Structural requirements for intracellular targeting of SP-C proprotein

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Russo, Scott J., Wenjing Wang, Catherine A. Lomax, and Michael F. Beers. Structural requirements for intracellular targeting of SP-C proprotein. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L1034–L1044, 1999.—Rat surfactant protein (SP) C is synthesized as a 194-amino acid proprotein that is proteolytically processed to a 35-amino acid mature form in subcellular compartments distal to the medial Golgi compartment. To identify domains of SP-C proprotein (proSP-C) necessary for endoplasmic reticulum translocation and for targeting to cytosolic processing compartments, we characterized expression patterns of heterologous SP-C fusion proteins in A549 lung epithelial cells and in the rat pheochromocytoma cell line PC-12. cDNA constructs were produced; these constructs encoded fusion proteins consisting of enhanced green fluorescent protein (EGFP) and wild-type proSP-C (EGFP/SP-C1–194), mature SP-C (EGFP/SP-C24–59), or progressive deletions of the NH2- or COOH-terminal flanking domains. By fluorescence microscopy, EGFP/SP-C1–194 transfected into A549 cells was translocated and expressed in acidic cytoplasmic vesicles. By deletional analysis, a functional signal peptide was mapped to the domain Phe24 to His59, whereas a motif for targeting to cytosolic vesicular compartments was localized to the NH2 flanking domain Met10 to Gln23. Truncations of the distal COOH terminus were retained in the endoplasmic reticulum/Golgi compartment; however, the COOH flanking region alone was insufficient for targeting. In PC-12 cells, EGFP/SP-C1–194 was expressed in peripheral cytosolic vesicles, whereas EGFP/SP-C24–194 and EGFP/SP-C23–59 were each translocated but not targeted. We conclude that two domains in the proSP-C sequence are required for targeting: mature SP-C (Phe24 to Leu58) contains a functional signal sequence active in epithelial and nonepithelial cells, whereas Met10 to Gln23, but not the COOH flanking peptide, is required for targeting to late vesicular compartments.

Rat SP-C is synthesized by the alveolar type II cell as a proprotein of 194 amino acids (6, 12). Contained within the propeptide is the 35-amino acid residue mature SP-C protein (SP-C3.7), which is flanked by propeptides of 23 amino acids at the NH2 terminus and 136 residues at the COOH terminus. Unlike other SPs, the NH2 terminus of the primary translation product does not contain a cleavable signal sequence, there are no sites for asparagine-linked glycosylation (6), and the mature SP-C molecule is an integral membrane protein. Nonetheless, after translation, proSP-C must be translocated to the endoplasmic reticulum (ER) membrane and routed into the distal secretory compartments, where it has been shown to undergo proteolysis, leading to production of the 3.7-kDa alveolar form. Therefore, the SP-C molecule is unique, in that the primary translation product represents a bitopic, transmembrane protein (18) that is ultimately secreted via the regulated exocytic pathway of epithelial cells as a processed, extremely lipophilic, mature peptide.

In vitro and in vivo models have been used to partially characterize the processing events that lead to the production of SP-C in alveolar surfactant. Pulse-chase experiments in isolated type II cells have demonstrated processing of proSP-C21 to 16- and 6-kDa intermediate forms, which involves an initial two-step removal of the COOH-terminal domains followed by the cleavage of the NH2-terminal domains (7, 29). Posttranslational proteolysis can be blocked by the use of brefeldin A (7), by low-temperature incubation (20°C) (29), or by inhibitors of organelar acidification (methylamine, bafilomycin A1) (4), which indicates that proteolysis is occurring within acidic subcellular compartments in the distal secretory pathway.

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Relatively little is known of the role of specific peptide domains contained within the proSP-C sequence that influence posttranslational subcellular targeting and processing. Recently, our laboratory has evaluated the role of the COOH terminus of proSP-C in intracellular processing of the primary translation product (8). Synthesis and processing of SP-C were evaluated using a lung epithelial cell line (A549) transfected with the eukaryotic expression vector containing the full-length cDNA or one of several COOH-terminal truncated forms. In these cells, immunoprecipitation of \(^{35}\)S-labeled cell lysates demonstrated synthesis and processing of proSP-C\(_{21}\) through the SP-C\(_{16}\) and SP-C\(_{6}\) intermediates by cleavages of the COOH-terminal propeptide. The wild-type SP-C protein was targeted to cytoplasmic vesicles of A549 cells. In contrast, deletion of as few as 10 amino acids from the COOH terminus resulted in translation but no further processing of mutant proteins. Double-label fluorescence immunocytochemistry with fluorescein isothiocyanate-concanavalin A indicated restriction of COOH-terminal mutants to an ER/Golgi compartment, demonstrating an association between targeting and subsequent processing.

The present study further extends the findings of the previous studies (4, 7, 8, 29) by defining specific peptide domains of proSP-C necessary for translocation and for targeting to processing compartments. Domain-specific proSP-C deletional mutants were used to construct and transfec heterologous fusion proteins containing the enhanced green fluorescent protein (EGFP) into the previously described A549 epithelial cell model and the neuroendocrine cell line PC-12, which has been shown to contain a regulated secretory pathway for exocytosis (14). The results of this study now demonstrate that a functional, internal signal sequence contained within the mature SP-C domain is sufficient for translocation of EGFP/SP-C into the ER in A549 and PC-12 cells. In addition, we also show that the region Met\(^{10}\) to Gln\(^{23}\), contained within the NH\(_{2}\)-flanking region, is necessary for targeting of translocated proSP-C from proximal compartments to more distal sites. The presence of the distal portion of the COOH-terminal flanking domain, shown in the previous study (8) to be necessary for processing, was not sufficient for targeting alone or when coupled with the signal peptide domain of proSP-C.

