Endoplasmic Reticulum Stress Induced by Surfactant Protein C BRICHOS Mutants Promotes Proinflammatory Signaling by Epithelial Cells

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Chronic interstitial lung disease in both adults and children is associated with mutations of the surfactant protein C (SP-C) proprotein. Among these, mutations within the distal COOH propeptide, known as the BRICHOS domain, are associated with a severe disease phenotype. We showed that prolonged expression of the BRICHOS mutants, SP-C$^{\Delta exon4}$ and SP-C^{L188Q}, destabilizes endoplasmic reticulum (ER) quality-control mechanisms (the unfolded protein response, or UPR), resulting in the induction of ER stress signaling, an inhibition of the ubiquitin/proteasome system, and the activation of apoptotic pathways. Based on recent observations that the UPR and ER stress can be linked to the induction of proinflammatory signaling, we hypothesized that the epithelial cell dysfunction mediated by SP-C BRICHOS mutants would activate proinflammatory signaling pathways. In a test of this hypothesis, A549 and human embryonic kidney epithelial (HEK293) cells, transiently transfected with either SP-C$^{\Delta exon4}$ or SP-C^{L188Q} mutants, each promoted the upregulation of multiple UPR response genes, including homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (HERPUD1) and GRP78. Commensurate with these results, increases in IL-8 secretion occurred and were accompanied by the activation of c-Jun N-terminal kinase (JNK)/activating protein–1 signaling. The stimulation of IL-8 cytokine release was completely attenuated by treatment with the JNK-specific inhibitor, SP600125. In addition, SP-C$^{\Delta exon4}$, but not SP-C^{L188Q}, activated NFκB. The treatment of SP-C$^{\Delta exon4}$ transfected cells with 4–phenylbutyric acid, a small molecule chaperone known to improve protein folding, blocked the activation of NFκB, but not the release of IL-8. Taken together, the results support the role of JNK signaling in mediating SP-C BRICHOS-induced cytokine release, and provide a link between SP-C BRICHOS mutants and proinflammatory cytokine signaling.

Keywords: surfactant proteins; unfolded protein response; ER stress; interleukin-8; interstitial lung disease

Interstitial lung disease (ILD) represents a broad classification of diffuse parenchymal lung diseases characterized by varying degrees of pulmonary inflammation and fibrosis. The pathophysiology of ILD reflects a mosaic of events, including epithelial injury, alveolitis, proinflammatory cytokine/chemokine elaboration, inflammatory cell infiltration, fibroblast activation, and matrix deposition, resulting in a final common pathway of lung remodeling and fibrotic scarring (1–3). Although many diseases incorporated into the classification are of unknown etiology, recent progress has allowed a further categorization of certain subgroups. This is based upon associations, or demonstrable cause-and-effect relationships, with environmental exposures, systemic diseases, or genetic factors. Based on a growing number of reports, abnormalities in various protein components of the pulmonary surfactant system can now be included within the genetic category of ILD (4).

CLINICAL RELEVANCE

Idiopathic pulmonary fibrosis (IPF) has been associated with the induction of ER stress signaling, apoptosis, and release of proinflammatory cytokines by epithelial cells of affected individuals. In this study, we show that expression of Surfactant Protein C (SP-C) mutations associated with IPF (i.e. SP-C BRICHOS) up-regulated multiple ER stress genes, increased IL-8 secretion, and activated c-Jun N-terminal kinase (JNK) signaling *in vitro*. Taken together, the results provide a link between ER stress and proinflammatory cytokine signaling, and highlight a potential new therapeutic target for IPF.

Surfactant protein C (SP-C) is a hydrophobic protein component of pulmonary surfactant whose expression in the adult lung is restricted to alveolar Type II epithelial cells. Synthesized as a larger (191 or 197 amino acid) proprotein, the alveolar form of human SP-C is generated by proteolytic processing of the precursor form to a 3.7-kD mature protein deposited into the alveolar space by regulated exocytosis (5). More than 30 mutations were identified within the SP-C gene (SFTPC) linked to both familial and sporadic forms of ILD (6). SFTPC mutations can be classified into three subgroups: (*1*) those that reside within the Bri family (BRICHOS) domain, a homologous conserved C-terminal region of approximately 100 amino acids found in a number of proteins involved in degenerative or proliferative diseases, resulting in misfolding and aggregate formation; (*2*) mutations in the non-BRICHOS C-terminal domain that result in trafficking to the plasma membrane or endosomal system; or (*3*) mutations in the cytoplasmic (N-terminal) domain that result in retention of the endoplasmic reticulum (ER) (5).

To date, most reports that examined the relationship between SP-C mutations and chronic lung disease have focused on SP-C BRICHOS mutants, giving much attention to the splice-variant deletion of exon 4 (SP-C$^{\Delta exon4}$), as well as a missense substitution at residue 188 (SP-C^{L188Q}) (7–9). SFTPC BRICHOS mutations were shown to affect proper folding and trafficking of the proprotein, resulting in the formation of cytosolic aggregates (7). To preempt these events, the epithelial response to the expression of mutant SP-C isoforms can include an induction of the unfolded protein response (UPR) to resolve improper folding and

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limit new protein synthesis, and ER-associated degradation to enhance mutant protein disposal through utilization of the ubiquitin–proteasome system. However, prolonged or unregulated activation of these systems (i.e. ER stress) is associated with an induction of epithelial cell death through intricate apoptotic pathways that contribute to lung injury and remodeling (9).

In addition to the upregulation of compensatory pathways such as the UPR, a molecular link was documented between cellular stress responses and inflammation, ultimately augmenting the production of proinflammatory genes. Activation of one of several signaling cascades involving mitogen-activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), or p38 and/or NFκB was shown to integrate ER stress and inflammation in a number of pathologic conditions (10). To the best of our knowledge, the role of epithelial-based unfolded protein and ER stress responses in the integration of an inflammatory response in the distal lung was not previously addressed.

We reported that two different SP-C BRICHOS mutants ($SPC^{\Delta exon4}$ and SPC^{L188Q}) induce an ER stress response through the activation of a canonic UPR-sensing pathway using insulin response element 1 (IRE1) (9). The IRE1-mediated signaling cascade was shown to play a pivotal role in triggering inflammatory responses in other systems. Conformational changes in the cytosolic domain of IRE1, which allow for binding to TNFα receptor–associated factor 2 (TRAF2), can interface with both the NFκB and MAPK pathways. The IRE1–TRAF2 complex can recruit IκB kinase (IKK), which phosphorylates and removes the IκB inhibitory subunit of NFκB (11). The IRE1–TRAF2 complex can also activate JNK through phosphorylation, with phospho-JNK subsequently activating the transcription factor activating protein 1 (AP-1). The nuclear translocation of AP-1 or NFκB can each upregulate the transcription of a variety of target genes that include proinflammatory proteins such as cytokines, interferons, cell-adhesion molecules, and major histocompatibility (MHC) Class I molecules (12, 13).

Based on these data, we hypothesized that coupling of the UPR/ER stress response to inflammation could be induced in cells expressing SP-C BRICHOS mutants. We demonstrate here that mutant SFTPC expression promotes the release of the proinflammatory cytokine IL-8 by epithelial cells *in vitro*. Importantly, through the use of a variety of reporter techniques, whereas cytokine release occurs in association with activation of both JNK and NFκB signaling, only the pharmacologic inhibition of the JNK pathway blocks the release of IL-8. These results further underscore the emerging importance of the epithelial cell as a critical component in the pathogenesis of ILD through the selective induction of ER stress–mediated inflammatory pathways.

MATERIALS AND METHODS

Reagents

The pEGFP–C1 (enhanced green fluorescent protein) plasmid was originally purchased from Clontech, Inc. (Palo Alto, CA). The pcDNA3 eukaryotic expression plasmid was obtained from Invitrogen (San Diego, CA). The ER stress activated indicator (ERAI) (XBP-1/*venus*) expression plasmid for the detection of IRE1 activation (14) was a generous gift of Dr. Masayuki Miura (University of Tokyo, Tokyo, Japan).

Tissue culture medium was produced by the Cell Center Facility at the University of Pennsylvania. Precast gels and buffers for SDS-PAGE (Novex) were obtained from Invitrogen, Inc. Except where noted, all other reagents of analytical grade for electrophoresis and tissue culture were purchased from Sigma Chemical, Inc. (St. Louis, MO) or BioRad, Inc. (Melville, NY).

