Adaptation and increased susceptibility to infection associated with constitutive expression of misfolded SP-C

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Mutations in the gene encoding SP-C (surfactant protein C; SFTPC) have been linked to interstitial lung disease (ILD) in children and adults. Expression of the index mutation, SP-C^{Δexon4}, in transiently transfected cells and type II cells of transgenic mice resulted in misfolding of the proprotein, activation of endoplasmic reticulum (ER) stress pathways, and cytotoxicity. In this study, we show that stably transfected cells adapted to chronic ER stress imposed by the constitutive expression of SP-C^{Δexon4} via an NF-κB-dependent pathway. However, the infection of cells expressing SP-C^{Δexon4} with respiratory syncytial virus resulted in significantly enhanced cytotoxicity associated with accumulation of the mutant proprotein, pronounced activation of the unfolded protein response, and cell death. Adaptation to chronic ER stress imposed by misfolded SP-C was associated with increased susceptibility to viral-induced cell death. The wide variability in the age of onset of ILD in patients with SFTPC mutations may be related to environmental insults that ultimately overwhelm the homeostatic cytoprotective response.

Introduction

SP-C (surfactant protein C) is a transmembrane protein that is synthesized as a 191–197-amino acid proprotein by type II epithelial cells of the lung. Processing of the proprotein in the distal secretory pathway liberates a 35–amino acid peptide that is secreted into the airspaces as a component of pulmonary surfactant. Mutations in the gene encoding SP-C (SFTPC) have been associated with the development of sporadic and familial interstitial lung disease (ILD). In all cases to date, the mutation was present on only one allele consistent with a dominant-negative effect. The index mutation, a heterozygous base substitution of A for G at the first base of intron 4 (c.460 +1 G→A), led to the internal deletion of 37 amino acids from the ER luminal domain, generating a truncated proprotein (SP-C^{Δexon4}; Nogee et al., 2001). Two separate SFTPC mutations associated with ILD, SP-C^{Δ188Q} and SP-C^{Δ337}, were detected in three kindreds (Thomas et al., 2002a; Chibbar et al., 2004; Cameron et al., 2005). The age of onset and penetrance of ILD varied markedly in all three kindreds.

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Abbreviations used in this paper: ERAD, ER-associated degradation; ILD, interstitial lung disease; MFI, mean fluorescence intensity; MOI, multiplicity of infection; PI, propidium iodide; RSV, respiratory syncytial virus; SP-C, surfactant protein C; SR, super repressor; UPR, unfolded protein response.

The online version of this article contains supplemental material.

Studies in transiently transfected cells suggested that the c.460 +1 G→A mutation led to misfolding of the mutant proprotein, retention of SP-C^{wt} in the ER, activation of the unfolded protein response (UPR), and apoptosis (Bridges et al., 2003; Wang et al., 2003; Mulugeta et al., 2005). SP-C^{Δexon4} was also associated with cytotoxicity and lung dysmorphogenesis when expressed in type II cells of transgenic mice (Bridges et al., 2003). The UPR is activated by conditions that perturb ER homeostasis, including the accumulation of misfolded proteins (Schröder and Kaufman, 2005). This response encompasses translational and transcriptional changes within the cell to alleviate the stress and to promote restoration of ER homeostasis. A model for the time-dependent induction of the UPR has been proposed, suggesting that translational repression via PERK activation/eIF2α phosphorylation occurs first followed by the cleavage of ATF6, activation of IRE1/XBP-1, and expression of ATF6 and XBP-1 target genes (Yoshida et al., 2003). If ER homeostasis cannot be restored by these pathways or by the induction of adaptive responses, apoptosis may occur as a means of avoiding the untoward effects of cell necrosis. ER stress–induced apoptosis has been associated with induction of the transcription factor C/EBP homologous protein, activation of c-Jun amino-terminal kinase via IRE1, and activation of the ER stress–specific...
caspases 4 (human; Hitomi et al., 2004) and 12 (mouse; Nakagawa et al., 2000; Urano et al., 2000; Hetz et al., 2003; for review see Oyadomari and Mori, 2004). Although the effects of acute ER stress, which is imposed by xenotoxic agents such as thapsigargin and tunicamycin, are well established, little is known about the molecular pathways involved in adaptation to chronic ER stress imposed by a misfolded protein.

The variability in the age of onset and penetrance of disease in the SP-CL188Q and SP-CL73T pedigrees suggests that both genetic and environmental factors may influence the manifestation of lung disease. Based on the results of acute ER stress, which is imposed by xenotoxic agents such as thapsigargin and tunicamycin, are well established, little is known about the molecular pathways involved in adaptation to chronic ER stress imposed by a misfolded protein.

The variability in the age of onset and penetrance of disease in the SP-CL188Q and SP-CL73T pedigrees suggests that both genetic and environmental factors may influence the manifestation of lung disease. Based on the results of the aforementioned studies in human patients and transiently transfected cells, experiments were designed to test the hypotheses that (1) chronic ER stress imposed by misfolded SP-C promotes adaptation and cell survival and (2) adaptation increases susceptibility to environmental stress. Clonal cell lines stably expressing SP-C∆exon4 or SP-Cwt were generated to identify cytoprotective pathways that are associated with adaptation to the constitutive expression of misfolded SP-C and to assess the cytotoxic effects of environmental stress on adapted cells.