**EXPERIMENTAL PROCEDURES**

**Materials**

The pCDNA3 eukaryotic expression plasmid was obtained from Invitrogen (San Diego, CA). pEGFP-C\(_{1}\) and pEGFP-N\(_{1}\) plasmids and polyclonal anti-green fluorescent protein (GFP) antisera were produced from a synthetic peptide immunogen and have been previously characterized (7). Anti-NPSP-C (Met\(^{10}\) to Gln\(^{23}\)) recognizes proSP-C\(_{21}\) and all major intermediates but does not recognize mature SP-C.

**ProSP-C antisera.** Monospecific polyclonal rat proSP-C antisera were produced from a synthetic peptide immunogen and have been previously characterized (7). Anti-NPSP-C (Met\(^{10}\) to Gln\(^{23}\)) recognizes proSP-C\(_{21}\) and all major intermediates but does not recognize mature SP-C.

**SP-C cDNA Expression Constructs**

All procedures involving oligonucleotide and cDNA manipulations were performed essentially as described by Ausbel et al. (2).

**pcDNA3-rSP-C(-).** A full-length rat SP-C cDNA (816 bp) insert was previously subcloned into the pCDNA3 eukaryotic expression vector polylinker at the EcoRI site (site 12). The vector contains the human cytomegalovirus promoter (early promoter and enhancer region), bovine growth hormone polyadenylation sequence, \(\beta\)-lactamase and neomycin resistance genes, and T7 and SP6 promoters for sense/antisense in vitro transcription. Detailed characterization of pCDNA3-rSP-C(-) has been previously published (8).

**Chimeric EGFP fusion proteins.** A family of chimeric fusion proteins consisting of EGFP and wild-type rat SP-C (Met\(^{1}\) to Ile\(^{1035}\)) or mutant SP-C cDNA containing truncations of the COOH- and for NH\(_{2}\)-terminal flanking region of proSP-C generated by PCR are schematically illustrated in Fig. 1. In all cases, pCDNA3-rSP-C(-) was used as a template. Sequences encoding the indicated regions of proSP-C peptide were amplified by PCR with use of modified primers to create in-frame fusion proteins through introduction of 1) a BspEI site at the 5'-end and an XhoI site at the 3'-end for cloning into pEGFP-C\(_{1}\) (Fig. 1A) or 2) a KpnI site at the 5'-end and an AgeI site at the 3'-end for cloning into pEGFP-N\(_{1}\) (Fig. 1B). The oligonucleotide primers are listed in Table 1.

**Amplification reactions containing 0.2 \(\mu\)M primers, 1.25 \(\mu\)M dNTP mixture, 1.5 \(\mu\)M MgCl\(_{2}\), 10 ng of template, and 2.5 U of Vent DNA polymerase (New England Biolabs, Beverly, MA) consisted of 30 cycles as follows: denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, and primer extension at 72°C for 15 s. After the last cycle, the mixture was incubated at 72°C for 7 min. Purified PCR fragments were ligated into pEGFP-C\(_{1}\) after digestion with BspEI and XhoI or into pEGFP-N\(_{1}\) after digestion with KpnI and AgeI. For construction of EGFP/SP-C\(_{60–175}\) (Fig. 1C), deletion mutagenesis was performed by overlap extension PCR with a two-round, four-primer technique (11). In round 1, two PCR products (SP-C\(^{1–59X}\) and SP-C\(^{1–75X}\)) were generated in separate reactions carried out using pCDNA3-rSP-C(-) as the template with primers A and B or primers C and D (Table 2). Cycling conditions for round 1 were as follows: one cycle at 94°C for 1 min 30 s, 25 cycles at 94°C for 40 s, 50°C for 30 s, and 72°C for 40 s, and a final extension at 72°C for 7 min. The resulting products were purified using the Wizard PCR Prep DNA Purification System (Promega, Madison, WI). Purified segments SP-C\(^{1–59X}\) and SP-C\(^{1–75X}\) were added jointly along with primers A and D for round 2 of PCR. Fusion of the two intermediate segments is achieved as a result of overlapping complementary regions in the products produced in round 1, which pair during the annealing phase of PCR round 2 and are amplified by use of primers complementary to the 5'- and 3'-ends (primers A and D, respectively). Cycling conditions in round 2 were identical to those in round 1 described above. The resulting mutant insert, SP-C\(^{60–175}\), was purified and ligated into pEGFP-C\(_{1}\) after digestion with BspEI and XhoI.

**Automated DNA sequencing in both directions was performed at the Core Facility in the Department of Genetics at the University of Pennsylvania.** No nucleotide mutations in the coding region of full-length SP-C or any deletional con-
structs were detected with the exception of EGFP/SP-C\textsuperscript{1–72} (D2Q).

**Cell Lines and Transfection**

A549 cells. The lung epithelial cell line A549 utilized in transfection studies was originally obtained through the American Type Culture Collection (Manassas, VA) and made available as a gift of Dr. S. I. Feinstein. A549 cells were grown at 37°C in 5% CO\textsubscript{2} in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin, as previously described (8).