Rabbit monoclonal antisera included phospho-NFκB–p65 (3033), phospho-stress activated protein kinase (SAPK)/JNK (Cat# 4668), GFP (Cat# 632381) (Clontech, Mountain View, CA), and SAPK/JNK (Cat# 9258) (Cell Signaling Technologies, Beverly, MA). Polyclonal antisera included NFκB-p65 (Cat# 3034), phospho–c-Jun (Cat#

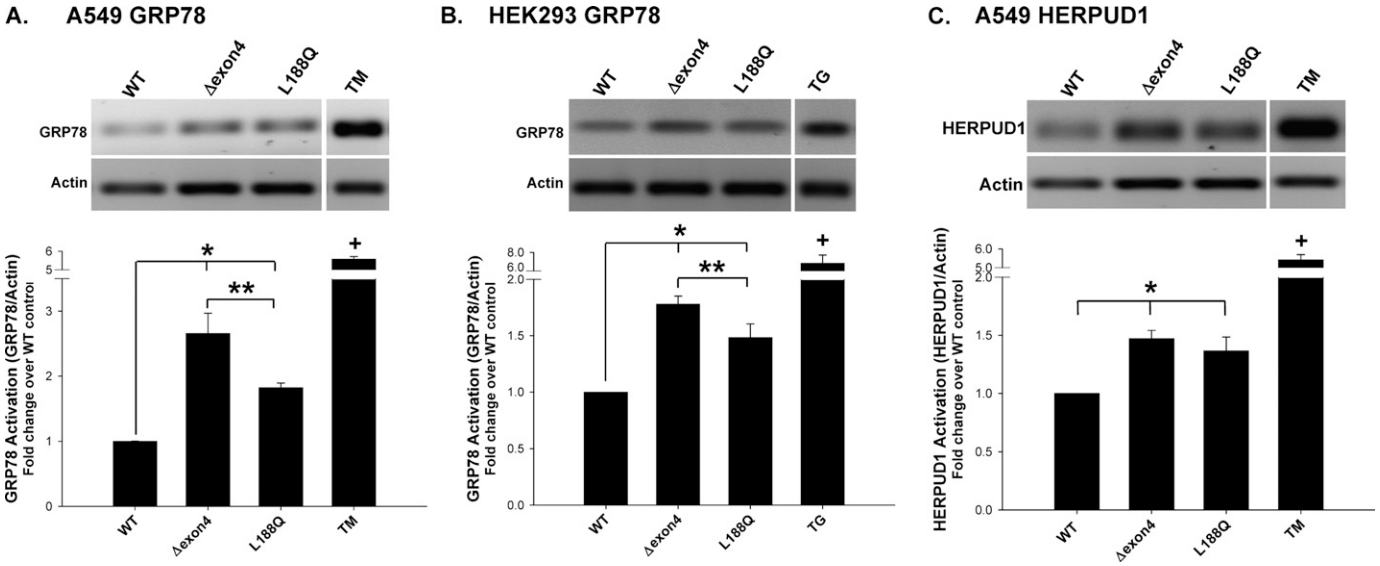

Figure 1. Unfolded protein response (UPR) gene induction in surfactant protein C (SP-C) BRICHOS mutant–expressing cells. A549 and human embryonic kidney epithelial (*HEK293*) cells, transiently transfected with EGFP–SP-C wild-type (*WT*), Δexon4, or L188Q constructs, were harvested 48 hours after transfection, and total RNA was prepared. Overnight treatments of 2 μM thapsigargin (*TG*) or 20 μg/ml tunicamycin (*TM*) were used for positive control samples. RT-PCR was performed with primers for GRP78/BiP (*A* and *B*) and homocysteine-inducible, endoplasmic reticulum stress–inducible, ubiquitin-like domain member 1 (HERPUD1) (*C*) expression, as described in MATERIALS AND METHODS. Band intensities were quantified by densitometry, and the ratio of either GRP78 or HERPUD1 to β-actin was determined. Data are expressed as fold change over WT control samples, and represent the mean ± SD of three separate experiments. Representative agarose gels appear above each graph. *$P < 0.05$ versus WT control. **$P < 0.05$ versus Δexon4. $^{+}P < 0.001$ versus WT control.

9261) (Cell Signaling Technologies), and β-actin (Sigma Chemical, Inc.). All primary antibodies were used at a dilution of 1:1,000. HRP-conjugated secondary antisera included goat anti-rabbit IgG or goat anti-mouse IgG, and were purchased from Jackson ImmunoResearch (West Grove, PA).

Cell Culture

Human lung epithelial (A549) and human embryonic kidney epithelial (HEK293) cell lines were obtained from ATCC (Manassas, VA), and grown as monolayers in Dulbecco's Modified Eagle Medium (DMEM) at 37°C, in humidified air equilibrated with 5% CO_2. All media were supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FBS.

SP-C Construct Generation

The subcloning of human SP-C (SP-C^{WT}), BRICHOS mutant SP-C (SP-$C^{\Delta exon4}$ and SP-C^{L188Q}), or non-BRICHOS mutant SP-C (SP-C^{I73T}) constructs into the pEGFP-C1 and pcDNA3 expression vectors was previously described (7, 9, 15). The hemagglutinin (HA) tag (YPYDVPDYA) was added to the NH_2 terminus of wild-type and mutant SP-C cDNAs by PCR, using a previously described method (9). For all constructs, the fidelity of PCR reactions and cloning was demonstrated by the automated DNA sequencing of cDNA coding regions, performed in both directions by the Core Facility of the Department of Genetics at the University of Pennsylvania.

Semiquantitative PCR

Total RNA was subjected to reverse transcription (RT) using a RETROscript Kit (Ambion, Inc., Austin, TX). Each RT reaction contained 2 μg of RNA, and was performed under conditions of heat denaturation at 80°C for 3 minutes, amplification for 1 hour at 44°C, and heat inactivation at 92°C for 10 minutes.

The resulting cDNAs were subjected to PCR using the PCR Master Mix (Promega Corporation, Madison, WI). Each reaction contained approximately 250 ng of cDNA template. DNA amplification was performed under conditions of denaturation at 94°C for 3 minutes, followed by the specified number of cycles at 94°C for 30 seconds, 62°C for 45 seconds, and 72°C for 1 minute. The products of reactions were then incubated at 72°C for 10 minutes to increase the yield of amplification. The homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (HERPUD1) gene was amplified with the primers 5′-CTACTCCTCCCTGAGCAGA TTC-3′ (forward) and 5′-GGTTGGGGTCTTCAGTTTCAGG-3′ (reverse) for 25 cycles. The Glucose Regulated Protein 78 (GRP78) gene was amplified with the primers 5′-GGAACACATGGTGCCTAC CAA-3′ (forward) and 5′-GGAGCAGGAGGAATTCCAGTCA-3′ (reverse) for 20 cycles. The β-actin gene was amplified with the primers 5′-TGGGTCAGAAGGATTCCTATGT-3′ (forward) and 5′-CAGCC TGGATAGCAACGTACA-3′ (reverse) for 22 cycles. All primer sequences were derived from Donati and colleagues, and cycle parameters were individualized to each cell line (16). The relative mRNA content of each gene was normalized to mRNA for β-actin.

Cell Transfection and Pharmacologic Treatments

A549 or HEK293 cells grown to 75% confluence were transiently transfected with the indicated plasmid constructs, using either $CaPO_4$ precipitation as previously described, or Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendations (17). Where indicated, positive control samples were generated by the treatment of nontransfected cells 15 hours before harvest with either thapsigargin (2 μM) or tunicamycin (20 μg/ml), which were shown to elicit ER stress (9, 18), or with TNF-α (10 ng/ml), which was shown to elicit the maximal expression of IL-8 (19).