**Results**

**Generation and characterization of stably transfected cell lines**

To determine the molecular mechanisms underlying SP-C∆exon4-induced cytotoxicity, HEK293 cell lines stably expressing SP-Cwt or SP-C∆exon4 were generated. Multiple clonal lines were obtained for each construct, and two lines were chosen for subsequent experimentation based on equivalent expression of SP-C mRNA, which was initially assessed by RT-PCR (Table I) and subsequently confirmed by microarray analysis (Fig. 1 b). These cell lines were morphologically indistinguishable by light microscopy (Fig. 1 a) or electron microscopy (not depicted) and exhibited similar doubling rates (not depicted). Basal SP-C protein levels were assessed by Western blot analysis of cell lysates with an antibody directed against the NH2-terminal peptide of the proprotein (proSP-C), a region which is unaffected by the ∆exon4 mutation. Despite equivalent mRNA levels, expression of the SP-C∆exon4 protein was barely detectable compared with SP-Cwt, which is consistent
| Fold change (microarray) | Fold change (real-time PCR) | Gene | Description | Function* | NF-κB-binding sites |
|-------------------------|----------------------------|------|-------------|-----------|---------------------|
| +4.8                    | +4.2                       | MYC  | V-myc myelocytomatisis viral oncogene homologue (avian) | Regulation of apoptosis | 1                   |
| +2.3                    | ND                         | SNCA | Synuclein, α | Anti      | 1                   |
| +2.1                    | ND                         | PROK2| Prokineticin 2 | Anti      | 1                   |
| +1.7                    | +2.9                       | IKBKG| Inhibitor of κ-light polypeptide gene enhancer in B cells, kinase γ | Pro | 1                   |
| +1.6                    | ND                         | PBEF | Pre-B cell colony–enhancing factor 1 | Anti | 1                   |
| +1.6                    | ND                         | BNIP1| Bcl2 adenovirus E1B 19-kD interacting protein 1 | Anti and pro | 2                   |
| +1.5                    | ND                         | MAPK14| Mitogen-activated protein kinase 14 | Anti | 2                   |
| +1.5                    | ND                         | MYB | V-myb myeloblastosis viral oncogene homologue (avian) | Regulation of apoptosis | 1                   |
| +2.1                    | ND                         | PDCD8| Programmed cell death 8 | Pro | 0                   |
| +2.0                    | ND                         | HRK | Harakiri, Bcl2-interacting protein | Pro | 0                   |
| +1.8                    | ND                         | HTATIP2| HIV-1 Tat interactive protein 2 | Anti | 0                   |
| +1.7                    | ND                         | BCA3| B cell receptor–associated protein 21 | Apoptosis | 0                   |
| +1.6                    | ND                         | PLAGL1| Pleiomorphic adenoma genelike 1 | Pro | 0                   |
| +1.6                    | ND                         | BAX | Bcl2-associated X protein | Pro | 0                   |
| +1.6                    | ND                         | P8  | P8 protein | Pro | 0                   |
| +1.6                    | ND                         | BAG2| Bcl2-associated anathogene 2 | Apoptosis | 0                   |
| +1.6                    | ND                         | MRPS30| Mitochondria ribosomal protein S30 | Apoptosis | 0                   |
| +1.5                    | ND                         | TNFRSF6| TNF receptor superfamily, member 6 | Anti | 0                   |
| −3.0                    | ND                         | SFRP1| Secreted frizzled rp1 | Anti | 2                   |
| −3.0                    | ND                         | CD24 | CD24 antigen | Pro | 1                   |
| −2.1                    | ND                         | FLI1 | Friend leukemia virus integration 1 | Cell death | 1                   |
| −2.0                    | ND                         | RUNX3| Run-related transcription factor 3 | Apoptosis | 2                   |
| −1.6                    | ND                         | PSEN1| Presenilin 1 | Anti | 1                   |
| −1.6                    | ND                         | CDC2L2| Cell division cycle 2-like 2 | Apoptosis | 1                   |
| −3.6                    | ND                         | DUSP6| Dual specificity phosphatase 6 | Apoptosis | 1                   |
| −3.0                    | ND                         | TGF-β1| Transforming growth factor β1 | Anti | 0                   |
| −2.1                    | ND                         | Bcl2 | B cell CLL/lymphoma 2 | Anti | 0                   |
| −1.9                    | ND                         | PHLD1| Pleckstrin homology-like DA2 | Apoptosis | 0                   |
| −1.9                    | ND                         | AKT1 | V-akt murine thymoma viral oncogene | Anti | 0                   |
| −1.7                    | ND                         | TIA1 | RNA-binding protein | Apoptosis | 0                   |
| −1.8                    | ND                         | PTPN6| Protein tyrosine phosphatase N6 | Apoptosis | 0                   |
| −1.7                    | ND                         | PRKCZ| Protein kinase C, ζ | Anti | 0                   |
| −1.7                    | ND                         | MCL1 | Bcl-2 related protein | Anti and pro | 0                   |
| +1.7                    | +2.3                       | IRAK1| IL-1 receptor-associated kinase 1 | NF-κB pathway | 0                   |

