Biosynthesis of surfactant protein C: characterization of aggresome formation by EGFP chimeras containing propeptide mutants lacking conserved cysteine residues

Albert F. Kabore, Wen-Jing Wang, Scott J. Russo and Michael F. Beers*

Lung Epithelial Cell Biology Laboratories, Pulmonary and Critical Care Division, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, USA

*Author for correspondence (e-mail: mfbeers@mail.med.upenn.edu)

Accepted 3 November 2000
Journal of Cell Science 114, 293-302 © The Company of Biologists Ltd

SUMMARY

Surfactant protein C (SP-C) is a lung-specific secreted protein, which is synthesized as a 21-kDa propeptide (SP-C_{21}) and then proteolytically processed as a bitopic transmembrane protein in subcellular compartments distal to the medial Golgi to produce a 3.7 kDa mature form. We have shown that initial processing of SP-C_{21} involves two endoproteolytic cleavages of the C terminus and that truncation of nine amino acids from the C-flanking peptide resulted in retention of mutant protein in proximal compartments. Because these truncations involved removal of a conserved cysteine residue (Cys^{186}), we hypothesized that intralumenal disulfide-mediated folding of the C terminus of SP-C_{21} is required for intracellular trafficking. To test this, cDNA constructs encoding heterologous fusion proteins consisting of enhanced green fluorescent protein (EGFP) attached to the N terminus of wild-type rat proSP-C (EGFP/SP-C^{1-194}), C-terminally deleted proSP-C (EGFP/SP-C^{1-185}; EGFP/SP-C^{1-191}) or point mutations of conserved cysteine residues (EGFP/SP-C^{C122G}; EGFP/SP-C^{C186G}; or EGFP/SP-$C^{C122/186G}$) were transfected into A549 cells. Fluorescence microscopy revealed that transfected EGFP/SP-C^{1-194} and EGFP/SP-C^{1-191} were expressed in a punctate pattern within CD-63 positive, EEA-1 negative cytoplasmic vesicles. In contrast, EGFP/SP-C^{1-185}, EGFP/SP-C^{C122G}, EGFP/SP-C^{C186G} and EGFP/SP-$C^{C122/186G}$ were expressed but retained in a juxtanuclear compartment that stained for ubiquitin and that contained γ-tubulin and vimentin, consistent with expression in aggresomes. Treatment of cells transfected with mutant proSP-C with the proteasome inhibitor lactacysteine enhanced aggresome formation, which could be blocked by coincubation with nocodazole. Western blots using a GFP antibody detected a single form in lysates of cells transfected with EGFP/SP-C cysteine mutants, without evidence of smaller degradation fragments. We conclude that residues Cys^{122} and Cys^{186} of proSP-C are required for proper post-translational trafficking. Mutation or deletion of one or both of these residues results in misfolding with mistargeting of unprocessed mutant protein, leading to formation of stable aggregates within aggresomes.

Key words: Surfactant protein C; Propeptide targeting; A549 cell; Green fluorescent protein

INTRODUCTION

Surfactant protein C (SP-C) is a 35 amino acid (aa) lung-specific peptide whose primary sequence represents one of the most hydrophobic secretory products known (Beers, 1998; Weaver, 1998). SP-C is a component of most replacement surfactants (Boncuk-Dayanikli and Taeusch, 1995) and the role of altered expression of SP-C in the pathophysiology of both congenital and acquired lung disease is becoming increasingly recognized (Atochina et al., 2000; Wert et al., 1998; Wiedmann, 1996).

The 3.7 kDa mature SP-C protein (SP-$C_{3.7}$) recovered from lung lavage is the result of synthesis and extensive post-translational processing of a 191-197 aa precursor by the type 2 pneumocyte of the alveolus (Fig. 1). The SP-C propeptide (SP-C_{21}) contains 23 aa residues at the N terminus and 133-139 residues at the C terminus, which flank the mature SP-C domain (Beers, 1998; Weaver, 1998). Unlike other surfactant proteins, the N terminus of the SP-C primary translation product does not contain a cleavable signal sequence. Importantly, in a lipid environment, the secondary structure of mature SP-C is predominantly α-helical (Szyperski et al., 1998) and in vitro, the primary translation product has been shown to be a bitopic, transmembrane protein, anchored by this region in a type II (N, cytosol/C, lumen) orientation (Keller et al., 1991) (Fig. 1). In contrast to other secreted proteins, most of which are lumenal, the biosynthesis of SP-C is atypical in that the SP-C proprotein is trafficked and cleaved as an integral membrane protein (Beers and Lomax, 1994). The resulting transmembrane anchor is then ultimately secreted with surfactant phospholipids and SP-B via the regulated exocytic pathway of type 2 epithelial cells (Beers, 1998; Weaver, 1998).

Prior studies using isolated rat type 2 cells and explants of human fetal lung indicate that post-translational processing of

SP-C_{21} involves an initial two-step removal of the C-propeptide to generate 16 kD and 7 kD intermediate forms (Beers et al., 1994; Beers and Lomax, 1995; Solarin et al., 1997). This is followed by two endoproteolytic cleavages of the N-propeptide to produce SP-$C_{3.7}$ (Johnson et al., 2000). Proteolysis can be blocked by the use of Brefeldin A (Beers and Lomax, 1995), by low-temperature incubation (20°C) (Vorbroker et al., 1995), or by inhibitors of organellar acidification (Beers, 1996), indicating that delivery of proSP-C from Golgi to acidic cytosolic organelles is essential for complete processing to mature SP-C.

The structural domains responsible for translocation and trafficking of proSP-C during SP-C biosynthesis have been partially elucidated. Deletional mutagenesis of the rat isoform in combination with the creation of enhanced green fluorescent protein (EGFP) fusion proteins has shown that the mature SP-C peptide functions as a non-cleavable signal domain for translocation of the proprotein from the cytoplasm (Russo et al., 1999). These studies have also shown that the region Met^{10}-Gln^{23} within the N-flanking propeptide contains a domain capable of targeting EGFP chimeras to cytoplasmic vesicles. Although this region in combination with the mature peptide was sufficient for routing of EGFP, it was also observed that maintenance of an intact C-propeptide terminus is an additional requirement for the normal trafficking of native proSP-C (Beers et al., 1998). Truncation of as little as nine amino acids from the C-propeptide (Leu^{185}-Ile^{194}) results in retention in Con A-labeled compartments, although this region alone is insufficient as a functional sorting signal (Russo et al., 1999). It is well established that disulfide bond formation in the oxidizing environment of the ER lumen often contributes to proper folding, sorting, processing, and intracellular trafficking of many proteins (Creighton, 1997; Stevens and Argon, 1999; Ellgaard et al., 1999). Within the C terminus of proSP-C, two conserved cysteine residues (rat isoform: Cys^{122}; Cys^{186}) are candidates for such disulfide bond formation and intrachain folding (Fig. 1) (Fisher et al., 1989). Since truncation of the C-flanking region of proSP-C results in deletion of one of these residues (Cys^{186}), disruption of disulfide-dependent folding represents a potential mechanism for the accumulation of mutant proSP-C in proximal compartments.