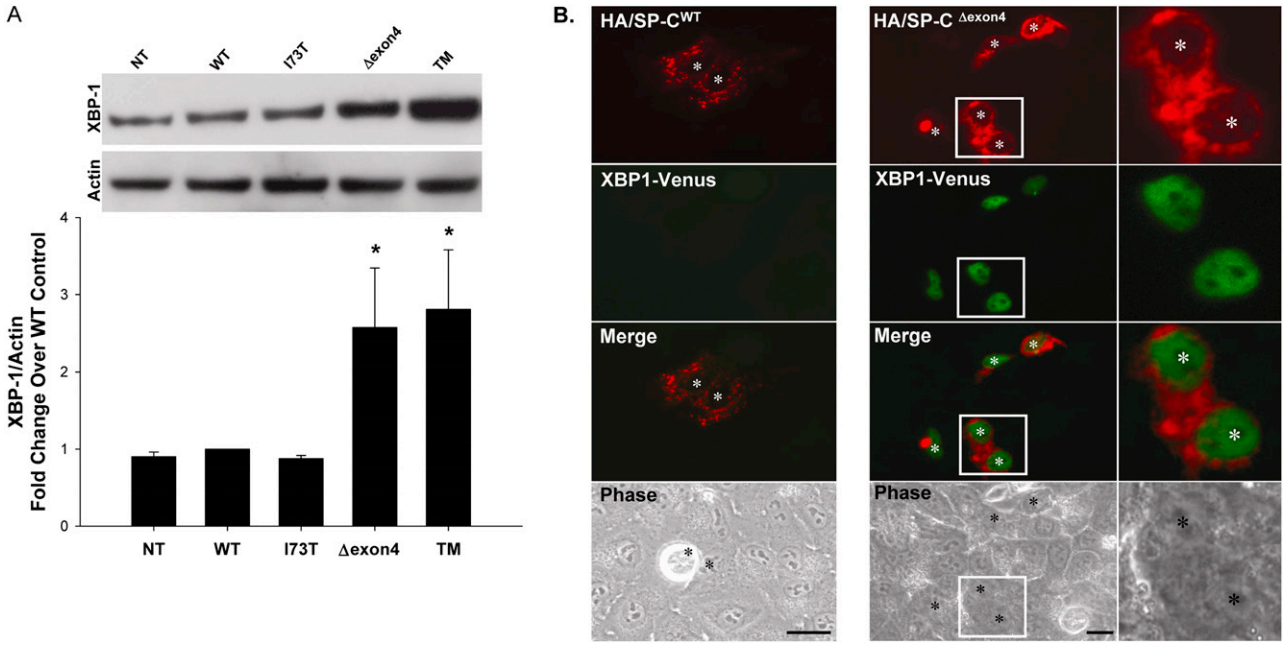

Figure 2. SP-C BRICHOS mutants activate the insulin response element 1 (IRE1) signaling pathway. (*A*) A549 cells, either nontransfected (NT) or transiently transfected with the indicated control or EGFP–SP-C constructs (WT, I73T, Δexon4 were harvested 48 hours after transfection. Overnight treatment with 10 μg/ml tunicamycin (*TM*) was used for a positive control sample. Cell lysates were analyzed for XBP-1 protein expression by Western blotting, as described in Materials and Methods. An anti–β-actin antibody was used as a loading control. A representative immunoblot appears above the graph. Band intensities were quantified by densitometry, and the ratio of XBP-1 to β-actin was determined. Data are expressed as fold change over WT control, and represent the mean ± SD of three separate experiments. *$P < 0.05$ versus WT control. (*B*) Hemagglutinin (HA)-tagged SP-C^{WT} (*left*) or SP-$C^{\Delta exon4}$ (*right*) were cotransfected with the ERAI plasmid (XBP-1/*venus*) developed by Iwawaki and colleagues (14) into A549 cells. Forty-eight hours after transfection, cells were fixed and stained with an anti-HA monoclonal antibody to label the HA–SP-C fusion protein, as described in Materials and Methods. Fluorescence microscopy shows XBP-1/*venus* expression in SP-$C^{\Delta exon4}$ but not in SP-C^{WT} transfected cells. Merged and phase contrast images are shown, and individual nuclei are identified by *asterisks*. Magnifications of selected areas are seen in the *rightmost* column.

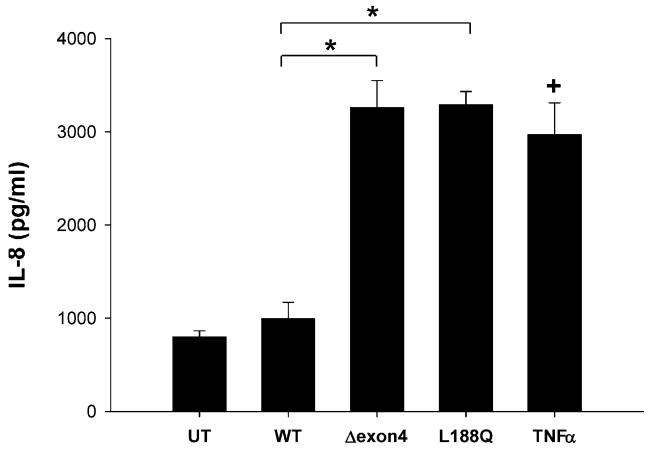

Figure 3. SP-C$^{\Delta exon4}$ and SP-C^{L188Q} induce IL-8 cytokine release. A549 cells were transiently transfected with the indicated EGFP-tagged SP-C WT or mutant construct, using Lipofectamine 2000 reagent (Invitrogen). Untreated (*UT*) cells were used as a negative control sample, and overnight treatment of 10 ng/ml TNFα was used for a positive control sample. Media were collected 48 hours after transfection, and IL-8 was measured by ELISA, as described in MATERIALS AND METHODS. Data are expressed as mean IL-8 concentration ± SD of triplicate samples, and are representative of three separate experiments. The intra-assay coefficients of variation (%CV) for the three experiments were 2.7%, 3.0%, and 2.1%, whereas the interassay %CV was 12.2%. $^{*}P < 0.05$ and $^{+}P < 0.05$ versus WT control.

To harvest cells, media were removed from cells, and 100 μl of 1× sample loading buffer containing DTT was added to each well of a six-well dish. Cells were scraped with a "rubber policeman" and sonicated for 10 seconds. After incubation at 95°C for 5 minutes and centrifugation at 12,000 rpm for 5 minutes to pellet the cellular debris, the supernatants were used for SDS-PAGE. Consistent with our previous results (7, 9, 15, 18), using either Western blotting for protein expression or cell counting for GFP-positive cells, the transfection efficiencies of the wild-type and mutant SP-C isoforms were nearly equivalent in both HEK and A549 cells (as shown in Figure E1 in the online supplement).

Immunocytochemistry

Immunocytochemistry and fluorescence imaging were preformed according to a previously described protocol (9). After permeabilization, cells on coverslips were immunolabeled with primary anti-HA antibody (Roche, San Francisco, CA) for 1 hour at room temperature at a 1:100 dilution. Texas red–conjugated secondary goat anti-mouse IgG monoclonal antibody (Jackson ImmunoResearch Laboratories) was used for visualization at a 1:200 dilution.

Luciferase Reporter Gene Assays

The A549–NFκB–luciferase (luc) cell line was used to determine NFκB activity. This cell line stably expresses a Firefly luciferase reporter construct regulated by six copies of the NFκB response element (Panomics, Inc., Freemont, CA). SP-C and control plasmids were transiently introduced into the cell line, using Lipofectamine 2000 reagent (Invitrogen). The pRL-SV40 *Renilla* luciferase vector was used to normalize transfection efficiencies. Forty-eight hours after transfection, cells were lysed and assayed for Firefly and *Renilla* luciferase activities, using a dual luciferase kit (Promega Corporation).

SDS-PAGE and Western Blotting

Cell lysate protein was separated by SDS-PAGE, as described by Laemmli (20), using a 4% stacking gel and a 10% resolving gel, transferred to nitrocellulose (O/N, 20 V), and incubated with antibody in either 5% BSA in Tris-buffered saline (TBS) and 0.1% Tween-20 for phospho-specific antibodies, or 1% nonfat dry milk in TBS and 0.1% Tween-20 at 4°C overnight. After sequential incubation with primary and HRP-conjugated secondary antisera at the indicated dilutions, bands were detected by chemiluminescence, using X-Omat AR film (Kodak, Rochester, NY). Specific band intensities were quantitated using Kodak1D software, version 3.0.

ELISA for Human IL-8

A549 cells were transiently transfected with the indicated SP-C mutant construct using Lipofectamine 2000, or were treated with the indicated stimulator. Media collected 12–48 hours after transfection were used for the determination of released IL-8, measured using the DuoSet Human IL-8 ELISA Assay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Intra-assay coefficients of variation (%CV) were accepted at a value of less than 10%, whereas interassay %CV were accepted at a value of less than 20%.