The clonal cell lines expressing SP-Cwt, SP-CΔexon4, or empty vector were subjected to transcriptional profiling using the human U133 gene chip set from Affymetrix. Genes that were specifically increased or decreased in SP-CΔexon4 over both SP-Cwt and an empty vector control (i.e., mutant-specific changes) are reported as fold changes from microarray analysis (first column). Select targets were validated by real-time PCR (second column). The last column lists the number of putative NF-κB-binding sites located in the 5′ flanking sequence (1 kb) of the gene on the left. Data represent the mean of three analyses for each genotype.

*Anti and pro designations signify anti- and pro-apoptosis; evidence of apoptosis is from the Gene Ontology database.

with rapid proteasome-dependent turnover of the mutant pro-protein (Fig. 1 c).

SP-CΔexon4 protein was previously shown to be rapidly degraded in a proteasome-dependent manner and failed to be exported from the ER when transiently expressed in HEK293 cells (Bridges et al., 2003). To determine the subcellular localization of the SP-C variants in stably transfected cell lines, cells were stained with antibodies directed against proSP-C and LAMP-1 and analyzed by confocal microscopy. A bright, punctuate staining pattern was observed for proSP-C in SP-Cwt cells, whereas cells expressing SP-CΔexon4 showed faint, diffuse staining that was detected only in the presence of proteasome inhibitor...
The clonal cell lines expressing empty vector (EV), SP-Cwt, or SP-C∆exon4 (Fig. 1d). In contrast, SP-C∆exon4 demonstrated efficient export of wild-type protein from the ER (open bars) or SP-Cwt (closed bars) clonal cell lines were transiently transfected with pELAM-Luc, pRL-TK, and increasing amounts (10 pg–1 ng) of a luciferase reporter construct (Fig. 2a). Cotransfection of a stabilized, nonphosphorylatable form of IκBα (super repressor [SR]) with the NF-κB reporter resulted in a dose-dependent decrease in luciferase activity in SP-C∆exon4 cells that was completely suppressed to levels detected in untreated SP-Cwt cells (Fig. 2b). IκBα protein in SP-C∆exon4 cells was decreased compared with SP-Cwt cells, which is consistent with increased NF-κB activity (Fig. 2c).

Treatment of SP-C∆exon4 cells with SN50 peptide to inhibit the nuclear translocation of NF-κB resulted in cellular retraction and a significant increase in cell death, as indicated by propidium iodide (PI) staining (Fig. 3a) and MTS reduction assay (Fig. 3c). Treatment of SP-C∆exon4 cells with a control peptide, SN50mut, at equivalent doses and duration had no effect (Fig. 3b). Cells expressing SP-Cwt were unaffected by SN50 or SN50mut treatment (not depicted). Treatment of SP-C∆exon4 cells with SN50 decreased basal NF-κB activity as measured by a NF-κB reporter (Fig. 3d) and inhibited nuclear translocation of the p65 subunit of NF-κB (Fig. 3e). Altogether, these results suggest that NF-κB, mediated in part by the p65 subunit, plays an important cytoprotective role in adaptation to the constitutive expression of SP-C∆exon4.