Conventionally, two major mechanisms underlie the recognition and disposal of misfolded proteins. One involves the ubiquitin-proteasome pathway shown to selectively catalyze the intracellular degradation of mutant proteins (Haas and Siepmann, 1997; Kopito, 1997). Targeting to lysosomes is considered a second quality control mechanism used for disposal of mutant proteins that accumulate in compartments other than the ER (Ellgaard et al., 1999; Johnston et al., 1998). Recent studies characterizing the intracellular fate of the cystic fibrosis transmembrane conductance regulator (CFTR) have demonstrated accumulation of mutant CFTR in a novel perinuclear compartment. These structures have been termed aggresomes and are believed to represent a third general cellular response to misfolded protein that can form when the capacity of the proteasome is exceeded either by overexpression of mutant protein or with inhibition of proteasome activity (Johnston et al., 1998; Wigley et al., 1999).

To explore the role of C-terminal cysteine residues in SP-C biosynthesis, fusion proteins consisting of EGFP and proSP-C mutants lacking Cys^{122}, Cys^{186} or both, were expressed in lung epithelial cells. Analysis of the trafficking of these proteins demonstrated that proSP-C cysteine mutants are retained in proximal compartments and, when transfected at high levels or in the presence of proteasome inhibitors, form stable, ubiquitinated aggregates that accumulate in a perinuclear, non-lysosomal compartment. These findings represent the first description of aggresome formation by a mutant bitopic transmembrane, secreted protein targeted for removal from the biosynthetic pathway.

MATERIALS AND METHODS

Materials

pEGFP-C_1 plasmid was purchased from Clontech (Palo Alto, CA, USA). Polyclonal anti-green fluorescent protein antisera were purchased from Molecular Probes (Eugene, OR, USA). Texas Red-conjugated monoclonal and polyclonal antibodies were obtained from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA, USA). Anti-CD 63 was purchased from Immunotech, Inc. (Marseille Cedex, France). Anti-calnexin polyclonal antibody was purchased from StressGen (Victoria, BC, Canada). Anti-ubiquitin monoclonal antibody was purchased from Chemicon (Temecula, CA, USA). Monoclonal antisera against γ-tubulin and vimentin (clone V9) were

Table 1. Primer sets used in PCR reactions for generation of EGFP rat SP-C fusion constructs

Construct name	Rat SP-C amino acid*	PCR primers	
		5′ primer (forward)‡	3′ primer (reverse)§,¶
EGFPC_1/SP-C^{1-185}	Met^1-Leu^{185}	dTACAAGTCCGGAATGGACATGGGTAGCAAAGAG	dCC*CTCGAG*TG**TTA**CAAGGTGCTCACAGCAA
EGFPC_1/SP-C^{1-191}	Met^1-Leu^{191}	dTACAAGTCCGGAATGGACATGGGTAGCAAAGAG	dCC*CTCGAG*TG**TTA**CAGTGGTAGCTCTCCACACAAGGTGCT
EGFPC_1/SP-C^{1-194}	Met^1-Ile^{194}	dTACAAGTCCGGAATGGACATGGGTAGCAAAGAG	dTCTAGATGCATG*CTCGAG*CG
EGFPC_1/SP-C^{24-58}	Phe^{24}-Leu^{58}	dTACAAGTCCGGATTTCGCATTCCCTGCTGC	dCC*CTCGAG*TG**TTA**AAGGCCCATGAGCA
EGFPC_1/SP-C^{24-194}	Phe^{24}-Ile^{194}	dTACAAGTCCGGATTTCGCATTCCCTGCTGC	dTCTAGATGCATG*CTCGAG*CG
EGFPC_1/SP-C^{C186G}	Met^1-Ile^{194} (C186G)	dTACAAGTCCGGAATGGACATGGGTAGCAAAGAG	dGCACCCTGACTCGAGCTAGATATAGTACAGTGGTAG CTCTCCACCCAAGGTGCTCACAGC

In all cases pcDNA3-rSP-C (+) (Beers and Lomax, 1995) was used as template.

*Numbers correspond to nucleic acids in the rat SP-C cDNA (Fisher et al., 1989).

‡Each 5′ primer contains a *Bsp*EI site (5′-TCCGGA-3′) for in-frame ligation.

§For constructs containing C-terminal truncations, the 3′ primer contains an *Xho*I site (5′-*CTCGAG*-3′) for ligation into the EGFP-C1 polylinker and **TTA** for production of a stop codon immediately adjacent to the terminal codon of the truncated coding sequence.

¶For constructs containing Ile^{194}, the 3′ primer matches the polylinker of pcDNA3 and contains an *Xho*I site; use of this primer resulted in inclusion of the 3′ untranslated region in these constructs.

obtained from Sigma Chemical Co., Inc. (St Louis, MO, USA). Clasto-lactacystin β-lactone and Mowiol were purchased from Calbiochem-Novabiochem (La Jolla, CA, USA). All electron microscopy reagents were purchased from EM Sciences (Fort Washington, PA, USA). Except where noted, other reagents were of electrophoretic grade and were purchased from Bio-Rad (Melville, NY, USA) or Sigma.

EGFP/SP-C cDNA expression constructs

EGFP/SP-C cDNA fusion constructs generated used in this study were generated by polymerase chain reaction. All procedures involving oligonucleotide and cDNA manipulations were performed essentially as described (Ausbel et al., 1995). Primers used for PCR amplification are listed in Tables 1 and 2.

A chimeric fusion protein consisting of EGFP and wild-type rat SP-C (Met^{1} to Ile^{194}) was previously generated using PCR amplification of a full-length rat SP-C cDNA clone (pcDNA3-rSP-C(+); Fisher et al., 1989) and subcloning into $pEGFPC_1$ (Russo et al., 1999).

EGFP truncations

Mutants containing truncations of the C-terminal flanking region of proSP-C were generated by PCR with pcDNA3-rSP-C(+) as a template, as previously described (Russo et al., 1999). Primer sets are shown in Table 1. For both EGFP/SP-$C^{1\text{-}185}$ and EGFP/SP-$C^{1\text{-}191}$, the 5′ primer contains a *Bsp*EI site (5′-TCCGGA-3′) for in-frame ligation into EGFP-C_1 whereas the 3′-end of primer contains an *Xho*I site (CTCGAG).

Site-directed mutagenesis

For construction of EGFP/SP-C^{C122G}, point mutagenesis was performed by overlap-extension PCR with a two-round, four-primer technique **(**Table 2) (Russo et al., 1999). In round 1, two PCR products (SP-$C^{1\text{-}122X}$ and SP-$C^{Y122\text{-}194}$) were generated in separate reactions carried out using pcDNA3-rSP-C(+) as the template with primers A and B or primers C and D. Cycling conditions for round 1 were as follows: one cycle at 94°C for 1 minute 30 seconds, 25 cycles at 94°C for 40 seconds, 50°C for 30 seconds, and 72°C for 40 seconds, and a final extension at 72°C for 7 minutes. The resulting products were purified using GenClean II purification System (Bio101, Viata, CA, USA). Fusion of the two intermediate segments (SP-$C^{1\text{-}122X}$ and SP-$C^{122\text{-}194}$) with amplification was achieved as a result of overlapping complementary regions in the products produced in round 1, which pair during the annealing phase of a second round of PCR using primers complementary to the 5′ and 3′ ends (primers A and D, respectively). Cycling conditions in round two were identical to those in round 1 described above. The resulting mutant insert, SP-C^{C122G}, was purified and ligated into pEGFP-C_1 after digestion with *Bsp*EI and *Xho*I.

