2002; Tirasophon et al., 2000; Yoshida et al., 2001) (Figure 2), the ATF6 pathway identified using ATF6-driven luciferase constructs (Haze et al., 1999; Wang et al., 2000) (Figure 3), and PERK activation as manifested by eIF2 α phosphorylation (Harding et al., 1999; Harding et al., 2000) (Figure 4).

Idiopathic pulmonary fibrosis, also known as cryptogenic fibrosing alveolitis, is a progressive interstitial lung disease of unknown etiology. In traditional models of IPF, toxins, infectious agents, or environmental antigens have each been suggested as triggers and presumed to interact with resident pulmonary immune cells to generate inflammation and subsequent lung remodeling by activated myofibroblasts. However, based on a variety of evidence, a resulting paradigm shift now suggests a central role for the alveolar (lung) epithelium in disease pathogenesis, whereby IPF is believed to result from repeated episodes of alveolar epithelial cell (AEC) injury in conjunction with release of pro-inflammatory and profibrotic mediators that lead to abnormal wound repair. Within this model, induction of ER Stress through expression of misfolded protein has been implicated as one potential mechanism for the observed AEC cell dysfunction. Since SP-C represents an AT2 cell specific gene, cases of IPF associated with SP-C mutations offer new, compelling evidence in support of this model. In addition to the *in vitro* data presented here demonstrating both a

general UPR response and specific UPR pathway activation, recent reports have described evidence of UPR activation / ER stress in patients with ILD *in vivo*. The expression of the SP-C BRICHOS mutant (SPC^{L188Q}) elicits prominent expression of UPR markers in alveolar epithelial cells of lung biopsies from affected patients, including BiP, EDEM, and XBP-1 (Lawson et al., 2008). Similar results are also seen in lung homogenates and AT2 epithelial cells of non-familial (sporadic) IPF lungs. Chronic ER stress was identified as an activator of ATF6, ATF4, and XBP-1, and suggests that chronic UPR signaling centered in lung epithelia from yet to be defined stimuli (e.g. other protein mutations [ABCA3 or SP-A (Maitra et al., 2010)], telomerase dysfunction (Armanios et al., 2007), or oxidant stress {1940}) is a common pathogenic mechanism in these patients.

Using two different endpoints, transient transfection of SP-C folding mutants was shown to produce apoptotic cell death (Figure 5). Since ER stress has been shown to be a major inducer of intrinsic (non-FAS ligand mediated) apoptosis, we sought to link these two observations using our model system. Four main intrinsic apoptosis pathways have been identified (Adams, 2003). Figure 10 illustrates the interactions of these pathways in inducing stress and apoptosis. The first being activation of ER associated caspase 4 (the human homologue of murine caspase 12 (Hitomi et al., 2004a)), secondly, initiation of a mitogen activated protein kinase (MAPK) cascade resulting in JNK phosphorylation, thirdly, promotion of mitochondrial release of cytochrome c through either ER stress dependent or independent mechanisms, and finally ATF4 mediated activation of the transcription factor CHOP (C/EBP homologous protein). Both JNK and caspase 4 are activated through the ER stress component pathway, IRE1. Under normal conditions, a complex is formed between IRE1, caspase 4, and TRAF2 at the ER membrane. ER stress promotes the release of caspase 4 from TRAF2, and the caspase is autoprocessed through homodimerization (Hitomi et al., 2004b; Nakagawa and Yuan, 2000; Yoneda et al., 2001). Similarly, the IRE1/TRAF2 complex promotes activation of ASK1 (apoptosis signal-regulating kinase 1), which activates JNK by phosphorylation (Nishitoh et al., 2002; Urano et al., 2000). We have previously demonstrated caspase 4 activation in response to expression of SP-C BRICHOS mutants (Maguire et al., 2011; Mulugeta et al., 2007). In addition, the ASK1/JNK pathway is also activated by expression of SPC^{Δexon4} in two different cell lines (Figure 8). Through the use of pharmacologic manipulation, our data suggests that both pathways contribute to the observed apoptosis. Chemical inhibition of both caspase 4 and JNK alone, or in combination, significantly blunted caspase 3 cleavage and cell death, but was not additive and did not completely abolish the signal (Figure 9 and Suppl. Fig. 1). The data suggest that these two IRE1 based pathways may be interdependent through a yet to be defined mechanism, but that other non-IRE1 based mechanisms may also contribute to the cytotoxicity.