Statistical Analysis

Parametric data were analyzed with Instat, version 3.0 (Graph Pad Software, Inc., La Jolla, CA), and were expressed as mean ± SD. Experimental groups were compared according to an unpaired, two-tailed Student *t* test for comparing two samples, or ANOVA between sample groups using the Tukey–Kramer multiple-comparison *post hoc* test. Significance was accepted at $P < 0.05$.

RESULTS

Expression of SP-C BRICHOS Mutants Results in UPR Induction

The expression of misfolded mutant proteins is associated with a generalized cell response aimed at promoting protein refolding and limiting cell toxicity. We assessed the ER stress response using semiquantitative RT-PCR for known UPR genes in our model system. Compared with cells transiently transfected with cDNA encoding EGFP–SP-C^{WT}, EGFP fusion constructs containing the BRICHOS mutant SP-C$^{\Delta exon4}$ induced a significant elevation of the well-recognized UPR gene product, GRP78/binding immunoglobulin protein (BiP), in both A549 and HEK293 cells (Figures 1A and 1B). Another BRICHOS mutant, SP-C^{L188Q}, also increased the expression of GRP78 in each cell line, but to a lesser extent than SP-C$^{\Delta exon4}$. Both SP-C BRICHOS mutants also increased the expression of HERPUD1, another UPR quality-control protein in A549 cells (Figure 1C). Taken in combination with previously reported data from our group and others (18, 21), these results support the notion that the expression of SP-C BRICHOS mutations induces a UPR response that is cell line–independent, and global in promoting the upregulation of a wide array of ER stress gene products.

Expression of SP-C BRICHOS Mutants Results in IRE1 Activation

The response of the ER to a mutant or increased protein load can be sensed by at least three distinct transducers, IRE1, pancreatic ER kinase (PERK), and activating transcription factor 6 (ATF6). Of these, the IRE1–XBP-1 pathway was most closely linked with ER stress inflammatory signaling (13). The splicing and expression of the X-box binding protein 1 (XBP-1) transcription factor can be used as a surrogate for IRE1 activity. Therefore, the expression of XBP-1 protein was assessed by Western blotting of cell lysates, 48 hours after the transient expression of SP-C mutant constructs and controls. Significant increases in XBP-1 protein concentrations were evident in A549 epithelial cells expressing SP-C$^{\Delta exon4}$, but not in cells expressing SP-C^{I73T}, an ILD-associated SP-C mutation located outside the BRICHOS domain (Figure 2A). To demonstrate functional IRE-1 activity directly, A549 cells were cotransfected with HA–SP-C constructs and the ERA1 expression plasmid developed by Iwawaki and colleagues (14). ERA1 contains an insert encoding for a fusion protein of XBP-1 and *venus*, a GFP-like reporter protein. The XBP-1 cDNA

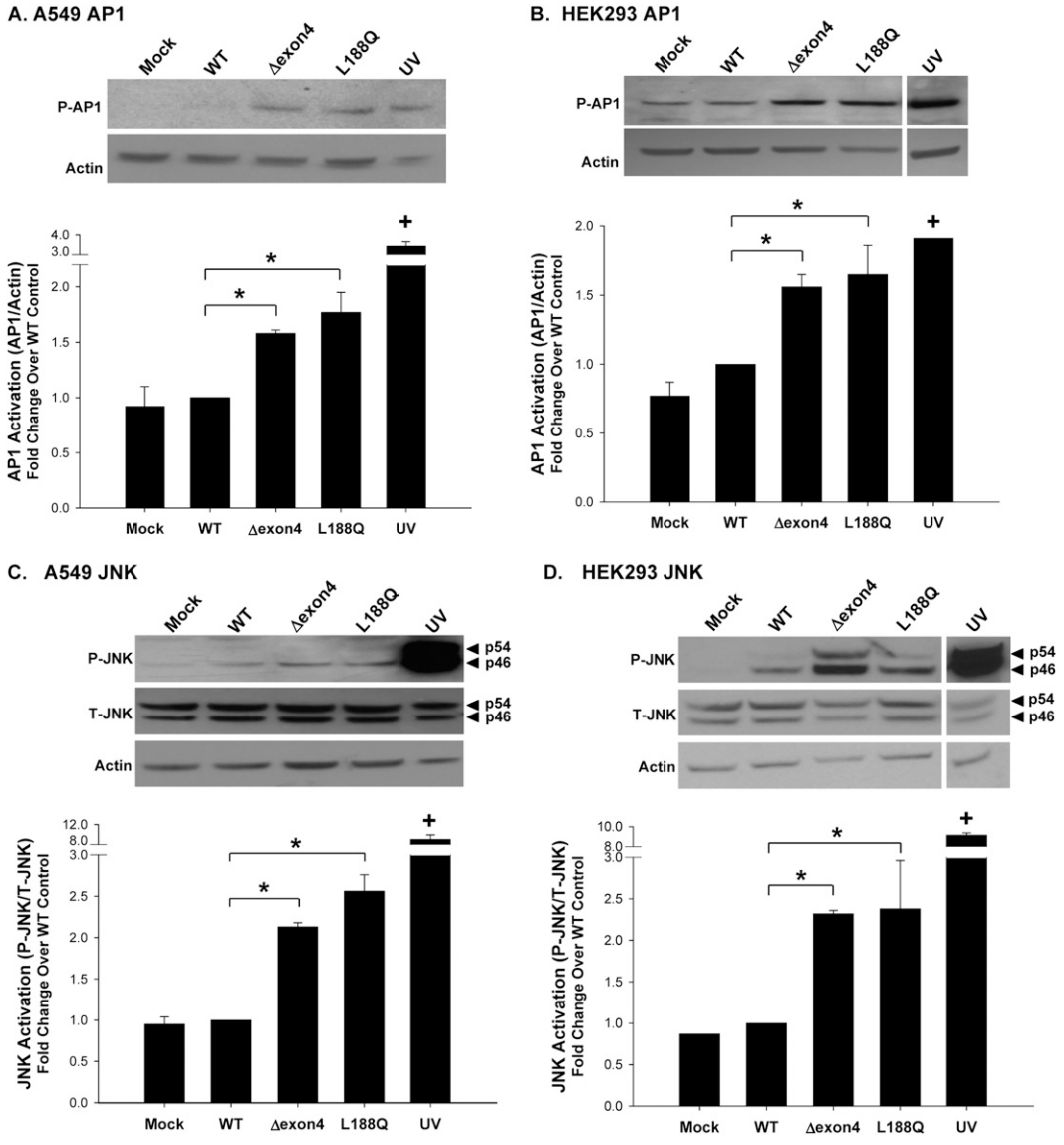

Figure 4. SP-C BRICHOS mutants upregulate activating protein 1 (*AP1*) and c-Jun N-terminal kinase (*JNK*) activation. A549 and HEK293 cells were transiently transfected in the absence (*Mock*) or presence of the indicated EGFP–SP-C constructs, and harvested 48 hours after transfection. Ultraviolet light (*UV*) treatment (40 mJ/cm^2) was used for a positive control sample. Cell lysates were analyzed for phospho–c-Jun (*A* and *B*), phospho–SAPK/JNK, and total SAPK/JNK expression, using monoclonal antibodies (*C* and *D*). An anti–β-actin antibody was used as a loading control. Band intensities were quantified by densitometry, and the ratio of either c-Jun to β-actin or phospho-JNK to total JNK was determined. Data are expressed as fold change over WT control, and represent the mean ± SD of three separate experiments. Representative immunoblots appear above each graph. *$P < 0.05$ versus WT control. $^+P < 0.001$ versus WT control.

contains a stop codon that prevents a full read-through unless spliced out by IRE1 endo-RNase activity, which occurs during IRE-1 dimerization/activation with ER stress. As shown in Figure 2B, cells staining for HA-SP-C$^{\Delta exon4}$ co-expressed XBP-1/*venus* in a nuclear pattern consistent with the transcription, translation, and nuclear translocation of XBP-1.

SP-C BRICHOS Mutants Induce Release of IL-8 by Epithelial Cells

Exaggerated production of the proinflammatory CXC chemokine IL-8 was found in patients, mice, and epithelial cell lines expressing mutant cystic fibrosis transmembrane conductance regulator (CFTR) (22, 23). IL-8 is also significantly elevated in specimens from patients with idiopathic pulmonary fibrosis (IPF), and is associated with increased neutrophilia and poorer outcomes (24). To investigate whether the expression of SP-C BRICHOS mutants could trigger the release of IL-8 by epithelial cells, an IL-8–specific ELISA was performed on media collected from transiently transfected A549 cells expressing wild-type or mutant SP-C isoforms. The pharmacologic treatment of nontransfected cells with TNF-α served as a positive control, and resulted in a substantial increase in cytokine elaboration. Similarly, an increased release of IL-8 occurred in cells expressing either BRICHOS mutant (SP-C$^{\Delta exon4}$ or SP-C^{L188Q}), but not in cells transfected with wild-type SP-C (Figure 3), an effect that was time-dependent, reaching a maximum at 48 hours (Figure E2).