For mutant EGFP/SP-C^{C186G}, a two-primer single reaction PCR technique was used with pcDNA3-rSP-C(+) serving as template with the primers shown in Table 1. For the double mutant EGFP/SP-$C^{C122/186G}$, a single PCR was performed using EGFP/SP-C^{C122G} as template with a primer set identical to that used to generate EGFP/SP-C^{C186G}.

Automated DNA sequencing in both directions was performed at the Core facility in the Department of Genetics at the University of Pennsylvania, USA. No nucleotide mutations in the coding region of full-length SP-C or any of the deletional constructs were detected.

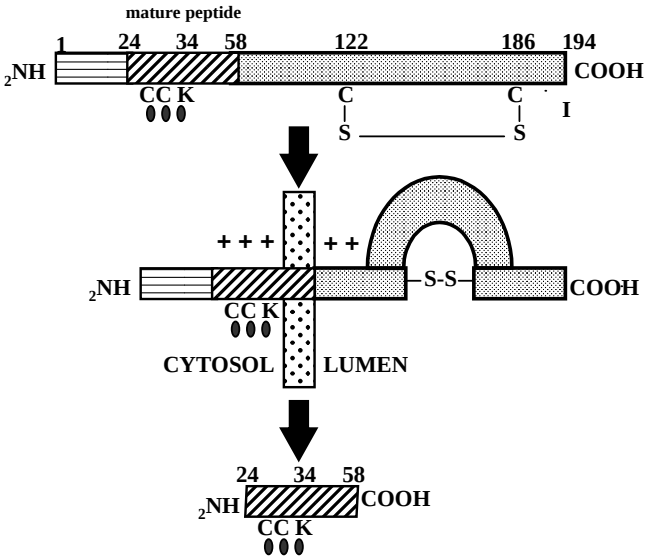

Fig. 1. Biosynthesis of SP-C. Rat SP-C is synthesized by the alveolar type 2 cells as a proprotein of 194 aa (SP-C_{21}). Contained within the propeptide is the 35-aa mature SP-C protein (SP-$C_{3.7}$), which is flanked by propeptides of 23 amino acids at the N terminus and 136 residues at the C terminus. After translation, SP-C_{21} is translocated to the endoplasmic reticulum (ER) and inserted into the ER membrane as a bitopic protein in a type II membrane orientation, which is anchored by the α-helical domain Leu^{36}-Leu^{55} contained within the mature SP-C sequence. The proSP-C C terminus contains conserved cysteine residues, which could participate in disulfide-mediated folding in the oxidizing environment of the ER lumen. Proteolytic removal of the C- and N-flanking domains is Brefeldin A-sensitive, indicating that processing is dependent upon delivery of SP-C_{21} to post-Golgi processing compartments. Within the mature peptide, aa residues Cys^{28}, Cys^{29}, and Lys^{34} (CCK) have been shown to contain covalent palmitic acid..

A549 cell line and transfection

The lung epithelial cell line A549 utilized in transfection studies was

Table 2. Primer sets used in PCR reactions for generation of SP-C^{C122G} insert construct

SP-C insert name (amino acid)	PCR primers			
	5′ primer (forward)*		3′ primer (reverse)‡	
Primary PCR				
Met^{1}-XXX^{122}	(A) dTACAAGTCCGGAATGGACATGGGTAGCAAAGAG	(B)	dG**CCGTAGGTTCCTGGAGCTGGCTTATA**	
Gly^{122}-Ile^{194}	(C) d**TATAAGCCAGCTCCAGGAACCTACGGC**TACATC ATGAAGATGGCTCCAGAG	(D)	dTCTAGATGCATG*CTCGAG*CG	
Secondary PCR				
SP-C^{C122G}	(A) dTACAAGTCCGGAATGGACATGGGTAGCAAAGAG	(D)	dTCTAGATGCATG*CTCGAG*CG	

For both reactions of primary PCR, pcDNA3-rSP-C(+) (Beers and Lomax, 1995) was used as template.

*Primer A contains a *Bsp*EI site (5′-TCCGGA-3′) for in-frame ligation into EGFP-C_1.

‡The 3′ prime end of primer D matches the polylinker of pcDNA3 and contains an *Xho*I site (*CTCGAG*); use of this primer resulted in inclusion of the 3′ untranslated region in the mutant construct.

Overlapping regions of primers B and C that permit complementation in the secondary PCR are shown in bold.

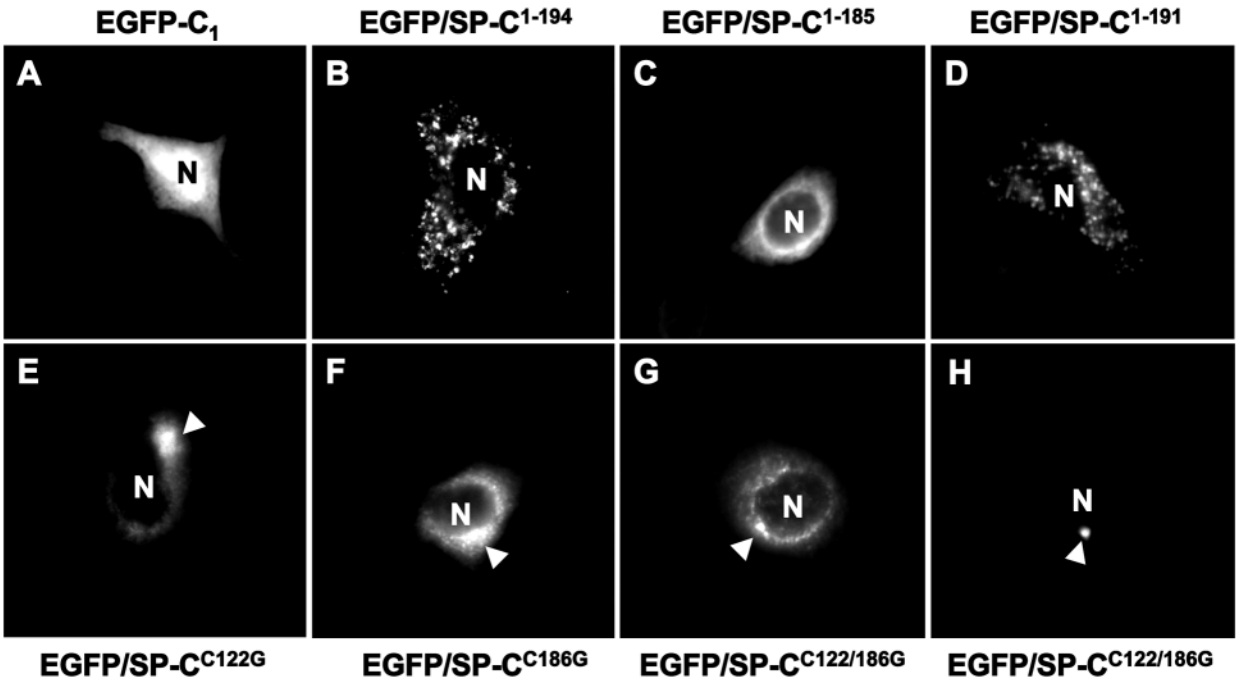

Fig. 2. C-mutants lacking Cys^{186} and/or Cys^{122} are retained in proximal compartments. A549 cells were transiently singly transfected either with EGFP-C_1 (A), EGFP-C_1/SP-C^{1-194} (B), the C-truncation mutants EGFP-C_1/SP-C^{1-185} (C) and EGFP-C_1/SP-C^{1-191} (D), or the C-cysteine mutants EGFP-C_1/SP-C^{C122G} (E), EGFP-C_1/SP-C^{C186G} (F) and EGFP-C_1/SP-$C^{C122,186G}$ (G,H), using $CaPO_4$. Images for EGFP expression were acquired 48 hours after transfection by fluorescence microscopy with a High Q fluorescein isothiocyanate (FITC) filter package (excitation at 480 nm, emission at 535/550 nm). Expression of EGFP-C_1/SP-C^{1-194} as well as EGFP-C_1/SP-C^{1-191} was in cytoplasmic vesicles. In contrast, EGFP-C_1/SP-C^{1-185} is translocated but failed to reach cytoplasmic vesicles. As with EGFP-C_1/SP-C^{1-185}, all three cysteine mutants (E-H) were restricted to a juxtanuclear region and showed variable degrees of aggregation (arrowheads).