SP-C∆exon4 expression increases susceptibility to viral-induced death

The increased expression of pro-apoptotic transcripts in SP-C∆exon4 cells (Table 1) suggested that adapted cells may...
be more susceptible to secondary stress. Infection with respiratory syncytial virus (RSV) preceded the onset of ILD in several patients carrying the SP-C<sup>L188Q</sup> mutation. To determine whether the expression of SP-C<sup>Δexon4</sup> increased susceptibility to RSV, the clonal cells lines were infected at a multiplicity of infection (MOI) of 10 pfu/cell, and cell viability was assessed 24 h after infection. Although cell viability was minimally affected in SP-C<sup>wt</sup> cells, as indicated by the low level of PI staining (Fig. 4 a [b and f]), the infection of cells expressing SP-C<sup>Δexon4</sup> resulted in significant cell death (Fig. 4 a [d and h]). Western blot analysis demonstrated that SP-C<sup>Δexon4</sup> protein accumulated in a manner directly correlated with the viral titer, whereas SP-C<sup>wt</sup> protein levels were unaffected (Fig. 4 b). Several viruses, including adenovirus, influenza, and hepatitis B and C viruses, have been reported to induce ER stress/UPR pathways (Meyer et al., 1992; Pahl and Baeuerle, 1995; Pahl et al., 1996; Tardif et al., 2004). To determine whether RSV activated the UPR in cells stably expressing SP-C, an UPR element luciferase reporter construct was transiently transfected into cells.
Figure 4. **SP-C∆exon4 increases susceptibility to RSV-induced cell death.** (a) Cells expressing SP-Cwt (panels a, b, e, and f) or SP-C∆exon4 (panels c, d, g, and h) were infected with RSV at 10 MOI (panels b, f, d, and h). Cells were stained with propidium iodide (PI) 24 hr after infection, and phase (panels a–d) or fluorescent (panels e–h) images were captured directly from the culture plate. Bars, 50 μm. (b) Immunoblot analysis of cells expressing SP-Cwt or SP-C∆exon4 after infection with 0.1, 1, or 10 MOI RSV for 24 h. Immunoblots were probed with an anti-proSP-C antibody or an anti-actin antibody (loading control). (c) Clonal cell lines expressing SP-Cwt or SP-C∆exon4 were transiently transfected with a reporter construct consisting of five UPR elements driving luciferase (UPRE-Luc) and pRL-TK. Cells were infected with 5 MOI RSV 24 h after transfection, and luciferase activity was assessed 24 h after infection. Results are presented as means ± SD (error bars) of the ratio of firefly/renilla, expressed as relative luciferase units; each determination was performed in triplicate, and the experiment was repeated four times. (d) Cell lines were transiently transfected with pELAM-Luc and pRL-TK vectors and infected with RSV at indicated doses 24 h after transfection. (e) SP-C∆exon4 cells were transiently transfected with the UPRE-Luc and pRL-TK vectors. Cells were treated with SN50 or SN50mut peptide 24 h after transfection at indicated doses. Cells were treated with 10 μg/ml tunicamycin for 12 h for positive control. (d and e) Luciferase activity was assessed 48 h after transfection and normalized to renilla luciferase activity. Results are presented as means ± SD; each determination was performed in triplicate, and the experiment was repeated three times. Data indicate fold change relative to untreated control for each genotype. (c–e) #, P < 0.05 compared with SP-Cwt unRx; *, P < 0.05 compared with SP-C∆exon4 unRx; ◆, P < 0.05 compared with RSV-infected SP-Cwt.

expressing SP-C∆exon4 or SP-Cwt before infection with RSV (Fig. 4 c). Baseline levels of luciferase activity were similar between the cell lines, which is consistent with the microarray data, indicating that the UPR was not activated in cells stably expressing SP-C∆exon4. However, luciferase activity was increased in both cell lines after RSV infection, and the effect was exacerbated in SP-C∆exon4 cells (2.5-fold increase in SP-Cwt vs. 4.4-fold increase in SP-C∆exon4; Fig. 4 c).
Furthermore, RSV-induced cell death appeared to be independent of NF-κB, as NF-κB activity was sustained in both RSV-infected SP-C\textsuperscript{Δexon4} and SP-C\textsuperscript{wt} cells (Fig. 4 d). Consistent with this finding, UPR activity was not induced in SP-C\textsuperscript{Δexon4} cells when NF-κB activity was inhibited with SN50 (Fig. 4 e). Collectively, these data indicate that RSV infection was associated with accumulation of the mutant proprotein, pronounced activation of the UPR, and increased cell death independent of NF-κB activity.

Proteasome function is decreased in RSV-infected SP-C\textsuperscript{Δexon4} cells

Accumulation of mutant SP-C proprotein in RSV-infected SP-C\textsuperscript{Δexon4} cells suggested that proteasome function was
inhibited. To assess the impact of RSV infection on proteasome activity, a proteasome reporter construct, pZsProSensor-1, was transiently transfected into the clonal cell lines. Cells were infected with 1–10 MOI of RSV and analyzed 24 h later by fluorescence microscopy and FACS analysis. Fluorescence in untreated SP-C\textsuperscript{Δexon4} cells was higher than that observed in untreated SP-C\textsuperscript{wt} cells, indicating a modest inhibition of basal proteasome activity in the presence of the mutant proprotein (Fig. 5, a vs. b). Infection of SP-C\textsuperscript{Δexon4} cells with RSV resulted in a dose-dependent increase in fluorescence detected by microscopy and FACS (Fig. 5 a vs. h). In contrast, fluorescence in untreated SP-C\textsuperscript{wt} cells was inhibited by RSV infection (Fig. 5 b vs. h). Transfection efficiency was similar between the two cell lines, and the proteasome reporter was activated in SP-C\textsuperscript{wt} cells treated with MG-132 (Fig. 5 c). These results demonstrate that basal proteasome function is decreased in SP-C\textsuperscript{Δexon4} cells and that this perturbation is exacerbated during RSV infection.