For transfection studies, A549 cells were grown to 80% confluence in 35-mm\textsuperscript{2} plates (Corning). EGFP/SP-C constructs (10 µg/dish) were transiently transfected into A549 cells by CaPO\textsubscript{4} precipitation: 0.18 ml of 0.25 M CaCl\textsubscript{2} was added dropwise to 0.18 ml of plasmid DNA dissolved in 2× HEPES-buffered saline (50 mM HEPES, 280 mM NaCl, and 1.5 mM NaPO\textsubscript{4}, pH 7.1) (2). The medium was replaced at 24 h, and cells were maintained for up to 48 h.

PC-12 cells. PC-12 cells (rat pheochromocytoma cells, clone 251), a gift from Dr. Harry Ischiropoulos (University of Pennsylvania), were grown in 75-cm\textsuperscript{2} culture flasks containing RPMI 1640 supplemented with 10% horse serum and 5% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO\textsubscript{2}. Under these conditions, PC-12 cells are not dependent on nerve growth factor for survival.

For transfection, PC-12 cells were grown to 60% confluence in 35-mm\textsuperscript{2} plates; 20 µg of plasmid DNA were combined with 10 µl of LipofectAMINE and applied to cells in Optimem.
Table 1. Primer sets used in PCRs for generation of EGFP rat SP-C fusion constructs

<table>
<thead>
<tr>
<th>Construct Name</th>
<th>Rat SP-C Amino Acid</th>
<th>5’ Primer (forward)</th>
<th>3’ Primer (reverse)</th>
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<tr>
<td>EGFP-C1/SP-C1–23</td>
<td>Met1–Gln23</td>
<td>ATGGAATGGACATGGGTAGCAAAGAG</td>
<td>dTACAAAGTCCGGAAATGGGACATGGGATGCAACAGCAAGAG</td>
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<tr>
<td>EGFP-C1/SP-C1–72</td>
<td>Met1–His32</td>
<td>ATGGAATGGACATGGGTAGCAAAGAG</td>
<td>dTACAAAGTCCGGAAATGGGACATGGGATGCAACAGCAAGAG</td>
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<td>EGFP-C1/SP-C1–175</td>
<td>Met1–Leu175</td>
<td>ATGGAATGGACATGGGTAGCAAAGAG</td>
<td>dTACAAAGTCCGGAAATGGGACATGGGATGCAACAGCAAGAG</td>
</tr>
<tr>
<td>EGFP-C1/SP-C1–185</td>
<td>Met1–Leu185</td>
<td>ATGGAATGGACATGGGTAGCAAAGAG</td>
<td>dTACAAAGTCCGGAAATGGGACATGGGATGCAACAGCAAGAG</td>
</tr>
<tr>
<td>EGFP-C1/SP-C1–194</td>
<td>Met1–Ile194</td>
<td>ATGGAATGGACATGGGTAGCAAAGAG</td>
<td>dTACAAAGTCCGGAAATGGGACATGGGATGCAACAGCAAGAG</td>
</tr>
<tr>
<td>EGFP-C1/SP-C1–24–59</td>
<td>Phe24–His59</td>
<td>ATGGAATGGACATGGGTAGCAAAGAG</td>
<td>dTACAAAGTCCGGAAATGGGACATGGGATGCAACAGCAAGAG</td>
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<tr>
<td>EGFP-C1/SP-C1–24–175</td>
<td>Phe24–Ile175</td>
<td>ATGGAATGGACATGGGTAGCAAAGAG</td>
<td>dTACAAAGTCCGGAAATGGGACATGGGATGCAACAGCAAGAG</td>
</tr>
<tr>
<td>EGFP-C1/SP-C1–175–194</td>
<td>Leu175–Ile194</td>
<td>GTGAAGGCCCATGAGCA</td>
<td>dGACCCAAGCTTGCTCATGGGCCTTCACGAG</td>
</tr>
<tr>
<td>SP-C1–59/EGFP-N1</td>
<td>Met1–His59</td>
<td>GTGAAG GCCCATGAGCA</td>
<td>dGACCCAAGCTTGCTCATGGGCCTTCACGAG</td>
</tr>
<tr>
<td>SP-C24–59/EGFP-N1</td>
<td>Phe24–His59</td>
<td>GTGAAG GCCCATGAGCA</td>
<td>dGACCCAAGCTTGCTCATGGGCCTTCACGAG</td>
</tr>
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</table>

Amino acid numbers correspond to nucleic acids in rat surfactant protein C (SP-C) cDNA (Ref. 12). In all cases, pcDNA3-rSP-C(+) (Ref. 12) was used as template. For enhanced green fluorescent protein (EGFP)-C1, each 5’ primer contains a BspEI site (5’-TCCGGA-3’) for in-frame ligation; for constructs containing COOH-terminal truncations, 3’ primer contains an XhoI site (5’-CTCGAG-3’) for ligation into EGFP-C1 polynucleotides and TTA for production of a stop codon immediately adjacent to terminal codon of truncated coding sequence; for constructs containing Ile34, 3’ primer matches polylinker of pcDNA3 and contains an AgeI site (5’-ACCGGT-3’) for in-frame cloning into EGFP-N1 for SP-C1–59, 5’ primer corresponds to polylinker sequence of pcDNA3 with XhoI site (GTACC) included; for SP-C24–59, 5’ primer contains a KpnI site (GTACC), start codon (ATG), and Kozak sequence.

medium (Life Technologies). The cells were incubated for 16 h with DNA-LipofectAMINE mixture, then the medium was supplemented with an equal volume of RPMI 1640 plus 20% horse serum and 10% fetal bovine serum. Cells were maintained for up to an additional 48 h.