originally obtained through the American Type Culture Collection (Manassas, VA, USA) and has been used in prior studies (Beers et al., 1998; Russo et al., 1999). A549 cells were grown at 37°C in 5% CO_2 in DMEM supplemented with 10% fetal calf serum (FCS), 100 i.u./ml penicillin and 100 μg/ml streptomycin, as previously described. A549 cells, grown to 50% confluence on glass coverslips in 35 mm plastic dishes, were transiently transfected with EGFP/SP-C constructs (10 μg/dish) by $CaPO_4$ precipitation as previously described (Russo et al., 1999). The medium was replaced at 24 hours, and cells were maintained for up to 48 hours.

Immunohistochemistry

A549 cells grown on glass coverslips and transfected with EGFP chimeric constructs were subjected to indirect immunofluorescence microscopy as previously described (Beers et al., 1998). Following washing with phosphate-buffered saline (PBS) (Ca^{2+}- and Mg^{2+}-free), plated cells were fixed by immersion of coverslips in 4% paraformaldehyde at room temperature for 20 minutes, and were then permeabilized by incubation for 30 minutes with 0.3% Triton X-100 in blocking buffer (5% BSA and 10% normal goat serum in PBS). Immunostaining was performed by incubation with the primary antibody for 1 hour at room temperature at the indicated dilutions. Coverslips were washed, incubated for 1 hour at room temperature with a 1:200 dilution of secondary goat anti-mouse IgG monoclonal or secondary goat anti-rabbit IgG polyclonal antibodies, each conjugated to Texas Red. Cells were washed in PBS, air-dried at room temperature, and mounted on slides with Mowiol.

Fluorescence images were viewed on an Olympus I-70 inverted

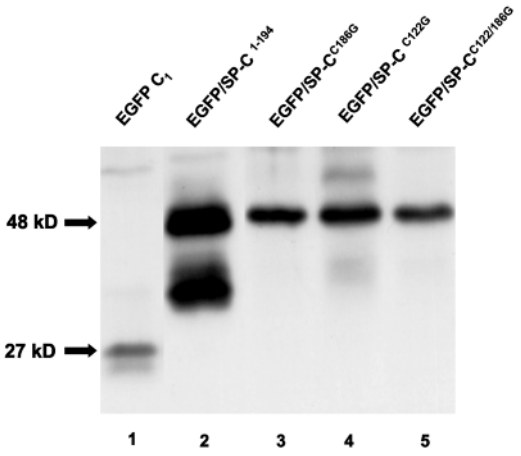

Fig. 3. Western blot of A549 cell lysates for detection of EGFP proteins. A549 cells (1×10^6) were transfected with 10 μg of EGFP-C_1, EGFP-C_1/SP-C^{1-194}, EGFP-C_1/SP-C^{C122G}, EGFP-C_1/SP-C^{C186G} or EGFP-C_1/SP-$C^{C122/186G}$. 48 hours later, cells were harvested by scraping and centrifugation for 10 minutes at 300 ***g***. Nuclear-free lysates were prepared from the cell pellet by incubation with 50 μl of PBS containing 1% Triton X-100 and protease inhibitors, and then centrifuged for 30 seconds at 12,000 ***g***. Half of each nuclear-free supernatant was subjected to 12% SDS-PAGE. Separated proteins were transferred to nitrocellulose and immunoblotted with primary rabbit polyclonal anti-GFP (Clontech, Palo Alto, CA, USA). Bands were visualized using enhanced chemiluminescence. Analysis of EGFP-C_1/SP-C^{1-194} fusion protein expression demonstrated three bands of 48, 42 and 33 kD. EGFP-C_1 was expressed as a major product of 27 kD. EGFP-C_1/SP-C^{C122G}, EGFP-C_1/SP-C^{C186G} or EGFP-C_1/SP-$C^{C122/186G}$ expressed a single and same major product of 48 kD.

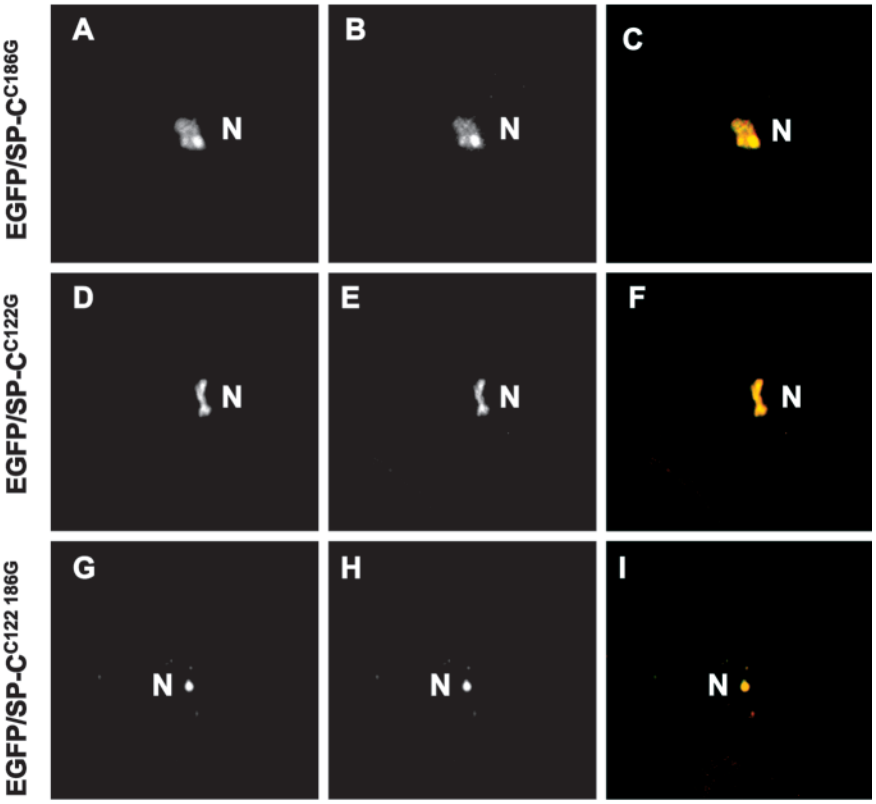

Fig. 4. ProSP-C containing C-terminal cysteine mutations forms ubiquitinated aggregates. A549 cells grown on coverslips were transfected with either EGFP-C_1/SP-C^{C122G} (A-C), EGFP-C_1/SP-C^{C186G} (D-F) or EGFP-C_1/SP-$C^{C122,186G}$ (G-I) using $CaPO_4$. 48 hours after introduction of plasmid DNA, cells were fixed, and stained with monoclonal anti-ubiquitin antibody (1:500) and secondary goat anti-mouse IgG conjugated to Texas Red. Double-label fluorescence images were acquired using High Q FITC filter package (excitation at 480 nm, emission at 535/550 nm) for EGFP (A,D,G) and a High Q Texas Red filter package (excitation at 560/555 nm, emission at 645/675 nm) for ubiquitin staining (B,E,H). Images were colorized (EGFP-green/ Texas Red-red) and overlaid using Image 1 software (C,F,I). Yellow denotes the colocalization of EGFP/proSP-C and ubiquitin.