**SP-C\textsuperscript{Δexon4} sensitizes cells to proteasome inhibition and is associated with accumulation of mutant proprotein and activation of XBP-1**

To determine whether decreased proteasome function induced ER stress and activation of apoptosis independently of RSV infection, the clonal cell lines were treated with the proteasome inhibitor MG-132. The SP-C\textsuperscript{Δexon4} cell line was exquisitely sensitive to proteasome inhibition. The majority of cells retracted and detached from the plate after treatment with 250 nM MG-132 for 18 h, and a more pronounced effect was detected at the 500 nM of concentration (Fig. 6 a vs. h). Total RNA was isolated from cells and subjected to RT-PCR analysis with primers spanning the intron of unspliced XBP-1 mRNA. XBP-1(u) denotes unspliced, inactive XBP-1; XBP-1(s) denotes spliced, active XBP-1; products were separated on a 4% agarose gel.
was performed with a proSP-C antibody after MG-132 treatment. There was a dose-dependent increase in SP-C<sub>Δexon4</sub> protein, whereas levels of the SP-C<sub>wt</sub> protein were unaffected by MG-132 treatment (Fig. 6 c). Accumulation of unfolded proteins in the ER results in the activation of the IRE1–XBP-1 pathway (for review see Harding et al., 2002). Active XBP-1 was not detected in SP-C<sub>wt</sub> cells after MG-132 treatment; in contrast, robust activation of XBP-1 was detected in SP-C<sub>Δexon4</sub> cells after proteasome inhibition (Fig. 6 d). Collectively, these results demonstrate that proteasome inhibition resulted in dose-dependent cytotoxicity associated with the accumulation of SP-C<sub>Δexon4</sub> and the activation of XBP-1.

**Accumulation of SP-C<sub>Δexon4</sub> is associated with apoptosis**

To determine whether the accumulation of SP-C<sub>Δexon4</sub> after proteasome inhibition was associated with apoptosis, cell lines were treated with MG-132 and stained with the fluorescent DNA-intercalating dye H33342 to assess nuclear architecture. Cells expressing SP-C<sub>wt</sub> showed a diffuse, uniform staining of the nucleus typical of viable, healthy cells (Fig. 7 a, left panel and inset). Although the majority of cells expressing SP-C<sub>Δexon4</sub> detached from the plate after MG-132 treatment and were excluded from this analysis, the cells that remained attached exhibited punctuate staining and nuclear condensation that is consistent with apoptosis (Fig. 7 a, right panel and inset). Furthermore, treatment of SP-C<sub>Δexon4</sub>-expressing HEK293 cells with MG-132 for 18 h resulted in the activation of caspase-3 (Fig. 7 b). Caspase-3 activation was not detected in SP-C<sub>wt</sub> cells treated with MG-132, indicating that this event was specific for cells expressing mutant SP-C<sub>Δexon4</sub> (Fig. 7 b). As previously reported, the expression of SP-C<sub>Δexon4</sub> in the distal lung epithelium of transgenic mice resulted in cytotoxicity associated with lung dysmorphogenesis (Bridges et al., 2003). Immunohistochemistry was performed on fetal lung sections from a transgenic positive animal (Fig. 7 d, TG#2) and a wild-type littermate control (Fig. 7 d, WT) with an antibody that detects the cleaved isoform of caspase-3. Robust staining for active caspase-3 was positive animal (Fig. 7 d, TG#2) and a wild-type littermate control (Fig. 7 d, WT) with an antibody that detects the cleaved isoform of caspase-3. Robust staining for active caspase-3 was present in sloughed epithelial cells in the distal airway and isoform of caspase-3. Robust staining for active caspase-3 was detected in SP-C<sub>Δexon4</sub> cells after proteasome inhibition (Fig. 6 d). Collectively, these results demonstrate that proteasome inhibition resulted in dose-dependent cytotoxicity associated with the accumulation of SP-C<sub>Δexon4</sub> and the activation of XBP-1.

**Discussion**

The SP-C<sub>Δexon4</sub> mutation was chosen for these experiments because of its link to ILD in human patients (Nogee et al., 2001, 2002), the severity of this mutation on the structure of the proprotein, and its association with cytotoxicity and lung dysmorphogenesis when expressed in transgenic mice (Bridges et al., 2003). Results of experiments performed in this study using stably transfected cells indicated that the SP-C<sub>Δexon4</sub> cell line adapted to the constitutive expression of misfolded SP-C and that NF-κB played a pivotal role in the adaptive response. Infection of cells expressing SP-C<sub>Δexon4</sub> with RSV resulted in enhanced cytotoxicity associated with decreased proteasome function, accumulation of the mutant

![Figure 7. Accumulation of SP-C<sub>Δexon4</sub> is associated with cell death.](image-url)
proprotein, and cell death. Collectively, these results suggest that adaptation to chronic ER stress imposed by misfolded SP-C may promote resistance to ILD, whereas environmental insults, such as viral infection, may trigger the onset of disease in patients with mutations in SFTP C.