Fluorescence Microscopy

Vital fluorescence microscopy for localization of expressed EGFP/SP-C fusion proteins was performed on 35-mm² plastic dishes with an Olympus I-70 inverted fluorescence microscope with filter packages High Q fluorescein isothiocyanate for GFP (excitation at 480 nm, emission at 535/550 nm), High Q TR for Texas red (excitation at 560/555 nm, emission at 645/675 nm), and standard 4,6-diamidino-2-phenylindole for LysoTracker blue (excitation at 360/340 nm, emission at 460 nm) obtained from Chroma Technology (Brattleboro, VT). 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).

Analytic Methods

PAGE and immunoblotting. One-dimensional SDS-PAGE was performed in 12% polyacrylamide gels by the method of Laemmli (20). Proteins were transferred to nitrocellulose, and immunoblotting of transferred samples was performed as previously described (5) using primary polyclonal anti-GFP antiserum (1:2,000). Bands were visualized by enhanced chemiluminescence (ECL kit, Amersham, Arlington Heights, IL).

RESULTS

Expression of EGFP/SP-C1–194 Protein by Transfected A549 Cells

Expression of EGFP-C1 and EGFP-C2/SP-C1–194 fusion proteins was readily detected in transiently transfected A549 cells within 24–48 h after introduction of plasmids by CaPO4-DNA precipitation. In Fig. 2A,

Table 2. Primer sets used in PCRs for generation of EGFP-C1/SP-C1–194 fusion constructs

<table>
<thead>
<tr>
<th>SP-C Insert Name</th>
<th>5’ Primer (forward)</th>
<th>3’ Primer (reverse)</th>
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<tbody>
<tr>
<td>Met1–His59</td>
<td>ATGGAATGGACATGGGTAGCAAAGAG</td>
<td>dGACCCAAGCTTGCTCATGGGCCTTCACGAG</td>
</tr>
<tr>
<td>Leu175–Ile194</td>
<td>ATGGAATGGACATGGGTAGCAAAGAG</td>
<td>dGACCCAAGCTTGCTCATGGGCCTTCACGAG</td>
</tr>
<tr>
<td>SP-C1–60/EGFP-N1</td>
<td>Met1–His59</td>
<td>ATGGAATGGACATGGGTAGCAAAGAG</td>
</tr>
<tr>
<td>SP-C1–59/EGFP-N1</td>
<td>Phe24–His59</td>
<td>ATGGAATGGACATGGGTAGCAAAGAG</td>
</tr>
</tbody>
</table>

For both reactions of primary PCR, pcDNA3-rSP-C(+) (Ref. 8) was used as template. Primer A contains a BspEI site (5’-TCCGGA-3’) for in-frame ligation into EGFP-C1. The 3’-end of primer D matches polylinker of pcDNA3 and contains an XhoI site (CTCGAG); use of this primer resulted in inclusion of 3’-untranslated region in mutant construct. Overlapping regions of primers B and C, which permit complementation in secondary PCR, are boldface.
transfection of EGFP-C3 resulted in expression of a fluorescent signal in a diffuse pattern throughout the cell. In contrast, EGFP-C2/SP-C1–194 was expressed in a similar time frame but was spatially restricted to cytoplasmic vesicles of A549 cells (Fig. 2B). This pattern was similar to that seen with A549 cells transfected with wild-type proSP-C (pcDNA3-rSP-Cwt), fixed, and stained with anti-NPROSP-C antiserum (Fig. 2C). These results indicate that the presence of EGFP does not interfere with proSP-C trafficking and that SP-C1–194 contains motifs for ER translocation (signal peptide) and direction of a heterologous fusion protein (EGFP) into distal vesicular compartments.

To define the major forms of transfected EGFP/proSP-C fusion proteins expressed, Western blotting was performed using a polyclonal anti-GFP antibody (Fig. 3). Transfection of A549 cells with EGFP resulted in expression of a major product matching the predicted relative molecular weight (Mr) of 27,000 (Fig. 3, lane 1). A minor band migrating at Mr 33,000 but constituting <10% of the total signal also appeared (+). Neither band was detected in cell lysates of mock-transfected cells, demonstrating specificity of the antiserum (lane 3). When EGFP-C2/SP-C1–194 was transfected, three bands were detected (Fig. 3, lane 1). A band with Mr 48,000, representing the predicted Mr of the primary translation product of the fusion protein, and two major intermediate forms of Mr 42,000 and 32,500 were identified (arrowheads). Bands with Mr 27,000–32,500 were not found, indicating that EGFP-C2/SP-C1–194 was partially processed by complete removal of the proSP-C COOH terminus.