fluorescence microscope with filter packages High Q fluorescein isothiocyanate for EGFP (excitation at 480 nm, emission at 535/550 nm), and High Q TR for Texas Red (excitation at 560/555 nm, emission at 645/675 nm) obtained from Chroma Technology (Brattleboro, VT, USA). Fluorescent and phase images were captured using a Hamamatsu 12-bit coupled-charge device camera. Image processing and overlay analysis were performed using IMAGE 1 software (Universal Imaging, West Chester, PA, USA).

Electron microscopy

Transmission electron microscopy was performed by the Imaging Core Facility in the Department of Pathology at the University of Pennsylvania School of Medicine using the method of Hayat (Hayat, 1986). Both transfected and control A549 cells grown on plastic dishes were rinsed in PBS and then fixed overnight at 4°C in 2% glutaraldehyde with 0.1 M sodium cacodylate buffer, pH 7.4. Cells harvested by scraping were pelleted by centrifugation and osmicated for 60 minutes at 4°C in 2% osmium tetraoxide in 0.1 M sodium cacodylate buffer. Following washing with sodium cacodylate buffer to remove osmium, samples were stained en bloc with 2% aqueous uranyl acetate for 30 minutes. Following dehydration in graded alcohol, pellets were embedded in epon 812 and cured at 70°C for 48 hours. 70 nm thick sections were generated with a Leica Ultracut S ultramicrotome and collected on 200 mesh copper grids, stained in 50% alcoholic uranyl acetate and counterstained with bismuth subnitrite. Air-dried samples were subsequently examined on a JEOL JEM 1010 electron microscope. Images were collected using a Hamamatsu CCD camera.

Polyacrylamide gel electrophoresis and immunoblotting

Cells pellets collected by scraping and centrifugation at 300 ***g*** were solubilized with 40 µl of 50 mM Tris, 190 mM NaCl, 6 mM EDTA, 2% Triton X-100, 1 mM PMSF, pH 7.4. Following centrifugation at 8,000 ***g*** for 30 seconds to remove nuclei, proteins were separated by electrophoresis on a 12% polyacrylamide gel and transferred to nitrocellulose (Laemmli, 1970).

Immunoblotting of transferred samples was done using successive incubations with primary polyclonal GFP antisera (1:5000) for 2 hours and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature. Bands were

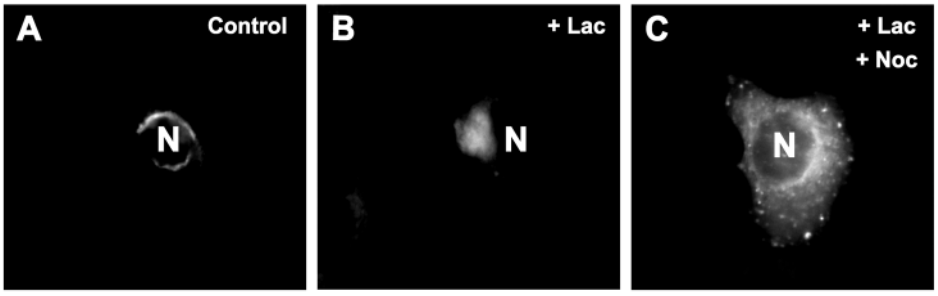

Fig. 5. Proteasome inhibition enhances aggregate accumulation that is dependent upon microtubules. A549 cells grown on coverslips were transfected with EGFP-C_1/SP-$C^{C122/186G}$ using $CaPO_4$. 24 hours later, the medium was changed and cells were treated either with 10 µM lactacystin (A), with DMSO alone (B) or with 10 µM lactacystin plus 10 µM nocodazole for 18 hours, and then fixed. Representative fluorescence images of >25 cells examined for each group were acquired using a High Q FITC filter package. Cells treated with lactacystin alone developed large perinuclear aggresomes; this was prevented by cotreatment with nocodazole, resulting in a peripheral dispersion.

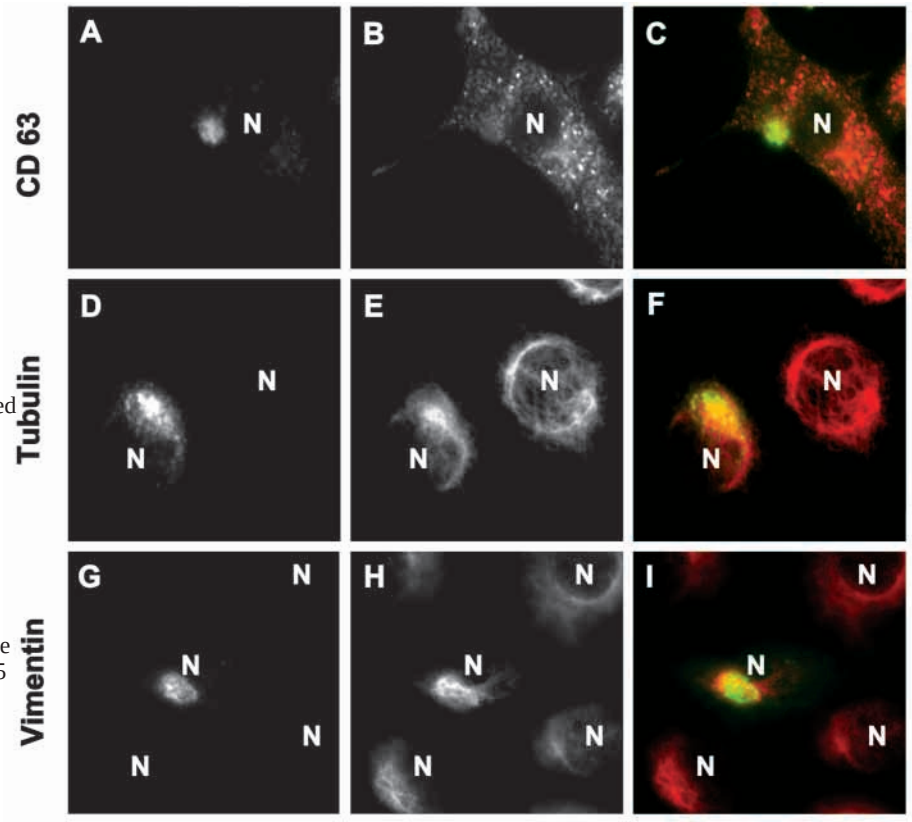

Fig. 6. Aggresomes in A549 cells are non-lysosomal structures associated with the microtubule-organising centre (MTOC) and intermediate filaments. A549 cells grown on coverslips were transfected with EGFP/SP-$C^{C186Gly}$ using $CaPO_4$. 24 hours after introduction of plasmid DNA, cells were treated for 18 hours with 10 μM lactacystin in DMSO and then fixed. Staining was performed using monoclonal antiserum against either CD-63 (1:100) (A-C), γ tubulin (1:100) (D-F) or vimentin (1:100) (G-I) and visualized with secondary goat anti-mouse IgG-Texas Red. Fluorescence images were acquired using a High Q FITC filter package for EGFP (A,D,G) and a High Q Texas Red filter package (excitation at 560/555 nm, emission at 645/675 nm) (B,E,H). Images were colorized (EGFP-green/ Texas Red Red) and overlaid using Image 1 software (C,F,I). Yellow denotes the colocalization of EGFP/SP-$C^{C186Gly}$ with tubulin and vimentin but not with CD-63.

visualized by enhanced chemiluminescence using a commercially available kit (Amersham Inc., Arlington Heights, IL, USA).