Although the effects of acute ER stress, imposed by xenotoxic agents such as thapsigargin and tunicamycin, are well established, little is known about the molecular pathways involved in adaptation to chronic ER stress imposed by a misfolded protein. The lack of a cytotoxic response coupled with the absence of ER stress induction in SP-CΔexon4 clones suggested that these cells successfully adapted to the constitutive expression of misfolded SP-C. Several lines of evidence support this hypothesis. First, the level of SP-C mRNA in the SP-CΔexon4 cell line was comparable with that in the SP-C wild-type cell line, thus excluding low expression of the mutant protein as a reason for the survival of SP-CΔexon4 cells. Second, in contrast to the wild-type protein, SP-CΔexon4 protein was barely detectable, indicating that the misfolded proprotein was recognized as terminally misfolded and rapidly degraded via the ERAD pathway. Furthermore, chronic expression of SP-CΔexon4 was associated with modestly reduced proteasome function that is consistent with near saturation of this degradative pathway. Third, components of the ER stress/UPR pathways were not transcriptionally up-regulated in the clonal SP-CΔexon4 cell line, which is in marked contrast to results in transiently transfected HEK293 cells (Bridges et al., 2003; Mulugeta et al., 2005). Fourth, chronic expression of SP-CΔexon4 was associated with differential expression of both pro- and anti-apoptotic genes, approximately half of which contained putative NF-κB-binding sites in their promoters. Lastly, the inhibition of NF-κB, which is known to promote cell survival in response to a variety of stresses, resulted in the death of SP-CΔexon4 cells. Collectively, these results are consistent with an NF-κB-dependent adaptive response to chronic ER stress imposed by the constitutive expression of SP-CΔexon4. Although the induction of other molecular pathways involved in cell survival may occur, the activation of NF-κB appears to be critical for survival in HEK293 cells that constitutively express SP-CΔexon4. Conclusively, NF-κB–dependent adaptive response to chronic ER stress imposed by the constitutive expression of SP-CΔexon4 results in the differential expression of apoptosis-related genes with an apparent balance shifted toward NF-κB–dependent anti-apoptotic responses. Identification of environmental triggers that tip the balance toward apoptosis/cell death are clearly important for understanding disease pathogenesis in humans with SFTP C mutations. ILD is often associated with infection and/or inflammation (Vassallo, 2003; Noble and Homer, 2004). The marked variability in severity and age of onset of lung disease in the SP-CL188Q pedigrees suggested that environmental factors might be involved in triggering the onset of ILD (Thomas et al., 2002a; Chibbaro et al., 2004). Consistent with this hypothesis, five individuals in the SP-CΔexon4 kindreds were diagnosed with viral infection before the manifestation of lung disease. Importantly, cells stably expressing SP-CΔexon4 were much more susceptible to viral-induced cell death, suggesting that infection may be an environmental trigger for ILD.

Although RSV infection in vivo is associated with epithelial cell damage and cellular desquamation, these cytotoxic effects are primarily mediated by an augmented immune response initiated by the infected host cells rather than viral-induced cell death. In support of this hypothesis, mice depleted of CD4 and CD8 cells exhibited persistent viral replication without illness (Graham et al., 1991). Furthermore, RSV infection of cultured airway epithelial cells (MOI of ~20; i.e., twice the dose used in this study) did not result in cytopathology (Zhang et al., 2002). Consistent with these findings, HEK293 cells constitutively expressing SP-C wild-type exhibited relatively little cell death in response to RSV infection (Fig. 4 a). In marked contrast, RSV infection of cells expressing SP-CΔexon4 resulted in extensive cell death associated with proteasome inhibition and accumulation of mutant proprotein. In vivo, injury of alveolar epithelial cells is an early event in progressive pulmonary fibrosis. Impaired reepithelialization leads to sustained proliferation/activation of interstitial fibroblasts and myofibroblasts, resulting in the accumulation of extracellular matrix and, ultimately, fibrosis (Thannickal et al., 2004; Noble and Homer, 2005). The increased susceptibility of some
individuals to severe RSV pneumonia may further contribute to variability in the severity and onset of the disease (Peebles and Graham, 2005).

Although RSV infection was specifically cytotoxic for SP-C$\Delta$exon4-expressing cells, the molecular pathway leading to cell death is not clear. Cell death did not appear to be apoptosis dependent, as caspase-3 cleavage was undetectable in the RSV-infected cells (Fig. 7 c). RSV has been shown to inhibit apoptosis of cultured epithelial cells via the activation of EGF receptor–extracellular signal-regulated kinase and PI3K–AKT signaling pathways, thereby promoting self-replication (Thomas et al., 2002b; Monick et al., 2005). In this study, RSV-induced cytotoxicity was associated with proteasome dysfunction, accumulation of the mutant proprotein SP-C$\Delta$exon4, and pronounced activation of the UPR. Induction of ER stress and proteasome inhibition in RSV-infected SP-C$\Delta$exon4 cells may be related, in part, to forced synthesis of viral membrane proteins, including the F, G, and SH proteins. UPR activation was higher in SP-C$\Delta$exon4 cells than in SP-Cwt cells, suggesting that the production of RSV membrane proteins superimposed on the expression of misfolded SP-C$\Delta$exon4 protein may overwhelm the degradative capacity of the proteasome, leading to accumulation of cytotoxic forms of SP-C$\Delta$exon4 and subsequent cell death. Therefore, it is possible that proteasome dysfunction and accumulation of SP-C$\Delta$exon4 drives the cell down a nonapoptotic cell death pathway in the presence of RSV. In contrast, accumulation of SP-C$\Delta$exon4 in transgenic mice and MG-132–treated cells (i.e., in the absence of RSV infection) resulted in caspase-3 activation, which is typical of apoptosis (Fig. 7 c; Bridges et al., 2003).