To further characterize the compartment in A549 cells to which EGFP-C2/SP-C1–194 fusion protein was being directed, fluorescence microscopy of transfected EGFP-C2/SP-C1–194 was performed in combination with markers for acidic compartments (Fig. 4). The EGFP-C2/SP-C1–194 fluorescent fusion protein could be colocalized with the LysoTracker blue probe, a fluorescence marker that accumulates in acidic subcellular compartments (Fig. 4). Thus EGFP-C2/SP-C1–194 is expressed and targeted to acidic cytoplasmic vesicles of A549 cells.
terminal position. SP-C^{24-59}/EGFP-N1, a construct containing mature SP-C in frame with the NH2 terminus of EGFP, was expressed in a pattern identical to EGFP-C1/SP-C^{24-59} (Fig. 5C).

Role of the COOH Flanking Peptide

To investigate the role of the COOH flanking peptide in the targeting proSP-C, EGFP fusion proteins containing truncations of the distal proSP-C COOH terminus were constructed and transfected into A549 cells (Figs. 6 and 7). In contrast to fusion proteins containing wild-type SP-C (Fig. 6A), expression of these two mutants was restricted to more proximal compartments. Expression of EGFP-C1/SP-C^{1-175} (Fig. 6B) and EGFP-C1/SP-C^{1-185} (Figs. 6C and 7B) was limited to early compartments. With use of BODIPY TR ceramide to identify ER/Golgi compartments in A549 cells (Fig. 7), fluorescence overlays demonstrated colocalization of EGFP-C1/SP-C^{1-185} with ceramide-labeled compartments (Fig. 7D).

In contrast to removal of the distal COOH terminus, more extensive truncation of the COOH domain restored targeting. When EGFP-C1/SP-C^{1-72} was transfected, localization of the fusion product to cytoplasmic vesicles was observed (Fig. 6E). To determine whether the domain His^{59} to Ser^{72} contained a specific motif, an additional construct was created in which His^{59} to Ser^{72} was replaced with the distal COOH terminus. The resulting construct, EGFP-C1/SP-C^{60-175}, also appeared in cytoplasmic vesicles (Fig. 6F). As expected, transient transfections of EGFP and the distal COOH terminus alone (EGFP-C1/SP-C^{175-194}) produced an expression pattern similar to EGFP-C1 (Fig. 6D). This pattern of diffuse fluorescence appearing throughout transfected A549 cells was recapitulated with the use of EGFP-C1/SP-C^{185-194} (data not shown). Taken together, these results indicate that the COOH flanking peptide of proSP-C does not contain specific targeting motifs for delivery to cytoplasmic vesicles.

Fig. 4. EGFP-C1/SP-C^{1-194} is expressed in acidic cytoplasmic compartments. After transfection for 48 h with EGFP-C1/SP-C^{1-194} (10 µg DNA/35-mm² dish), A549 cells were incubated at 37°C for 120 min with 1 µM LysoTracker blue (LTB). Cells were then washed with DMEM and photographed using a standard diamidino-2-phenylindole filter for LysoTracker blue (excitation at 360/340 nm, emission at 460 nm; A) and High Q FITC filter for GFP (excitation at 480 nm, emission at 535/550 nm; B). Images were assigned pseudocolor (LysoTracker blue = blue; GFP = green). LysoTracker blue identifies acidic subcellular compartments in all A549 cells (A), which exhibit a subcellular distribution similar to that of EGFP-C1/SP-C^{1-194} (B). C: overlay of A and B, resulting in extensive colocalization appearing as aqua (blue-green).

Fig. 5. Mature SP-C contains a functional signal peptide. Phase (A, B, and C) and fluorescence (A’, B’, and C’) images of A549 cells were transfected for 36 h with EGFP-C1/SP-C^{1-194} (A and A’), EGFP-C1/SP-C^{24-59} (B and B’), or SP-C^{24-194}/EGFP-N1 (C and C’) by CaPO4 method (10 µg DNA/35-mm² dish). Images were acquired by video fluorescence microscopy with a High Q FITC filter (excitation at 480 nm, emission at 535/550 nm) and are representative of >150 cells for each construct derived from 6 separate transfections. *, Nucleus.
Met\textsuperscript{10} to Gln\textsuperscript{23} Is Required for ProSP-C Targeting

We next evaluated domains of the NH\textsubscript{2} terminus for the presence of targeting motifs. Deletion of the entire NH\textsubscript{2} flanking domain resulted in limitation of expression of the truncated fusion protein to early compartments (Fig. 8A). As with EGFP-C\textsubscript{1}/SP-C\textsubscript{1–175} (B), EGFP-C\textsubscript{1}/SP-C\textsubscript{1–185} (C), EGFP-C\textsubscript{1}/SP-C\textsubscript{175–194} (D), EGFP-C\textsubscript{1}/SP-C\textsubscript{1–72} (E), or EGFP-C\textsubscript{1}/SP-C\textsubscript{160–175} (F) by CaPO\textsubscript{4} method. Images were acquired 36 h after transfection by video fluorescence microscopy with a High Q FITC filter (excitation at 480 nm, emission at 535/550 nm). Expression of distal COOH truncations is restricted to perinuclear compartments of A549 cells, inasmuch as EGFP-C\textsubscript{1}/SP-C\textsubscript{1–185} and EGFP-C\textsubscript{1}/SP-C\textsubscript{1–175} are translocated from cytoplasm but fail to target cytoplasmic vesicles. In contrast, EGFP-C\textsubscript{1}/SP-C\textsubscript{1–72} (E) and EGFP-C\textsubscript{1}/SP-C\textsubscript{160–175} (F) appear in cytoplasmic vesicles.