RESULTS

Expression of proSP-C C-terminal truncation mutants

Expression of EGFP-C_1 and fusion proteins was readily detected in transiently transfected A549 cells 24-48 hours after introduction of plasmids. Transfection of EGFP-C_1 resulted in a diffuse distribution of fluorescent signal throughout the cell **(**Fig. 2A). Although maximal expression of EGFP/SP-C^{1-194} occurred in a similar time frame, the pattern of expression differed and was restricted to cytoplasmic vesicles of A549 cells, indicating successful translocation and export from the Golgi (Fig. 2B). EGFP/ SP-C^{1-194} containing vesicles were acidic and partially colocalized with CD63 (data not shown), a marker antigen associated with lamellar bodies and multivesicular bodies of type II cells (Voorhout et al., 1992; Voorhout et al., 1993;Vorbroker et al., 1992). In contrast, EGFP/SP-C^{1-185}, a C-terminal truncated fusion protein lacking Cys^{186}, was restricted to a juxtanuclear location (Fig. 2C). Cytoplasmic vesicular targeting was again observed when EGFP/SP-C^{1-191} was used (Fig. 2D), indicating that absence of residue Cys^{186} from the C-propeptide domain alters trafficking.

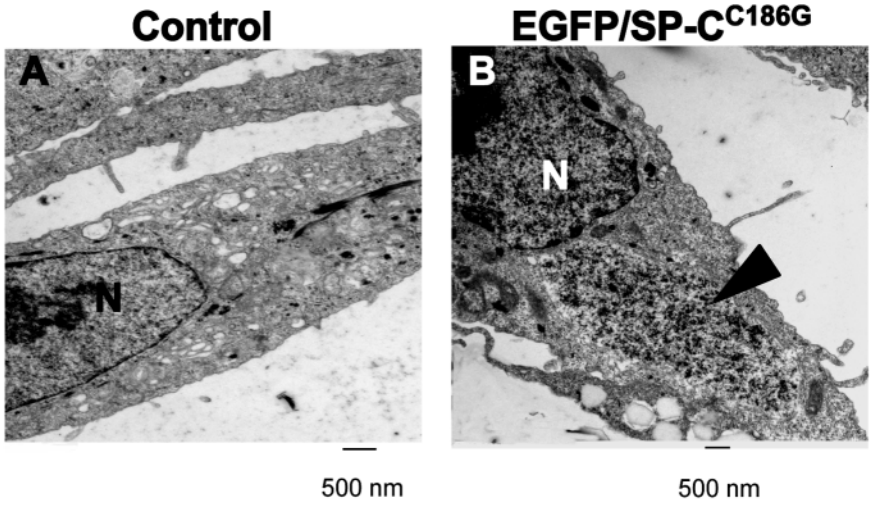

Fig. 7. Ultrastructure of aggresomes in A549 cells. A549 cells were transfected with EGFP-C_1/SP-C^{C186G} using $CaPO_4$. Non-transfected cells were used as control. 48 hours later, cells were processed for transmission electron microscopy as described in Materials and Methods. In contrast to non-transfected cells (A), cells transfected with EGFP-C_1/SP-C^{C186G} showed an aggregate of particulate material close to the nucleus (B, arrowhead).

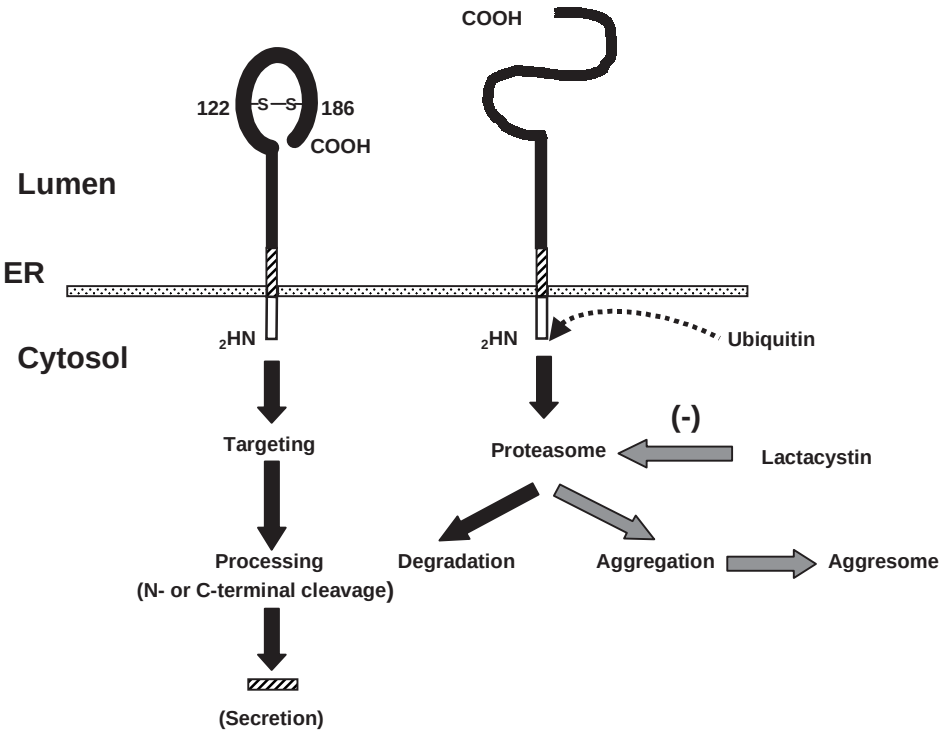

Fig. 8. Mechanism for aggresome formation by C-terminal mutant proSP-C. Schematic diagram summarizing the fates of synthesized proSP-C. (Left) Wild-type proSP-C. In the presence of an intact disulfide bridge at Cys^{122} and Cys^{186}, proSP-C is committed to fold properly into its native conformation, released from the ER, targeted and processed. (Right) C-terminal mutant. The alteration of disulfide bond formation by a single or double point mutation at Cys^{122} or Cys^{186} or both cysteine residues results in proSP-C misfolding, release from the ER, ubiquitination in the cytoplasm and aggregate formation. The ubiquitinated aggregates are targeted for proteasomal degradation. When the ubiquitin-proteasome pathway is inhibited or if the misfolded mutant is expressed in excess of the capacity of the proteasome for degradation, the formation of large aggresomes can result.