Based on the findings of this study, the following model is proposed. SP-Cwt protein is correctly folded and exported from the ER, whereas SP-C$\Delta$exon4 is terminally misfolded and degraded by a proteasome-dependent pathway. Constitutive expression of terminally misfolded SP-C$\Delta$exon4 results in chronic ER stress and an NF-κB–dependent cytoprotective response. Superimposition of a secondary stress, such as RSV infection, on the constitutive expression of SP-C$\Delta$exon4 leads to proteasome dysfunction, accumulation of SP-C$\Delta$exon4, and cytotoxicity. Accumulation of SP-C$\Delta$exon4 and cytotoxicity may contribute to the pathogenesis of ILD associated with mutations in SFTPC.

Materials and methods

Reagents

Recombinant TNFα protein was obtained from PeproTech. The MTS reduction assay kit and Dual-Luciferase Reporter Assay System were purchased from Promega. MTS reduction assays were performed in 96-well plates according to the manufacturer’s protocol. MG-132, SN50, and control peptide (SN50mut) were obtained from EMD Biosciences. MG-132, SN50, and control peptides were obtained from Promega; MTS reduction assays were performed in 96-well plates according to the manufacturer’s protocol. The MTS reduction assay kit was obtained from Promega. The MTS reduction assay kit was obtained from Promega. The MTS reduction assay kit was obtained from Promega. The MTS reduction assay kit was obtained from Promega. The MTS reduction assay kit was obtained from Promega. The MTS reduction assay kit was obtained from Promega.

SP-C cDNA constructs and generation of stably transfected cell lines

Full-length human wild-type SP-C (SP-C$^\text{wt}$) cDNA and SP-C$\Delta$exon4, generated as previously described (Bridges et al., 2003), were subcloned into pTRE2-Hyg (BD Biosciences) to generate SP-C$^\text{wt}$/pTRE2-Hyg and SP-C$\Delta$exon4/pTRE2-Hyg. To generate stably transfected cell lines, HEK293 Tet-Off cells (CLONTECH Laboratories, Inc.) were transiently transfected with SP-C$^\text{wt}$/pTRE2-Hyg or SP-C$\Delta$exon4/pTRE2-Hyg using LipofectAMINE 2000 (Invitrogen) in the presence of 250 μg/ml hygromycin B as the selection agent. Doxycycline was present in the media during the selection process to obtain stably transfected lines (i.e., expression of SP-C$^\text{wt}$ or SP-C$\Delta$exon4 was dependent on doxycycline withdrawal). However, all lines that were initially regulatable eventually reverted to constitutive expression of the transgene. Therefore, SP-C$^\text{wt}$ or SP-C$\Delta$exon4 cells from this study were cultured in the absence of doxycycline and G418 (selection agent for the tTA cassette). Clonal colonies were isolated, amplified, and screened for integration of the transgene by RTPCR using SP-C–specific primers and Western analyses using an antibody directed against the NH2-terminal peptide of proSP-C (Bridges et al., 2003).

RNA isolation, RT-PCR, and microarray analysis

Total RNA for RTPCR and real-time PCR analysis was isolated using the acidified guanidinium method (Chomczynski and Sacchi, 1987), treated with DNase I (DNA free; Ambion), and reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen). Forward and reverse primer sequences for human XBP-1 are 5′-GGGATTAAGAGACCGGCTG-3′ and 5′-TGAGAGGGTGCTTCCTCCGATT-3′. PCR reactions for XBP-1 were performed for 35 cycles, and products were separated on 4% agarose gels. Verification of microarray data for selected genes was performed by real-time PCR analysis with a SmartCycler (Cepheid) using reaction conditions as previously described (Hyatt et al., 2004).

RNA samples for microarray analysis were prepared as previously described (DeFelice et al., 2003) and hybridized to the GeneChip Human Genome U133 Set (HG-U133A and HG-U133B; Affymetrix, Inc.) according to the manufacturer’s protocol. Affymetrix Microarray Suite 5.0 was used to scan and quantitate the gene chips under default scan settings. Normalization was performed using the Robust Multichip Average model (Irizarry et al., 2003a,b). Data were further analyzed using Significance Analysis Of Microarrays (Tusher et al., 2001) and GeneSpring 7.2 (Silicon Genetics). Detection of differential expression was performed using random permutation and Welch’s approximate F test for mutant and control groups if P ≤ 0.01, false discovery rate ≤ 10%, a minimal of twofold changes in absolute ratio, and a minimum of two present calls by Affymetrix algorithm in three samples with the relative higher expression. Gene ontology analysis was performed using the database for annotation, visualization, and integrated discovery (Dennis et al., 2003). Potential protein–protein interactions were identified using PathwayAssist (Ariadne Genomics). NF-kB-binding sites (GGGACCTTCCC) were scanned in ~2-kb promoter regions of all differentially expressed genes from microarray analysis using MathInspector (Genomatix), allowing a maximum of one mismatch.