Fig. 6. Expression of proSP-C COOH-terminal deletional mutants. A549 cells were transfected with EGFP-C\textsubscript{1}/SP-C\textsubscript{1–194} (A), EGFP-C\textsubscript{1}/SP-C\textsubscript{1–175} (B), EGFP-C\textsubscript{1}/SP-C\textsubscript{1–185} (C), EGFP-C\textsubscript{1}/SP-C\textsubscript{175–194} (D), EGFP-C\textsubscript{1}/SP-C\textsubscript{1–72} (E), or EGFP-C\textsubscript{1}/SP-C\textsubscript{160–175} (F) by CaPO\textsubscript{4} method. Images were acquired 36 h after transfection by video fluorescence microscopy with a High Q FITC filter (excitation at 480 nm, emission at 535/550 nm). Expression of distal COOH truncations is restricted to perinuclear compartments of A549 cells, inasmuch as EGFP-C\textsubscript{1}/SP-C\textsubscript{1–185} and EGFP-C\textsubscript{1}/SP-C\textsubscript{1–175} are translocated from cytoplasm but fail to target cytoplasmic vesicles. In contrast, EGFP-C\textsubscript{1}/SP-C\textsubscript{1–72} (E) and EGFP-C\textsubscript{1}/SP-C\textsubscript{160–175} (F) appear in cytoplasmic vesicles.

Targeting of EGFP/SP-C Fusion Proteins in Neuroendocrine Cells

To determine whether the targeting epitopes in the proSP-C molecule are recognized by sorting machinery of nonlung epithelial cells, the neuroendocrine cell line PC-12 was transfected with EGFP-SP-C fusion proteins (Fig. 9). As for A549 cells, EGFP alone was expressed diffusely throughout the cytoplasm of PC-12 cells (not shown). In contrast, EGFP-C\textsubscript{1}/SP-C\textsubscript{1–194} was directed to vesicles throughout the cytoplasm, in neurites, and beneath the plasma membrane (Fig. 9, A and A'). The mature SP-C sequence was capable of effecting translocation into ER/Golgi compartments but was insufficient for direction of fusion proteins to more distal compartments in the secretory pathway (Fig. 9, B and B'). Similar to A549 cells, the deletion of the NH\textsubscript{2} flanking domain of proSP-C resulted in retention in early compartments (Fig. 9, C and C').

DISCUSSION

The complete synthesis of SP-C by the alveolar type II cell must include translation and translocation of proSP-C to the ER, palmitoylation, sorting, and exit of the proprotein from the Golgi compartment, deavage of flanking propeptide domains, and assembly of mature SP-C with surfactant phospholipids and SP-B in the

Fig. 7. Expression of COOH-terminal deletional mutant proSP-C colocalizes with a Golgi marker. A549 cells were transfected with EGFP-C\textsubscript{1}/SP-C\textsubscript{1–185} (A–D) or EGFP-C\textsubscript{1}/SP-C\textsubscript{1–194} (E–H) by CaPO\textsubscript{4} method. Twenty-four hours after introduction of plasmid DNA, cells were labeled for 30 min with N-((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxyl)acetyl)sphingosine [BODIPY TR ceramide (Cer-TR), 2 µM] at 37°C. Images were sequentially acquired by video fluorescence microscopy with a High Q FITC filter (excitation at 480 nm, emission at 535/550 nm) for GFP (B and F) and a High Q TR filter (excitation at 560/555 nm, emission at 645/675 nm) for Cer-TR (C and G). Fluorescence images were assigned pseudocolor (red = TR; green = GFP), and 2 channels were combined using an IMAGE 1 software overlay protocol. Extensive areas of colocalization of EGFP-C\textsubscript{1}/SP-C\textsubscript{1–185} and Cer-TR appear as yellow (D). EGFP-C\textsubscript{1}/SP-C\textsubscript{1–194} is localized in cytoplasmic vesicles (green) distinct from Cer-TR compartment (H). A and E represent corresponding phase images.
lamellar body followed by exocytosis (6). Comparative analysis of numerous secreted proteins has failed to identify consistently conserved amino acid sequences that mediate translocation of the primary translation product or selective targeting to secretory granules. Although by deletional analysis and construction of fusion proteins, functional targeting determinants have been localized for several secretory proteins, including SP-B (1, 21, 22), such detailed information regarding SP-C has been lacking. Previously, we showed that synthetic processing of SP-C by alveolar epithelial cells was dependent, in part, on an intact distal COOH propeptide terminus (8). The present study extends these observations by use of alveolar epithelial and neuroendocrine cell lines to define sequence motifs important for correct translocation and targeting. We have now identified two functional domains in the primary sequence that are responsible for the normal trafficking of synthesized proSP-C protein. The mature SP-C sequence has been shown to function as a non-cleavable signal peptide, whereas the direction of EGFP fusion proteins to cytoplasmic targets is dependent on the presence of the region Met10 to Gln23 found in the NH₂-terminal flanking domain. In addition, although disruption of the distal COOH terminus results in mistargeting, the COOH-terminal flanking region does not appear to contain targeting motifs.