Cysteine mutation results in proSP-C aggregates

To further test the hypothesis that C-terminal cysteine residues are required for normal trafficking of proSP-C, the role of Cys^{122} and of Cys^{186} was evaluated by replacement of each residue with glycine. Fluorescence microscopy of cells transfected with either EGFP/SP-C^{C122G} (Fig. 2E) or EGFP/SP-C^{C186G} (Fig. 2F) demonstrated that expression of the resulting fusion proteins showed a consistent pattern of perinuclear accumulation, varying somewhat in shape as well as size (arrowheads). In order to exclude dimerization via interchain disulfide bonding between unpaired cysteine residues as a mechanism, the double mutant EGFP/SP-$C^{C122/186G}$ was also studied and likewise failed to reach cytosolic vesicular targets (Fig. 2G,H).

Cysteine-substituted proSP-C mutants retained in proximal compartments represented steady state accumulation of unprocessed, non-degraded fusion protein. Using an anti-GFP antibody, western blotting of A549 cells transfected with $EGFPC_1$ showed expression of a major product matching the predicted size of EGFP of 27 kD (Fig. 3, lane 1). This was not detected in cell lysates of mock-transfected cells (data not shown). When EGFP/SP-$C^{1\text{-}194}$ was introduced, lysates contained three anti-GFP positive bands. A form of 48 kD, corresponding to the predicted size of the primary translation product of the fusion protein, as well as two smaller intermediates, was identified (Fig. 3, lane 2). Bands of 27 kD (indicating liberation of free EGFP) were not found, showing that EGFP/SP-$C^{1\text{-}194}$ was partially processed in this cell-type by two-step cleavage of the C terminus. In contrast to wild-type proSP-C, expression of each of the three C cysteine mutants yielded only a single band of 48 kD, representing the primary translation product of the fusion protein indicating a lack of processing or of degradation of these forms (Fig. 3, lanes 3-5). Together the cytochemistry and western blotting data demonstrate that C-mutant proteins accumulate in perinuclear compartments without degradation.

Mutant proSP-C is ubiquitinated and accumulates following treatment with a proteasome inhibitor

Since the recognition of abnormal and misfolded proteins in eukaryotes is mediated by the ubiquitin machinery that promotes delivery of substrates to the 26S proteasome for degradation (Haas and Siepmann, 1997), we performed immunocytochemical staining of transfected A549 cells grown on coverslips to determine if perinuclear EGFP aggregates were deposited as ubiquitin-protein conjugates. In control experiments with cells transfected with EGFP/CP-$C^{1\text{-}194}$, expressed protein appearing in cytoplasmic vesicles did not stain for ubiquitin (data not shown). In contrast to wild-type SP-C, aggregates formed following transfection with all three EGFP/proSP-C cysteine mutants were ubiquitinated (Fig. 4A-I).

The formation of juxtanuclear, ubiquinated, mutant EGFP/proSP-C aggregates suggested that proteasome function might be a critical factor in this event and that this process represents an effort by the cell to handle misfolded mutant proSP-C protein when the capacity of proteasome degradation is exceeded. Cotreatment of cells transfected with EGFP/SP-C^{C186G} for 18 hours with lactacystin, a specific inhibitor of proteasome activity, resulted in significant expansion of juxtanuclear aggregates (Fig. 5A,B). The enhancement of aggregate formation by lactacystin was abrogated by coincubation of transfected cells with nocodazole, indicating that this process is dependent upon microtubules (Fig. 5C).

Characterization of EGFP-proSP-C aggregates

The subcellular pattern of distribution of ubiquinated, mutant proSP-C was defined using double-label immunofluorescence.

Immunostaining with an anti-CD 63 antibody carried out on A549 cells transfected with EGFP/SP-C^{C186G} and treated with lactacystin demonstrated that while CD-63 staining was distributed in a punctate vesicular pattern in the cytoplasm (Fig. 6B), the expression of EGFP/SP-C^{C186G} was limited to the perinuclear region (Fig. 6A) and failed to colocalize with CD-63 (Fig. 6C). In addition, this compartment was negative for calnexin (not shown). Both EGFP/SP-C^{C122G} and EGFP/SP-$C^{C122/186G}$ exhibited similar patterns (data not shown).

Previous investigations have reported that once formed, misfolded protein aggregates refractory to intracellular proteolysis are delivered to perinuclear, centrosome-associated structures termed aggresomes (Johnston et al., 1998). To address whether mutant proSP-C aggregates are centrosome-associated, A549 cells transfected with EGFP/SP-C^{C186G} (Fig. 6D) were immunostained for tubulin, an integral centrosome protein associated with microtubules. This revealed a network of tubulin-positive microtubules distributed near the nucleus of A549 cells (Fig. 6E), with EGFP/proSP-C aggregates surrounded by filaments of these microtubules in a cage-like structure (Fig. 6F). Adjacent, non-transfected cells failed to show redistribution of tubulin staining. In addition, aggresome formation was accompanied by reorganization of intermediate filaments. Immunostaining for vimentin of A549 cells transfected with EGFP/SP-C^{C186G} and treated with lactacystin demonstrated the collapse and intimal association of vimentin with proSP-C/EGFP aggregates (Fig. 6G-I).

Taken in total, these data indicate that expression of EGFP/proSP-C mutants results in accumulation of ubiquitinated protein aggregates in a non-ER, CD-63 negative, centrosome-associated juxtanuclear structure of lung epithelial cells, which is compatible with the accepted definition of an aggresome.

Ultrastructure of aggresomes

Ultrastructural analysis of transfected cells was performed by electron microscopy. Compared to nontransfected cells (Fig. 7A), cells expressing EGFP/SP-C^{C186G} exhibited a relatively large (approx. 2 μm) perinuclear structure of electron-dense particles that were surrounded by filamentous material (Fig. 7B). Cells transfected with EGFP/SP-C^{1-194} showed ultrastructure features similar to non-transfected control cells (data not shown).

DISCUSSION

The recognition and removal of incompletely folded or misfolded proteins in the cell is an important quality control mechanism to prevent accumulation of potentially toxic proteins (Ellgaard et al., 1999). Several recent reports have described the characterization of a novel subcellular structure, the aggresome, which appears in response to the expression of mutant protein products (Garcia-Mata et al., 1999; Johnston et al., 1998; Notterpek et al., 1999; Vanslyke et al., 2000; Wigley et al., 1999). Aggresomes form by collection and deposition of misfolded proteins into a large heterogeneous structure surrounding the microtubular organizing center (MTOC). The formation of aggresomes has been observed in cells expressing altered forms of multitopic, integral membrane proteins (CFTR, PS-1) (Johnston et al., 1998; Wigley et al., 1999), or a soluble cytosolic protein-GFP chimera (GFP-250) (Garcia-Mata et al., 1999), or a small peripheral, hydrophobic plasma membrane protein (PMP-22) (Notterpek et al., 1999) or connexin 32 (Vanslyke et al., 2000). In this study, new data for another class of proteins (secreted, membrane associated peptides) demonstrates that the selective alteration of a cysteine-mediated disulfide bridge in the C-flanking domain of the surfactant protein C propeptide impairs normal trafficking, leading to accumulation of non-degraded, ubiquinated protein within aggresomes. This is the first report of the use of this pathway by the cell for the targeted removal of a mutant hydrophobic, membrane-associated secretory protein from the biosynthetic pathway.