We have previously shown that SP-C BRICHOS mutant expression prompts the cytosolic release of the key mitochondrial electron transport chain protein, cytochrome c (Mulugeta et al., 2007), which elicits formation of the apoptosome, in turn activating incorporated caspase 9, and ultimately leading to caspase 3 activation, DNA fragmentation and apoptosis (Crompton, 1999; Riedl S.J. and Salvesen G.S., 2007). In other model systems, permeabilization of the outer mitochondrial membrane can result from a variety of events including ER / calcium dependent *Apaf1* activity (Shiraishi et al., 2006), alterations in *Bax/Bcl2* balance (Nechushtan et al., 2001), or direct injury from protein aggregates. Thus, while it is likely that mitochondrial based signaling is contributing to the residual apoptosis signal observed in the mutant expressing cells treated with both caspase 4 and JNK inhibitors, further interrogation of these pathways will be required.

While IRE1 signaling and mitochondrial dysfunction could account for the majority of the apoptosis we observed, the transcriptional activation of CHOP, which functions as an

amplifier of the death pathway in other systems such as pancreatic islet cells (Oyadomari and Mori, 2004), did not appear to be directly involved in the cell death response to SP-C BRICHOS mutants in our model systems (Figure 7A, 7B). In contrast, signatures of CHOP expression have been demonstrated in homogenates and epithelial cells from sporadic IPF patient lung samples (Korfei et al., 2008), and in AT2 cells in Hermansky-Pudlak syndrome-associated interstitial pneumonia (Mahavadi et al., 2010). Although CHOP appears to be a key participant in certain apoptotic events, it has not been proven to be a universal trigger. CHOP is not upregulated in PERK^{-/-} and eIF2 α mutant cells after chemical induction of ER stress, yet cells treated in this fashion still undergo apoptosis (Harding et al., 2003; Scheuner et al., 2001). Given that transient transfection of SP-C BRICHOS mutants into primary human AT2 cells also failed to acutely induce CHOP upregulation (Figure 7C), it is unlikely that our findings are an artifact of transformed cell lines, but instead suggests that CHOP expression could be a relatively late epiphenomenon to fibrotic lung remodeling.

In summary, we have shown that expression of Surfactant Protein C mutants associated with human interstitial lung disease produce significant epithelial cell dysfunction through activation of multiple ER-stress associated signaling pathways that contribute to cell death, including both caspase 4 and JNK. In addition, our previous studies demonstrate that mitochondrial mediated (cytochrome c) events also appear to contribute to the observed epithelial cytotoxicity. Taken together, our findings lend additional support to the hypothesis that the pathogenesis of interstitial lung disease involves primary epithelial dysfunction and abnormal wound repair. Chronic lung injury / remodeling from expression of mutant *SFTPC* can be thought of as part of a unified view of the cellular and molecular pathogenesis of a larger family of conformational diseases. When this is combined with the complexity of AT2 cell biology and their central role as a progenitor cell in alveolar repair, the secondary disruption of cellular function or induction of cell death are now key components in ILD pathogenesis, and will permit improved recognition of new therapeutic strategies for interstitial lung disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by: NIH HL 19737 (MFB) and HL 090732 (SM); JAM received support from NIH 2T32 HL007748

ABBREVIATIONS

SP-C	Pulmonary Surfactant Protein C
EGFP	Enhanced Green Fluorescent Protein
ER	endoplasmic reticulum
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PBS	phosphate buffered saline
SDS	sodium dodecylsulfate
DMEM	Dulbecco's Modified Media

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A. A549

B. HEK293

C. A549

D. HEK293

Figure 1. SP-C Δ exon4 Induces an ER Stress Response

A549 (A) and HEK293 (B) cells were transiently transfected with the indicated control or EGFP-SP-C mutant constructs using the calcium phosphate transfection method. Overnight treatment with 2 μ M thapsigargin was used as a positive control. Cell lysates were analyzed for GRP78 protein expression using an anti-GRP78 polyclonal Ab. Anti- β -Actin was used as a load control. The ratio of mean GRP78 to β -Actin protein was determined, and expressed as fold change over the WT control. A549 (C) and HEK293 (D) cells were transiently transfected with a p5 $\times$ ATF6-GL3 ER stress reporter construct, the indicated control or SP-C construct, and pRL-SV40 for normalization of transfection efficiencies. Overnight treatment

with 2 μ M Thapsigargin was used as a positive control. Cells were harvested 48 hrs post-transfection, lysed with nonreducing lysis buffer, and Firefly or *Renilla* luciferase activities were measured using the Promega Dual Luciferase Kit. The ratio of mean Firefly to *Renilla* luciferase was determined and expressed as fold change over the WT control. Quantitation represents the mean $\pm$ S.D of three independent experiments. Significant differences are indicated by asterisks determined using the Tukey multiple comparison test. * $p < 0.05$