Transfection of a plasmid containing EGFP alone into A549 cells resulted in expression of fluorescent protein in a diffuse pattern throughout the entire cell, consistent with and predicted by the lack of a signal peptide in the EGFP sequence. The fusion protein consisting of full-length proSP-C in frame with EGFP was capable of ER translocation and targeting, as indicated by the resulting vesicular fluorescence pattern (Figs. 2, 4, 6, and 7). The pattern of EGFP/SP-C₁–₁₉₄ expression seen in vital fluorescence microscopy was

Fig. 8. NH₂ terminus of proSP-C contains motifs for targeting. Cells at 80% confluence were transfected with EGFP-C₁/SP-C₂₄–₁₉₄ (A), EGFP-C₁/SP-C₁–₂₃ (B), EGFP-C₁/SP-C₁₀–₁₉₄ (C), or EGFP-C₁/SP-C₁–₁₉₄ (D) by CaPO₄ method. After 48 h of transfection, images were acquired by video fluorescence microscopy with a High Q FITC filter (excitation at 480 nm, emission at 535/550 nm) and are representative of > 50 cells for each construct.

Fig. 9. Expression of EGFP/SP-C fusion proteins in PC-12 cells. PC-12 cells grown to 60% confluence were transfected with EGFP-C₁/SP-C₁–₁₉₄ (A and A'), SP-C₂₄–₅₉/EGFP-N₁ (B and B'), or SP-C₂₄–₅₉/EGFP-N₁ (C and C') with LipofectAMINE (20 µg DNA/35-mm dish). Phase images (A', B', and C') and corresponding fluorescence images (A, B, and C) were acquired by video microscopy. GFP expression was visualized using a High Q FITC filter (excitation at 480 nm, emission at 535/550 nm). Micrographs are representative of >50 cells for each construct.
similar to immunochemical staining patterns of fixed A549 cells transfected with SP-C1–194, indicating that EGFP does not affect the targeting of proSP-C (Fig. 2B) (8). Previously, in isolated rat type II cells, we demonstrated that endogenous proSP-C was targeted to acidic compartments distal to the Golgi compartment (4). Colocalization studies with the LysoTracker blue marker indicated that the vesicles in A549 cells (Fig. 4) were also acidic. As for primary type II cells, delivery of proSP-C (EGFP/SP-C1–194) to acidic cytosolic vesicles in A549 cells is associated with posttranslational proteolytic processing of proSP-C. Using metabolic labeling, we previously demonstrated synthesis of proSP-C21 and cleavage to proSP-C18 and proSP-C6 intermediates by transfected A549 cells, but the lack of a monospecific antibody against the mature SP-C epitope prevented us from definitively detecting the presence of mature SP-C (8). In the present study, the appearance of EGFP/SP-C1–194 in cytosolic vesicles is associated with processing of the fusion protein, as indicated by Western blotting patterns (Fig. 3). The banding pattern of cell lysates from transfections of EGFP/SP-C1–194 is consistent with complete cleavage of the COOH flanking sequence. However, the failure to liberate free EGFP (M, 27,000) or a product of M, 29,000 (EGFP + proSP-C NH2 terminus) indicates that these cells do not fully process the NH2 terminus of proSP-C, leaving a truncated fusion protein of EGFP/SP-C6.

Although the A549 cell line was originally derived from alveolar type II cells, the failure to fully process proSP-C may be due to limitations with this model. In contrast to type II cells, the A549 cell, as well as any available cell line model, does have potential limitations in attempting to mimic all functional aspects of surfactant metabolism. For A549 cells, these limitations include a lack of lamellar bodies, a lack of SP expression, and a lack of a stimulated (regulated) secretory pathway. Because in type II cell and perfused lung models final processing (NH2-terminal proteolysis) has been shown to occur at or near the lamellar body, we speculate that the lack of lamellar bodies in A549 cells (which also do not express SP-B) may contribute to the failure to completely process the NH2 terminus of proSP-C. Nonetheless, this model does appear useful in evaluating important early events, namely, translocation and targeting from the Golgi compartment, but may not be as useful in evaluating later secretory events. A recent preliminary report by Conkright et al. (9) has shown that primary isolated mouse type II cells can maintain a type II cell phenotype in culture. If transfectable with reasonable efficiency, this cell model may be of use in future studies.

Because of the potential limitations in relying on a single model, we chose to evaluate some of the findings in the neuroendocrine cell line PC-12. Although these cells have a regulated secretory pathway that has been shown to target transfected SP-B constructs (21), they do not process surfactant proprotein, do not synthesize or store phospholipid, and, in cases of overexpression of transfected protein, will target to a constitutive pathway. Importantly, we have found that the ability of specific motifs in proSP-C to target EGFP/SP-C1–194 in A549 cells could be recapitulated using PC-12 cells. The location of fusion proteins in cytosolic vesicles in neu-rite structures is consistent with targeting to the regulated pathway, although alternative direction of fusions to constitutive vesicles or lysosomes cannot be excluded from the present studies. With use of the LysoTracker reagent, acidic vesicles can be identified in similar locations within the neurites of PC-12 cells; this is consistent with the known pH of secretory granules (unpublished observations). Thus our approach has been to examine targeting in two separate but complementary models, and, taken together, results shown here support the notion that proSP-C signal and sorting domains are functional in epithelial and endocrine cells.