Cell culture and Western analysis

HEK293 stable cell lines were propagated as previously described (Bridges et al., 2003). Cell lysis preparation, protein standardization, and Western analysis were performed as previously described (Bridges et al., 2003). Nuclear extracts were isolated as previously described (Odoms et al., 2003). Antibody sources are as follows: anti–proSP-C (Bridges et al., 2003), anti–caspase-3 (Cell Signaling), antihistone H2B (Ingenex), anti–proSP-C (Bridges et al., 2003).

Confocal fluorescence

Cells for confocal microscopy were prepared as previously described (Conkright et al., 2001). Primary antibodies included a polyclonal anti–proSP-C (Bridges et al., 2003), a monoclonal anti-LAMP1/CD107A (Research Diagnostics, Inc.), and a monoclonal anti-KDEL (StressGen Biotechnologies). Secondary antibodies included anti-rabbit FITC-conjugated and anti–mouse Texas red–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Vectashield Hardset (Vector Laboratories) was used as the mounting medium. Fluorescence was visualized on a confocal microscope (LSM510; Carl Zeiss MicroImaging, Inc.) using FITC/Texas red filters and a 40× NA 1.3 objective. Fluorescence and phase microscopy of live cells was performed on a microscope (UX70; Olympus) equipped with a UV lamp, FITC/Texas red filters, and a 20× NA 0.40 objective. Images were captured with a digital camera (MagnaFire; Optronics) using MagnaFire software (Optronics). For cell death assays, 2 μl of 1 mg/ml PI solution was added to live cells for 10 min at room temperature before visualization.

Flow cytometry

HEK293 cells were detached from the culture plate 24 h after RSV infection/transient transfection by treatment with trypsin/EDTA, washed once with PBS, and resuspended in FACS buffer consisting of PBS with 0.1% FBS and 0.05% sodium azide. Cell-associated fluorescence was measured on a FACScalibur flow cytometer using CellQuest software (BD Biosciences). For each sample, 20,000 events were acquired.
Immunohistochemistry

Immunohistochemistry was performed on mouse fetal lung samples that were previously described (Bridges et al., 2003) using an antibody that detects the cleaved form of caspase-3 (R&D Systems) at a 1:5,000 dilution. Localization of antigen–antibody complexes was performed as previously described (Bridges et al., 2003).

RSV production and infection protocol

The A2 strain of RSV was amplified, purified, and quantitated in HEP2 as previously described (LeVine et al., 1999). Clonal cell lines were infected with RSV using a previously established protocol for adenoviral infection (Bridges et al., 2003). For studies in which proteasome activity was assessed after RSV infection, the proteasome sensor plasmid pZsProSensor-1 (CLONTECH Laboratories, Inc.) was transfected into the cells using LipofectAMINE 2000 24 h before RSV infection. The pZsProSensor-1 plasmid encodes a destabilized GFP consisting of amino acids 422–461 of the degradation domain of mouse ornithine decarboxylase protein fused to the COOH terminus of the naturally occurring reef coral Zoaanthus species protein ZsGreen. This fusion protein is rapidly degraded by the proteasome in an ubiquitin-independent manner.

Luciferase constructs and assays

The NF-κB–dependent ELAM-1–promoter-driven firefly luciferase plasmid pELAM-luc (obtained from R. Prywes, Columbia University) (Bridges et al., 2003). For studies in which proteasome activity was assessed after RSV infection, the proteasome sensor plasmid pZsProSensor-1 (CLONTECH Laboratories, Inc.) was transfected into the cells using LipofectAMINE 2000 24 h before RSV infection. The pZsProSensor-1 plasmid encodes a destabilized GFP consisting of amino acids 422–461 of the degradation domain of mouse ornithine decarboxylase protein fused to the COOH terminus of the naturally occurring reef coral Zoaanthus species protein ZsGreen. This fusion protein is rapidly degraded by the proteasome in an ubiquitin-independent manner.

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Statistics

Data were analyzed with Instat version 3.0 (GraphPad Software). Values are presented as means ± SD. Multiple comparisons were made by analysis of variance between groups using the Tukey-Kramer multiple comparison test, and paired samples were analyzed by t tests. In both cases, statistical significance was defined as P < 0.05 or less.

Online supplemental material

Fig. S1 is a model of functional associations between differentially expressed genes in SP-Cα knock-out cells associated with apoptosis using PathwayAssist software. Table S1 lists descriptive information of apoptosis-associated genes from the model in Fig. S1. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200508016/DC1.

The authors would like to thank Chen Xia Duan, Mei Wang, Senad Divanovic, AI Serfi, Mukund Raghavan, and Xiaofei Shangguan for technical assistance and/or advice.

This work is supported by National Institutes of Health awards PO1-HL61646 and P50-HL56387 to T.E. Weaver.

Submitted: 2 August 2005
Accepted: 22 December 2005

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