A. A549**B. HEK293**

Figure 2. SP-C^{Δexon4} Activates IRE1 UPR Signaling

A549 (A) and HEK293 (B) cells were transiently transfected with HA-tagged SP-C^{WT} or SP-C^{Δexon4} constructs, along with ERA1-ΔDBD plasmid (XBP1/ΔDBD-Venus) developed by Miaura (Iwawaki et al., 2004). 48 hours post-transfection, the cells were fixed and stained with an anti-HA monoclonal antibody to label the HA-SP-C fusion protein, as described in Methods. Fluorescence microscopy shows ERA1 expression in SP-C^{Δexon4} and not in SP-C^{WT} transfected cells.

Figure 3. SP-C Δ exon4 Activates the ATF6 UPR Pathway

A549 (**A**) and HEK293 (**B**) cells were transiently transfected with the indicated SP-C mutant construct, the GAL4-ATF6 and p5 $\times$ GAL4-E1b-GL3 luciferase reporter constructs (double construct system), and pRL-SV40 for normalization of transfection efficiencies. Overnight treatment with 2 μ M Thapsigargin was used as a positive control. Cells were harvested 48 hrs post-transfection, lysed with nonreducing lysis buffer, and Firefly or *Renilla* luciferase activities were measured using the Promega Dual Luciferase Kit. The ratio of mean Firefly to *Renilla* luciferase was determined and expressed as fold change over the WT control. Quantitation represents the mean $\pm$ S.D of three independent experiments. Significant differences are indicated by asterisks determined using the Tukey multiple comparison test. *p<0.05

A. A549 **B. HEK293** **Figure 4. SP-C^{Δexon4} activates PERK**

A549 (**A**) and HEK293 (**B**) cells were transiently transfected with the indicated control or EGFP-hSP-C mutant constructs using the calcium phosphate transfection method. 30 minute treatment with 1 μ M Thapsigargin was used as a positive control. Cell lysates were analyzed for phospho-eIF2 α and total eIF2 α protein expression using a phospho-specific eIF2 α and eIF2 α rabbit monoclonal antibodies, respectively. Anti- β -Actin was used as a load control. The ratio of mean phosphorylated eIF2 α /(total eIF2 α / β -Actin) protein was determined, and expressed as fold change over the WT control. Quantitation represents the mean $\pm$ S.D. of three independent experiments. Representative immunoblots appear above each graph. * $p < 0.05$.

A. A549 **B. A549** **C. HEK293** **Figure 5. SP-C^{Δexon4} induces Apoptotic Cell Death**

(A) A549 cells transiently transfected with the EGFP-hSP-C^{WT} or EGFP-hSP-C^{Δexon4} construct were fixed 72 hours after introduction of cDNA and immunostained with Alexa-647 tagged Annexin V. Plasma membrane phosphatidylserine binding to Annexin V was observed in SP-C^{Δexon4} expressing cells. Images represent data from at least three independent experiments.

(B) Parental A549 cells (untransfected = UT) or groups transiently expressing the indicated EGFP constructs were harvested 72 hours after introduction of plasmids. Overnight (18h) treatment of UT cells with 25 ng/ml TNFα was used as a positive control. Lysates were prepared as described and incubated with a caspase 3 substrate (Ac-DEVD-AFC). Caspase 3 activity was determined through colorimetric quantitation. Data represents the mean ± S.D. of three independent experiments. *p<0.05 vs. WT control, +p<0.001 vs. WT control.

(C) HEK293 cells were transiently transfected with a Myc-tagged human caspase 3 ORF construct (Origene) and the indicated EGFP-tagged SP-C construct. Overnight treatment with 25 ng/ml TNFα was used as a positive control. 48 hours post transfection, cells were harvested and lysates were analyzed for caspase 3 cleavage with an anti-myc antibody. Anti-β-Actin was used as a load control. Band intensities were quantified by densitometry, and the ratio of (cleaved caspase 3 / uncleaved caspase 3)/Actin was determined. Data represents the mean ± S.D. of three independent experiments. Representative immunoblots appear above each graph. *p<0.05 vs. WT control, +p<0.001 vs. WT control.