SP-C BRICHOS Mutants Activate AP-1/JNK Signaling

A growing body of evidence suggests that the signaling pathways involved in ER stress induction and inflammation are interconnected. The activation of AP-1 was shown to occur in response to ER stress, when an increased protein load is sensed and transduced by the IRE1–XBP-1 signaling pathway. Because AP-1 is an important transcriptional activator for the induction of a number of inflammatory genes, we sought to assess the effects of SP-C BRICHOS mutations on these pathways.

The phosphorylation of AP-1, shown to be necessary for its transcriptional activity, was evaluated via Western blotting. Cell lysates, harvested 48 hours after transfection, were immunoblotted with a phospho-specific c-Jun (subunit of the heterodimer complex of AP-1) antibody that recognizes the phosphorylation at Ser63. In both A549 and HEK293 cells, the expression of either the SP-C$^{\Delta exon4}$ or SP-C^{L188Q} mutant resulted in the increased phosphorylation of AP-1, compared with SP-C^{WT}–expressing cells (Figures 4A and 4B).

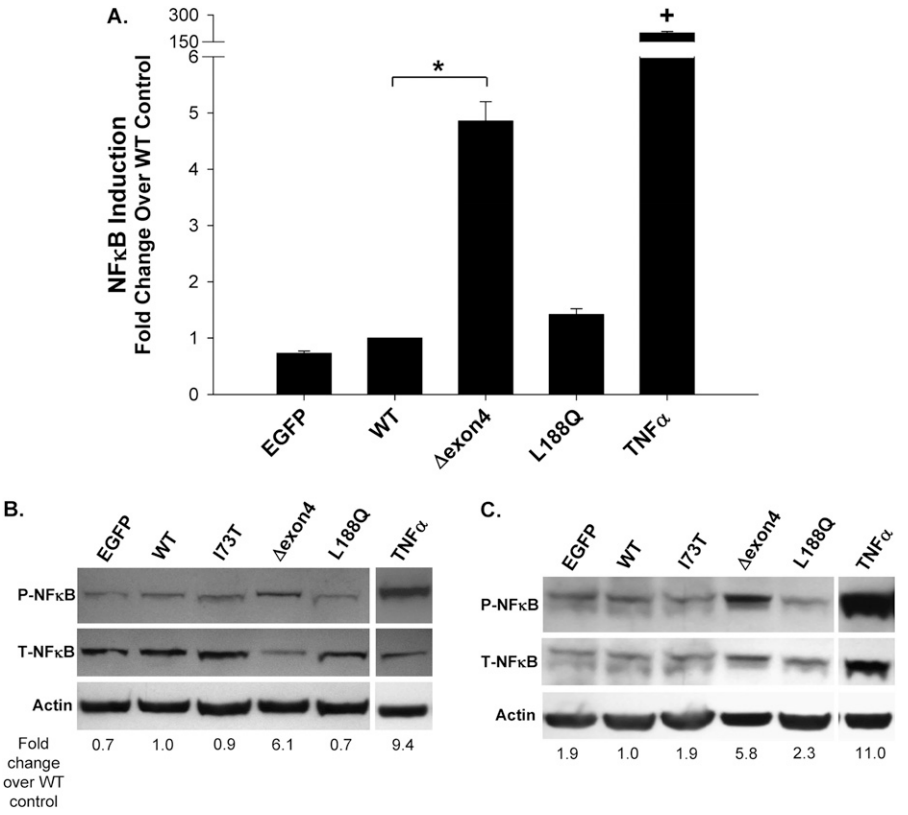

Figure 5. SP-C$^{\Delta exon4}$ induces an NFκB response. NFκB activation induced by SP-C BRICHOS mutants was assessed in A549–NFκB–luciferase (*luc*), A549, and HEK293 cell lines. (*A*) A549–NFκB–luc cells were cotransfected with the indicated control (*EGFP*) or EGFP–SP-C constructs, with pRL-SV40 (expressing *Renilla* luciferase) to normalize transfection efficiencies. Overnight treatment with 10 ng/ml TNFα before harvesting was used for a positive control sample. Cells lysates were prepared 48 hours after transfection, and Firefly and *Renilla* luciferase activities were measured, using luciferin substrates provided by the Dual Luciferase Kit (Promega). The ratio of mean Firefly to *Renilla* luciferase was determined, and expressed as fold change over WT control. These data represent the mean ± SD of three separate experiments. $^{*}P < 0.05$ versus WT control. $^{+}P < 0.001$ versus WT control. (*B* and *C*) Cell lysates from A549 and HEK293 cells transfected with the indicated control (*EGFP*), WT, non-BRICHOS SP-C mutant (*I73T*), or BRICHOS mutant constructs (Δ*exon4* and *L188Q*) were prepared 48 hours after the introduction of plasmid cDNA. Representative immunoblots of the activated/phosphorylated P65 subunit of NFκB (*P-NFκB*) and total cellular P65 subunit of NFκB (*T-NFκB*) from A549 (*B*) and HEK293 (*C*) cells are shown. Cell lysates were analyzed for phospho–NFκB–P65 and total NFκB-P65 expression, using a monoclonal phospho-specific antibody and a polyclonal NFκB antibody, respectively. An anti–β-actin antibody was used as a loading control. Band intensities were quantified by densitometry, and the ratio of phosphorylated to total NFκB–P65/actin was determined. Data are expressed as fold change over WT control, and are representative of at least two separate experiments.

MAPK/JNK activity occurs upstream of AP-1 activation (25). Numerous JNK isoforms were identified, resulting from the alternative splicing of three JNK genes: JNK1, JNK2, and JNK3 (Mol. Wt. = 46, 54, and 57 kD, respectively) (26). To assess the involvement of the MAPK/JNK pathway in the induction of inflammation by SP-C BRICHOS mutants, the phosphorylation of JNK was measured in mutant-expressing A549 and HEK293 cells (Figures 4C and 4D). Using an antibody that recognizes the p46 and p54 isoforms of JNK, both the SP-C$^{\Delta exon4}$ and SP-C^{L188Q} mutants were shown to significantly increase phosphorylation of the p46 subunit in A549 cells, and of the p46 and p54 subunits in HEK293 cells, compared with SP-C^{WT}–expressing cells.

SP-C BRICHOS Mutants Differentially Affect NFκB Signaling

In some systems, UPR–inflammatory coupling was also linked to the activation of NFκB (10). To assess this phenomenon, the activation of NFκB in response to SP-C BRICHOS mutant expression was evaluated with several methodologies. We transiently transfected an A549–NFκB–luc reporter cell line with the indicated control or mutant SP-C constructs. The fidelity of the reporter assay was confirmed by treatment of the cells with the known NFκB trigger, TNF-α, which induced a robust increase in luciferase activity (Figure 5A). Similarly, a selective fivefold increase in luciferase activity occurred with the expression of the SP-C$^{\Delta exon4}$ mutant, compared with either EGFP-C1 or SP-C^{WT} transfected cells. The expression of SP-C^{L188Q} did not activate NFκB signaling, with luciferase activity equivalent to that of control transfections.

The differential effect of the two BRICHOS mutants on NFκB activation was then corroborated biochemically, using Western blotting for the detection of the phosphorylated p65 subunit of NFκB. Forty-eight hours after the transient expression of SP-C constructs and control samples, a significant increase in NFκB-p65 phosphorylation was observed in A549 cells expressing the SP-C$^{\Delta exon4}$ mutant, in a pattern similar to that of nontransfected cells treated with TNF-α, but not with cells expressing other SP-C constructs (SP-C^{WT}, SP-C^{L188Q}, and SP-C^{I73T}; Figure 5B). A selectively substantial increase in phospho–NFκB–p65 was also observed in HEK293 cells expressing SP-C$^{\Delta exon4}$ or treated with TNF-α (Figure 5C). The increase in phospho-NFkB in HEK cells was accompanied by an increase in total NFkB, likely reflecting an additional effect on NFkB expression in this cell line. Taken together, these experiments indicate that the SP-C$^{\Delta exon4}$ mutant selectively induces an NFκB response in both cell lines.