Production of SP-C from a larger propeptide precursor has been shown to be a multistep process, which requires translocation of synthesized SP-C_{21} to the ER, sorting and exit from the Golgi, stepwise cleavage of flanking propeptide domains, and assembly of mature SP-C with surfactant phospholipids and SP-B in the lamellar body for secretion (Beers, 1998). Data generated using in vitro systems has shown that the SP-C precursor is a type II, integral, bitopic membrane protein anchored by a polyvaline α-helical domain in the mature peptide in which the C-flanking domain of propeptide extends into the ER lumen (Keller et al., 1991). Using transfected lung epithelial cells, we have found that a small (9 aa) truncation of the proSP-C C-flanking domain (EGFP/SP-C^{1-185}) results in translocation but restriction of expressed protein to a perinuclear compartment (Fig. 2C). The addition of six amino acids to C domain of EGFP/SP-C^{1-185} (generating EGFP/SP-C^{1-191}) restored the ability of proSP-C to target EGFP to CD-63 positive, EEA-1 negative cytoplasmic vesicles. The finding that EGFP/SP-C^{1-191} is targeted to a similar compartment as wild-type proSP-C eliminates the possibility that, in contrast to some type II bitopic proteins such as TGF-α (Briley et al., 1997), the carboxyl-terminal hydrophobic residue of proSP-C (Ile^{194}) is not utilized for targeting.

Based on the derived transmembrane topology of proSP-C, since the C-flanking propeptide is entirely within the ER lumen, it is unlikely that truncation mutations effect removal of a targeting motif. It appears more plausible that the retention of the C-truncated EGFP/SP-C^{1-185} chimera in a perinuclear compartment would be due to misfolding of the propeptide in the ER lumen. Results obtained by comparing the cellular localization of EGFP/SP-C^{1-194}, EGFP/SP-C^{1-191} and EGFP/SP-C^{1-185} are consistent with the notion that establishment of intrachain disulfide-dependent folding of the C-flanking propeptide is required for normal trafficking. Within this region of the rat proSP-C molecule, there are two cysteine residues (Cys^{122}; Cys^{186}), which are highly conserved across species. Site-directed mutagenesis, in which the selective substitution of glycine for either Cys^{122} or Cys^{186} resulted in an expression pattern that was similar to EGFP/SP-C^{1-185}, provided further support for this mechanism. Importantly, the double mutant EGFP/SP-$C^{C122/186G}$ yielded an identical pattern, indicating that this effect was not due to homotypic dimerization by interchain disulfide bonding between two mono-cysteine mutant proSP-C molecules.

By fluorescence microscopy, we found that a significant portion of the proSP-C/EGFP chimeras containing C-terminal cysteine mutations tended to form punctate aggregates adjacent to the nucleus (Fig. 4). We compared this pattern of expression

with a report that presented the initial characterization of a novel subcellular structure, the aggresome. The expression of mutant forms of either CFTR or PS-1 in HEK cells resulted in deposition of a pericentriolar membrane-free cytoplasmic inclusion containing ubiquinated, mutant protein surrounding the MTOC (Johnston et al., 1998). In addition, the size of these structures varied with the level of mutant protein expression. Based on these criteria, it is most likely that the localization of EGFP/SP-C mutants represents expansion of the aggresome compartment induced by the accumulation of mutant protein. Double-label immunofluorescence established that this compartment contains anti-ubiquitin positive material (Fig. 6B) but is CD-63 negative (non-lysosomal). In addition, transmission EM of A549 cells expressing EGFP/SP-C^{C186G} shows large juxtanuclear structures consisting of electron-dense particles surrounded by a filamentous network (Fig. 7). This pattern in lung epithelial cells is consistent with previous ultrastructural descriptions of aggresomes in other cell types, which indicated that these were not membranous organelles but rather aggregates of misfolded molecules wrapped by filamentous structures, such as vimentin or tubulin (Johnston et al., 1998). By immunofluorescence, we found that a majority of γ-tubulin staining appeared to be in a filamentous pattern surrounding the aggregates of EGFP/SP-C^{C186G} centered in the centrosome/microtubule-organizing center (MTOC) (Fig. 6E). It has been speculated that the dense network of filament encircling the aggresome serves to stabilize it by restricting the diffusion of aggregated protein. In addition, it appears that aggresome formation also induces rearrangement of intermediate filaments as identified by vimentin staining (Fig. 6H).

The formation of aggresomes by a mutant bitopic transmembrane protein such as proSP-C supports the emerging concept that aggresome formation is a general response by the cell to misfolding. As illustrated in Fig. 8, this could occur either as a reaction to overexpression of aggregation-prone protein in which the capacity of the proteasome machinery is overwhelmed, or in response to inhibition of the proteasome. We have therefore examined the consequences of the inhibition of proteasome activity on the capacity to degrade misfolded proSP-C mutants. Incubation of transiently transfected A549 cells with lactacystin β-lactone, a highly specific, cell-permeable proteasome inhibitor, led to an accumulation of ubiquinated mutants in larger perinuclear aggregates (Fig. 5). Such accumulations formed upon inhibition of proteolysis, which suggests that the 20S proteasome participates directly in the recognition of misfolded proSP-C and that its inhibition allows further aggregation and deposition of misfolded mutants in aggresomes.

Formation of aggregates of misfolded proteins within specialized cells has been linked to a number of pathological states in both animal models and in humans. Perinuclear inclusions composed of aggregated, ubiquitinated protein and intermediate filament proteins are present in amyloidosis and in several neurodegenerative diseases including Alzheimer's disease (Wetzel, 1994), as well as the peripheral myelin protein 22 (PMP-22) associated polyneuropathies (Notterpek et al., 1999). Similar lesions have been associated with mutant PMP-22 expression in Schwann cells from the Trembler-J mouse (Tobler et al., 1999). The exact role of aggresome formation in the pathogenesis of lung disease has not yet been investigated in paradigms of naturally occurring mutant proSP-C expression or under conditions of abnormal proSP-C trafficking. The present study predominantly concerns the formation of aggresomes in lung epithelial cells expressing mutant proSP-C proteins but does not address their fate or their long-term effect on type II cell function. The experimental conditions that fostered the formation of aggresomes in this study were overexpression and proteasome inhibition, which impose a significant stress on the cell's degradative capacity. Our experiments confirm the existence of a homeostatic mechanism in the lung epithelium for the handling of mutant proteins. We speculate that the transport of ubiquinated proSP-C mutants to aggresomes functions to clear the cytoplasm of potentially toxic aggregates or may serve as a staging ground for eventual removal by incorporation into autophagocytic structures. However, western blots of lysates (Fig. 3) from our cells confirm the accumulation of a single molecular mass form of mutant EGFP/proSP-C without evidence of any smaller degradation products, suggesting that the half-life of these structures could be long. Thus, although aggresome formation may provide a cytoprotective role, the toxicity of the long-term accumulation of non-degraded aggregates leading to disruption of intermediate filaments and the MTOC is undefined. Several neurodegenerative diseases attributed to abnormal expression and trafficking of a mutant protein are associated with inclusion formation, resulting in disruption of microtubule-based directional axonal transport (Naef and Suter, 1999) and with promotion of apoptosis (Gow et al., 1998). Therefore, given the recent in vitro results presented in this report, the expression of mutant forms of surfactant protein could contribute to lung pathogenesis through alterations in type 2 cell viability, function and/or homeostasis induced by the formation of aggresomes. These hypotheses await further investigations in appropriate in vitro and animal models, which are currently in progress.

We thank Seth Thomas Scanlon for assistance with image processing. This work was supported by NIH HL-19737 and P50-HL56401 (both to M.F.B.).

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