A. A549

B. HEK293

Figure 6. Apoptosis from SP-C^{Δexon4} is ATF4 Independent

A549 (A) and HEK293 (B) cells were transiently transfected with the indicated control or EGFP-hSP-C mutant constructs using the calcium phosphate transfection method. Overnight treatment with 2 μ M Thapsigargin was used as a positive control. Total RNA was isolated 48 hours post transfection using Trizol reagent, reverse transcribed, and analyzed with human ATF4 specific primers. Human β -Actin primers were used as a load control. The ratio of mean ATF4 to β -Actin was determined and expressed as fold change over the WT control. Quantitation represents the mean $\pm$ S.D. of three independent experiments. + $p < 0.05$.

A. **B.** **C.**

Figure 7. SP-C Δ exon4 Induces Apoptosis Through a CHOP Independent Pathway

A549 and HEK293 cells were transiently transfected with the indicated control or EGFP-hSP-C mutant constructs using the calcium phosphate transfection method. Cells were harvested 48 hours post-transfection, and lysates (**A**) or RNA (**B**) were analyzed for CHOP expression using an anti-GADD153/CHOP polyclonal antibody or human CHOP specific primers. (**C**) RNA was isolated from transiently transfected human primary AT2 cells and analyzed for CHOP expression. An anti- β -actin antibody or actin primers were used as a load control. Data represents results from three independent experiments.

A. A549 **B. HEK293**

Figure 8. SP-C BRICHOS Mutants Promote c-JUN N-terminal Kinase (JNK) Activation
 A549 (A) and HEK293 (B) cells were transiently transfected with the indicated EGFP-SP-C constructs and harvested 48 hours post-transfection. UV treatment (40 mJ/cm²) was used as a positive control. Cell lysates were analyzed for phospho-SAPK/JNK and total SAPK/JNK expression using monoclonal antibodies. An anti-β-actin antibody was used as a loading control. Band intensities were quantified by densitometry, and the ratio of Phospho-JNK to total JNK was determined. Data is expressed as fold change over wild type control, and represents the mean ± S.D. of three independent experiments. Representative immunoblots appear above each graph. *p<0.05 vs. WT control, +p<0.001 vs. WT control.

HEK293

Figure 9. Both JNK and Caspase 4 Contribute to Caspase 3 Dependent Cell Death

HEK293 cells were transiently transfected with a Myc-tagged human caspase 3 ORF construct (Origene), and the indicated EGFP-tagged SP-C construct. Cells were either untreated, treated with 5 μ M caspase 4 inhibitor (LEVD-CHO), or 10 μ M JNK inhibitor (SP600125) for ~18 hours. Overnight treatment with 25 ng/ml TNF α was used as a positive control. 48 hours post transfection, cells were harvested and lysates were analyzed for caspase 3 cleavage with an anti-myc antibody. Anti- β -Actin was used as a load control. Band intensities were quantified by densitometry, and the ratio of (cleaved caspase 3 / uncleaved caspase)/Actin was determined. Data represents the mean $\pm$ S.D. of three

independent experiments. Representative immunoblots appear above each graph. * $p < 0.05$ vs. WT control, + $p < 0.001$ vs. WT control.

Figure 10. ER Stress, Protein Aggregation, and Induction of Apoptosis

The presence of mutant SP-C protein in the ER induces the dissociation of BiP from three proximal sensors, IRE1, ATF6, and PERK to initiate transcription factor cascades for chaperone induction or translation attenuation. If uncorrected, SP-C misfolding sustains both a long-term UPR (= "ER stress") and an accumulation of SP-C aggregates which induces an ER-dependent apoptotic cascade. The linking pathways include the participation of the IRE1 sensor, which can independently trigger caspase 4 activation or ASK-1 / JNK upregulation. Previously published data (Mulugeta, et al 2007) also implicates mitochondrial release of cytochrome c (CytC) as a third pathway (---->) which is resistant to caspase4 and JNK inhibition. Apoptotic upregulation by BRICHOS SP-C mutants occurs independently of CHOP activation ("X"), a transcription factor known to induce apoptosis in some other systems.