Inhibition of JNK Blocks the Release of IL-8

To determine the relative contribution of JNK/AP-1 signaling in SP-C BRICHOS–induced inflammation, A549 cells, transiently transfected with the indicated control or mutant SP-C constructs, were treated with SP600125, a reversible ATP-competitive inhibitor of JNK, and assessed for the production of IL-8. As shown in Figure 6, the selective blockade of JNK signaling led to a complete inhibition of the IL-8 release induced by SP-C$^{\Delta exon4}$. This inhibition by SP600125 was specific to SP-C$^{\Delta exon4}$ expression, because no change in IL-8 secretion was evident in A549 cells exposed to TNF-α, or in A549 cells transfected with SP-C^{WT} constructs treated with the same dose of inhibitor (Figure E3).

4–Phenylbutyric Acid, a Facilitator of Protein Folding, Blocks NFκB Activation, but Not IL-8 Release

We previously showed that the treatment of cells expressing the mutant SP-C$^{\Delta exon4}$ with the small molecule chemical facilitator 4–phenylbutyric acid (4-PBA), a known modulator of protein

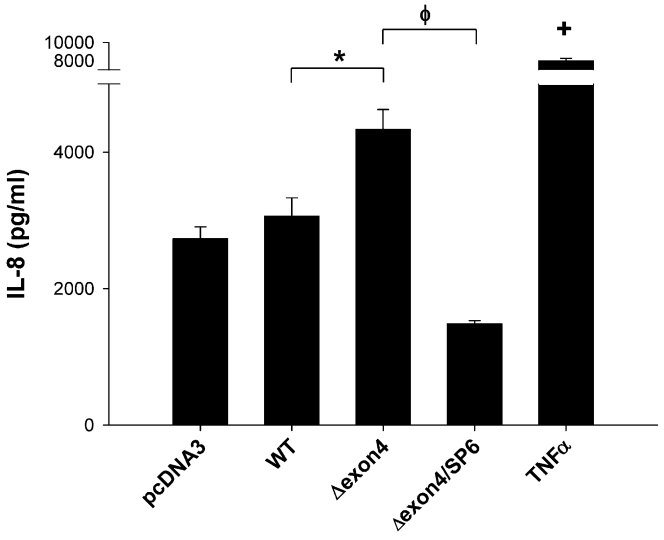

Figure 6. IL-8 release induced by SP-C BRICHOS mutants is blocked by the inhibition of JNK. A549 cells were transiently transfected with the indicated control or HA-tagged SP-C mutant construct, using Lipofectamine 2000 reagent. Twenty-four hours after transfection, 10 μM of the JNK-specific inhibitor SP600125 was added to the indicated samples. Overnight treatment with 10 ng/ml TNFα was used for a positive control sample. Media were collected 48 hours after transfection (18 hours after TNF-α treatment), and IL-8 was measured by ELISA, as described in MATERIALS AND METHODS. Data are expressed as mean IL-8 concentration ± SD of triplicate samples, and are representative of three separate experiments. The intra-assay %CVs for the three experiments were 2.1%, 1.5%, and 1.6%, whereas the interassay %CV was 6.0%. *$P < 0.05$ versus WT control. $^{\phi}P < 0.05$ versus SP-C$^{\Delta exon4}$ sample. $^{+}P < 0.0001$ versus WT control.

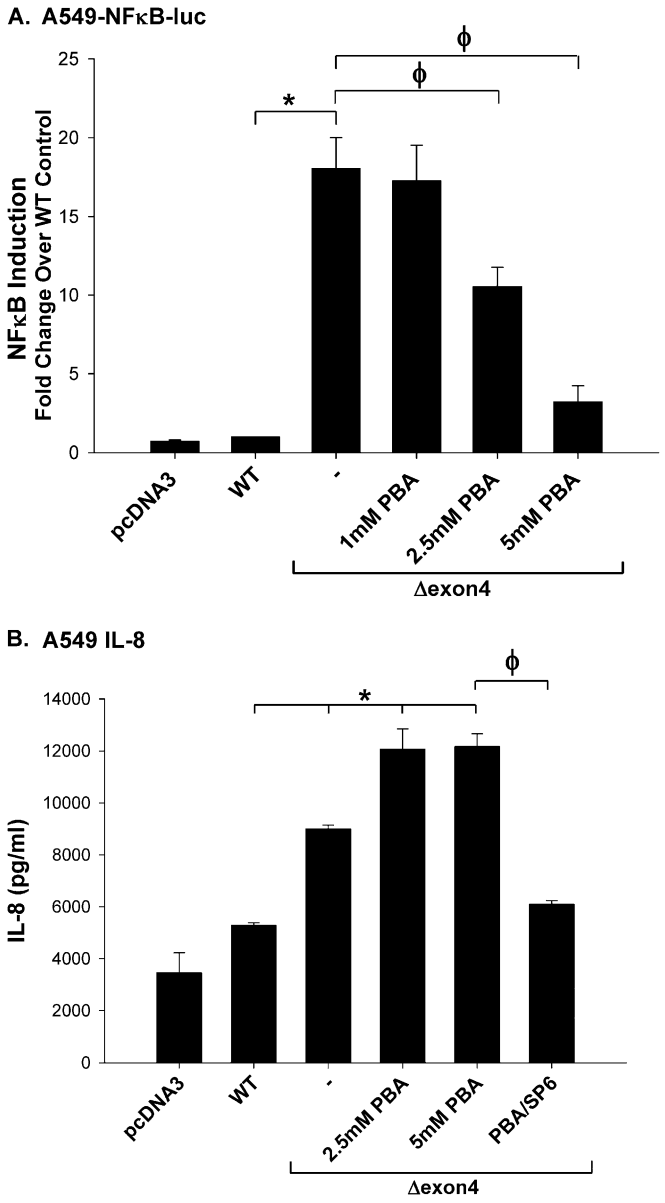

Figure 7. 4–Phenylbutyric acid (4-PBA) blocks NFκB activation, but not the release of IL-8. (*A*) The A549–NFκB–luc cell line was transiently transfected with the indicated control or SP-C constructs. pRL-SV40 was transfected into all samples as an internal control to normalize transfection efficiencies. Eight hours after transfection, increasing concentrations of 4-PBA (*PBA*) were added to SP-C$^{\Delta exon4}$–transfected cells, and overnight treatment with 10 ng/ml TNF-α was used for a positive control sample. Cells were harvested and lysed 48 hours after transfection. Firefly and *Renilla* luciferase activities were measured using luciferin substrates, as in Figure 5. The ratio of mean Firefly to *Renilla* luciferase was determined, and expressed as fold change over WT control. Graph represents the mean ± SD of triplicate experiments. (*B*) A549 cells were transiently transfected with the indicated control or EGFP–SP-C mutant constructs using Lipofectamine 2000 reagent. Eight hours after transfection, increasing concentrations of 4-PBA, or a combination of 5 mM PBA and 10 μM SP600125, was added to SP-C$^{\Delta exon4}$ transfected cells, as indicated. Media were collected 48 hours after transfection, and IL-8 was measured by ELISA as described in MATERIALS AND METHODS. Graph represents the mean ± SD of triplicate samples, and is representative of three separate experiments. The intra-assay %CVs for the three experiments were 1.4%, 3.4%, and 1.6%, whereas the interassay %CV was 2.3%. *$P < 0.05$ versus WT control. $^{\phi}P < 0.05$ versus SP-C$^{\Delta exon4}$ sample. $^{+}P < 0.0001$ versus WT control.

folding, results in the redirection or dispersion of aggregated mutant SP-C protein (7). To determine if 4-PBA also corrected the proinflammatory signaling induced by SP-C$^{\Delta exon4}$, the activation of NFκB and release of IL-8 were measured in transfected cells subjected to treatment with 4-PBA. As shown in Figure 7A, 4-PBA caused a dose-dependent decrease in luciferase activity in A549–NFκB–reporter cells transiently expressing SP-C$^{\Delta exon4}$. Despite this complete inhibition of NFκB activation, 4-PBA treatment actually enhanced the release of IL-8 by A549 cells expressing SP-C$^{\Delta exon4}$. When coadministered with 4-PBA, SP600125 completely antagonized the release of IL-8 induced by SP-C$^{\Delta exon4}$ and 4-PBA (Figure 7B). Moreover, the effect of 4-PBA in modulating phospho-JNK and phospho–AP-1 concentrations was minimal in A549 cells transfected with either SP-C$^{\Delta exon4}$ or SP-C^{L188Q} (Figures 8A and 8B). When the same studies were performed using HEK293 cells, 4-PBA actually stimulated both JNK and AP1 phosphorylation in this cell line (Figure E4). Taken together, these data indicate that, in contrast to JNK MAPK, the attenuation of NFκB-based signaling had no effect on proinflammatory cytokine generation by epithelial cells expressing SP-C BRICHOS mutants.

DISCUSSION

Recent observations indicate that pathologic conditions interfering with ER homeostasis result in the chronic activation of both the UPR and inflammation. We previously showed that the expression of mutant SP-C (BRICHOS domain mutants), associated with the development of pulmonary fibrosis in children and adults, is capable of inducing an ER stress response,

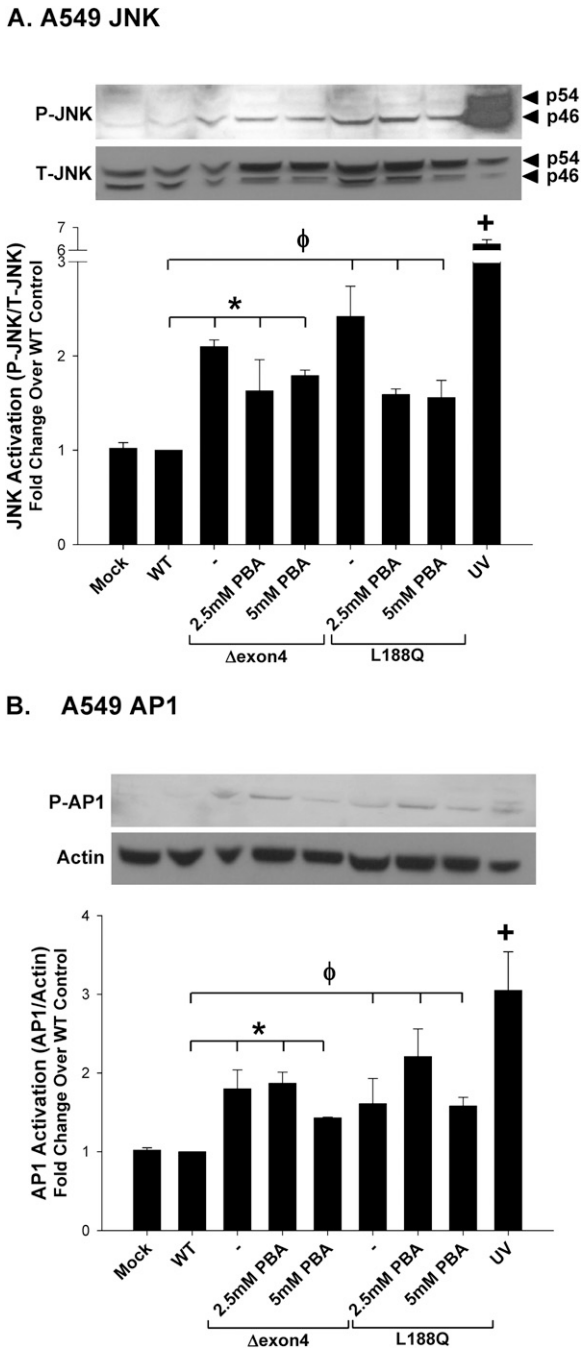

Figure 8. SP-C mutant induction of JNK and AP-1 is not altered by 4-PBA treatment. A549 cells were transiently transfected in the absence (*Mock*) or presence of the indicated EGFP–SP-C constructs, and harvested 48 hours after transfection. Eight hours after transfection, increasing concentrations of 4-PBA were added to SP-$C^{\Delta exon4}$–transfected cells and harvested 48 hours after transfection. UV treatment (40 mJ/cm^2) was used for a positive control sample. Cell lysates were analyzed for phospho-SAPK/JNK and total SAPK/JNK expression (*A*) and phospho–c-Jun (*AP1*) expression (*B*). An anti–β-actin antibody was used as a loading control. Band intensities were quantified by densitometry, and the ratio of either phospho-JNK to total JNK, or c-Jun to β-actin, was determined. Data are expressed as fold change over WT control, and represent the mean ± SD of three separate experiments. Representative immunoblots appear above each graph. $^*P < 0.05$ for SP-$C^{\Delta exon4}$ samples versus WT control. $^{\Phi}P < 0.05$ for SP-C^{L188Q} samples versus WT control. $^{+}P < 0.01$ for UV positive control versus WT control.

caspase activation, and ultimately cell death (9, 18). The present study extends these observations through an examination of the proinflammatory signaling mediated by the UPR in lung epithelia expressing SP-C BRICHOS mutants. In addition to demonstrating the induction of IL-8 by the expression of SP-C BRICHOS mutants, our results establish the first mechanistic link between activation of the UPR and inflammation in pulmonary epithelia. Given that the BRICHOS domain in SP-C has marked structural homology with other Bri family members associated with neurodegeneration and dementia, these results have broader implications for understanding the pathogenesis of a variety of conformational diseases (27). In addition to Bri-induced encephalopathy, these diseases include α-1 antitrypsin proteinopathy, Huntington's disease, and Parkinson's disease, all of which were shown to result from the significant cellular dysfunction induced by protein misfolding (28–30).

The rationale for studying the relationship between the UPR and proinflammatory signaling was based on several lines of evidence. In addition to previous *in vitro* studies demonstrating the induction of the UPR and ultimately ER stress in epithelial cells by mutant SP-C (8, 9, 18, 21, 31), markers of both ER stress and apoptosis were documented in lung epithelia *in vivo* in patients with classic IPF (usual interstitial pneumonitis, or UIP) and in patients with UIP expressing the SP-C BRICHOS domain mutation, L188Q (8, 32). Similarly, lung biopsies from patients with idiopathic pulmonary fibrosis demonstrated enhanced levels of expression of the CXC chemokine, IL-8, compared with control tissue (24). Although a direct link between these two events was previously lacking, this study demonstrates that the expression of SP-C BRICHOS mutants in alveolar epithelial cells results in the significant expression of IL-8 (Figure 3). Thus, taken together, our results complement previous *in vivo* studies, and support the concept of the alveolar epithelial cell as an important effector regulating the local inflammatory milieu in pulmonary fibrosis.

Crosstalk between the UPR and inflammation was suggested to occur in several cell types, including hepatocytes, pancreatic β-islet cells, neurons, and macrophages, all of which have in common either enhanced metabolic or immune function or the expression of misfolded mutant protein (10). Mechanistically, data from these studies infer that two well-known signaling pathways involved in inflammation, JNK/AP-1 and NFκB, can be induced by prolonged activation of the UPR, and are capable of serving as molecular integrators between the two processes. In addition, the IL-8 promoter was shown to contain both AP-1 and NFκB transactivation elements (33). Results from the present study recapitulate this theme, and are summarized in Figure 9. The expression of two different SP-C BRICHOS mutants resulted in the phosphorylation of JNK and its downstream target, the transcription factor AP-1. The release of IL-8 by cells transfected with either SP-$C^{\Delta exon4}$ or SP-C^{L188Q} was completely blocked by treatment with a JNK-specific inhibitor, SP600125 (Figure 6). Coupled with the findings that the activation of NFκB was differentially induced only by SP-$C^{\Delta exon4}$ (Figure 5), and that 4-PBA treatment blocked the activation of NFκB but not the release of IL-8 induced by SP-$C^{\Delta exon4}$ in lung epithelia (Figure 7), JNK signaling appears to represent the major pathway mediating crosstalk between the ER and the nucleus, to generate a proinflammatory cytokine response. Although the inhibition of JNK was shown to modulate many other ER stress–related cellular responses such as insulin resistance (34), to our knowledge, this is the first report of JNK inhibition attenuating proinflammatory responses to misfolded proteins.

Whereas previous studies established links between NFκB and the regulation of proinflammatory responses induced by

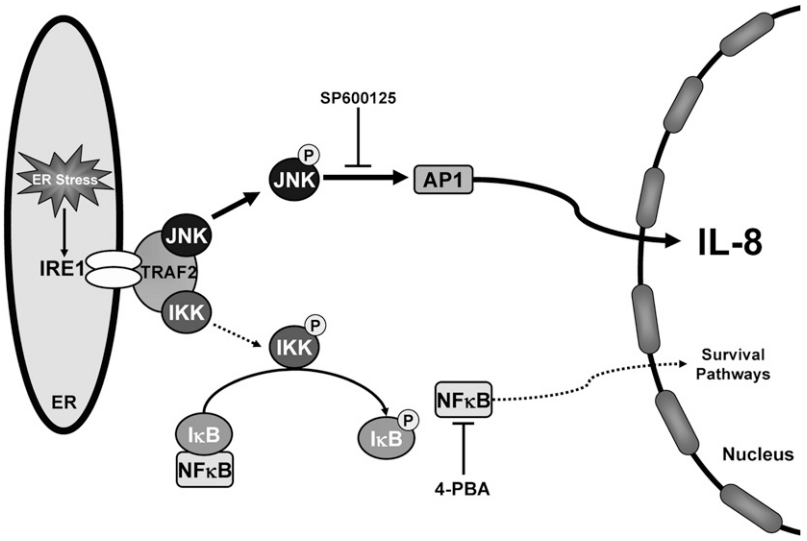

Figure 9. Proinflammatory signaling pathways induced by SP-C BRICHOS mutants. Expression of SP-C BRICHOS mutants (SP-$C^{\Delta exon4}$ and SP-C^{L188Q}) induce an unfolded protein response, in part via activation of a canonical endoplasmic reticulum stress–sensing pathway, using insulin response element 1 (*IRE1*). Conformational changes in the cytosolic domain of IRE1, which allow for binding to TNF-α receptor–associated factor 2 (*TRAF2*), can interface with both NFκB and mitogen-activated protein kinase (MAPK) pathways. IRE1–TRAF2 activates JNK through phosphorylation, with phospho-JNK subsequently activating the transcription factor, activating protein 1 (*AP1*). Nuclear translocation of AP-1 leads to an upregulation of IL-8 release. Inhibition of IL-8 release by SP600125 treatment indicates that JNK signaling represents the major pathway mediating the proinflammatory cytokine response. With more severe misfolding, as observed with SP-$C^{\Delta exon4}$, the IRE1–TRAF2 complex can also recruit IκB kinase (*IKK*), which phosphorylates and removes the IκB inhibitory subunit of NFκB. 4-PBA treatment downregulates NFκB, but not IL-8 release, and suggests that NFκB does not play a significant role in the inflammatory response of this system. P, phosphorylation modification.

cytokine stimulation (35), as well as between misfolded protein–induced ER stress and NFκB activation *in vitro* (36, 37), the upregulation of IL-8 by misfolded SP-C BRICHOS mutants could not be linked to the observed increase in NFκB activity. This finding suggests that the activation of NFκB in response to prolonged UPR or ER stress may play a different role. The constitutive activation of NFκB promotes survival by activating antiapoptotic genes in a wide range of cells, including malignant cells, B cells, and hepatocytes (38). Bridges and colleagues demonstrated that stably transfected HEK293 cells adapted to the chronic ER stress imposed by the constitutive expression of SP-$C^{\Delta exon4}$ via an NFκB-dependent pathway (31). Using gene array analysis, this adaptation was associated with the upregulation of antiapoptotic gene programs, and the inhibition of NFκB resulted in cell death. A similar response has not yet been reported for SP-C^{L188Q}. However, the differential response in the activation of NFκB by epithelial cells transfected with SP-$C^{\Delta exon4}$ versus SP-C^{L188Q} may reflect differences in the severity of mutations. Compared with SP-C^{L188Q}, the G > A mutation that produces SP-$C^{\Delta exon4}$ results in a 37 amino-acid deletion of the COOH flanking domain, resulting in a significant disruption of protein structure (39). Using macroaggregation as a biochemical readout, we previously reported that the Δexon4 mutation results in a more severe phenotype compared with the L188Q mutation (18), which is accompanied by an enhanced association with cytotoxicity *in vitro* (M.F.B., unpublished observations) and by a disruption of lung morphogenesis when SP-$C^{\Delta exon4}$ is expressed in transgenic mice (21). Thus, the severity of protein misfolding imparted by SP-$C^{\Delta exon4}$ may be more likely to trigger NFκB-mediated cytoprotective pathways than its SP-C^{L188Q} counterpart.

The pathogenesis of chronic lung remodeling via the expression of proSP-C BRICHOS mutants can be thought of as part of a unified view of the cellular and molecular pathogenesis of a larger family of conformational diseases. The realization that conformational diseases are attributable to either aberrant intermolecular aggregation or transport to non-native compartments makes possible both general and specific approaches for the correction of defects. Recent data suggest that preserving or restoring ER function may be of therapeutic value. Small molecules that are classified as chemical facilitators modulate protein folding and the UPR, potentially mitigating ER stress. One of these, 4-PBA, has attracted a great deal of attention as a therapeutic agent because of its capacity to restore proper trafficking of mutant CFTR to the plasma membrane (40). Previously, the addition of 4-PBA to A549 cells expressing the $SPC^{\Delta exon4}$ mutant was shown to prevent the formation of intracellular aggregates (7). Nonetheless, we show here that treatment with 4-PBA was characterized by a cell-dependent sustained or elevated release of IL-8, despite the attenuation of NFκB activation (Figure 7). Mechanistically, the lack of an anti-inflammatory effect of 4-PBA in our model system may have been attributable to off-target effects of 4-PBA, as described in other systems. These effects include histone deactylase inhibition (HDACi) (41–43) and enhanced MAPK (JNK) signaling (44). The treatment of cells with other inducers of HDACi *in vitro* (such as trichostatin A) was shown to modulate IL-8 production in both an NFκB-dependent and AP-1–dependent fashion, but this effect is highly cell-specific (33, 45). In addition, Roque and colleagues observed that 4-PBA enhances the production of IL-8 in lung epithelial cells expressing ΔF508–CFTR (44). The treatment of these cystic fibrosis cell lines with 4-PBA resulted in an activation of intracellular JNK signaling and the release of IL-8, despite a decrease in NFκB activation, a result similar to what we observed here in cells expressing BRICHOS mutants (Figures 7, 8, and E4).

Despite its effect on protein folding, it is unclear if 4-PBA exerts a consistent effect on ER stress. This conundrum was best described for Δ508 mutant CFTR, where a significant disparity also occurs in the effects of 4-PBA on folding and trafficking to the plasma membrane versus the UPR. Using IB3-1 cells expressing mutant Δ508 CFTR, 4-PBA actually increased the expression of ER stress markers such as GRP78 (ostensibly to promote folding) (46). In a preliminary report, 4-PBA treatment of HEK cells transiently expressing SP-C BRICHOS mutants was associated with the sustained expression of ER stress response proteins and cytotoxicity (47). Although we found no evidence of cytotoxicity from 4-PBA, we also observed in our models that GRP78/BiP concentrations were unaffected by treatment with 4-PBA (J.A.M., unpublished observations). Altogether, studies of CFTR and SP-C suggest that, for ER stress associated with mutant protein expression (versus chemically or metabolically induced ER stress), the beneficial effects seen with regard to protein folding mediated by 4-PBA are dissociated from the measured expression of key chaperones and UPR markers. Furthermore, the propensity for

4-PBA to decrease NFκB survival pathways, while promoting JNK-mediated inflammatory signaling, may obscure potential beneficial effects on protein folding and trafficking.

In conclusion, we demonstrate that SP-C BRICHOS mutants promote proinflammatory signaling in lung epithelial cells through the integration of UPR and inflammation via a JNK-dependent signaling cascade. Interestingly, in an emerging therapy aimed at improving protein folding, 4-PBA had deleterious effects on cytokine release in the same model system. Understanding these complex signaling pathways that integrate ER stress and inflammation may lead to effective new therapies for treating a previously untreatable, devastating disease. However, conformational diseases, as seen with mutant SP-C, will likely require combination therapies aimed at the restoration of protein folding, the enhanced clearance of protein aggregates, the blockade of inflammatory signaling, and the appropriate modulation of cytotoxicity and apoptosis. A number of studies showed that, in addition to 4-PBA, treatment with small molecule chaperones can restore proper protein folding and ER function. These chaperones include taurine-conjugated ursodeoxycholic acid, vaticanol B, and others, which were shown to decrease aggregate formation and prevent an ER stress response (48, 49). Although these treatments have shown promising results, they will likely have to be simultaneously evaluated for off-target effects on the many other cellular pathways that link ER stress and cellular dysfunction.

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