In contrast to SP-B, proSP-C does not contain a consensus sequence for a cleavable signal peptide (12). Deletional analysis mapped the functional signal sequence to amino acids 24–59, the sequence of the mature SP-C molecule, which was capable of inducing translocation of EGFP in A549 and PC-12 cells. From in vitro studies, proSP-C has been shown to be an integral membrane protein (27), with the mature SP-C sequence postulated to act as a membrane anchor (18). It now appears that this region is also required for ER translocation and that this function is independent of its position within the fusion protein. EGFP-C1 and EGFP-N1 plasmids each containing mature SP-C alone could be translocated, indicating that the mature SP-C sequence can function as a signal sequence regardless of placement in an NH2-terminal position (SP-C24–59/EGFP-N1), COOH-terminal position (EGFP-C1/SP-C24–59), or internal position (EGFP-C1/SP-C1–194).

Interestingly, the expression pattern of EGFP-C1/SP-C24–59 and SP-C24–59/EGFP-N1 shows clustering of mutant fusion protein in juxtanuclear structures of A549 (Fig. 5) and PC-12 (Fig. 9B) cells. The appearance of a localized “hot spot” image is consistent with a similar structure previously described by Johnston et al. (17) using HEK-293 cells stably transfected with a mutant form of another integral membrane protein, cystic fibrosis transmembrane regulator (CFTR). Overexpression of the ΔF508 form of CFTR or inhibition of cellular proteasome activity in transfected cells led to the accumulation of stable, aggregated, high-molecular-weight, detergent-insoluble, multiubiquinated forms of CFTR. Immunofluorescence demonstrated accumulation of degraded CFTR in a distinct pericentriolar structure, which they have termed the aggresome. Thus it appears that the EGFP/SP-C24–59 and SP-C24–59/EGFP are translocated but end up in juxtanuclear structures that are compatible with aggresomes. As for CFTR or any other aggregation-prone protein, this could occur if the high-level expression of these mutants is misfolded and/or aggregated and the levels of abnormal protein exceed the capacity of the proteasome.

Despite being necessary and sufficient for translocation, the mature SP-C molecule was incapable of direct-
ing fusion proteins to distal (cytoplasmic) compartments. Previously, data from our group and others (8, 19) demonstrated the importance of an intact COOH propeptide terminus for targeting and processing. Keller et al. (19) showed that deletion of 22 amino acids from the COOH terminus resulted in retention of mutant proSP-C protein within the ER of Chinese hamster ovary cells. We previously extended this observation by demonstrating that deletion of as few as 10 amino acids of this COOH terminus resulted in colocalization of the mutated protein with a concanavalin A-stained (ER/Golgi) compartment in A549 cells (8). In this study, we asked whether the distal COOH terminus could act as a sorting signal. EGFP fusion proteins containing only the domain Leu175 to Ile194 were not targeted (Fig. 6), which again is attributable to the absence of a signal peptide sequence. However, even if the entire COOH propeptide domain (His39 to Ile194) was combined with the mature SP-C molecule (EGFP-C1/SP-C1–194), expression was limited to early compartments. In combination with studies using EGFP-C1/SP-C1–175 and EGFP-C1/SP-C1–72, which were targeted to cytoplasmic vesicles (Fig. 6), these results demonstrate that the COOH flanking domain (His39 to Leu172, Leu172 to Leu175, and Leu175 to Ile194) does not contain regions that are sufficient alone or in the context of the signal peptide to target proSP-C. In this regard, proSP-C trafficking appears to be similar to SP-B, in which deletion analysis has shown that the NH2 flanking domain and mature peptide are sufficient to effect targeting (1). Although the COOH domain of SP-B appears to play a role in the genesis of the lamellar body, the function of the proSP-C COOH flanking sequence remains to be defined.

Although significantly shorter than the COOH flanking peptide, the NH2-terminal domain of proSP-C is highly conserved among all species (12). The lack of vesicular targeting of EGFP fusions containing NH2-terminal deletions (SP-C1–194 and SP-C1–59) is consistent with removal of targeting determinants. The restoration of targeting with the use of EGFP-C1/SP-C1–104 identifies the region Met10 to Gln23 as the COOH flanking sequence. Although proSP-C is targeted to acidic vesicles in type II and A549 cells and the lamellar body has been shown to contain lysosomal markers such as LAMP-1 (lgp-120) (32), there is no homology of this region with other known lysosomal targeting determinants such as dileucine- or tyrosine-based motifs found in integral bitopic membrane proteins such as LAMP-1 or CD-3y. BLAST search of the domain Met10 to Gln23 has failed to identify homology with other known domains and underscores the novelty of this motif.

Although the amino flanking sequence contains the targeting motif, the restriction to ER/Golgi compartments observed from the deletion of the distal COOH terminus is probably due to another mechanism. Misfolding of the natural conformation of proteins has been associated with aggregation and retention in the early stages of the secretory pathway (15). Within the COOH terminus of proSP-C, two conserved cysteine residues (C122 and C188) could promote intrachain folding. Generation of EGFP-C1/SP-C1–175 and EGFP-C1/SP-C1–185 mutants results in deletion of one of these residues (C188). Thus misfolding of nascent protein could serve as the mechanism for retention of distal COOH truncations in more proximal compartments. Additional studies utilizing site-directed mutagenesis of C120 and C188 are in progress to further define the role of the cysteines in the COOH-terminal flanking peptide.

The SP-C molecule is unique, in that the primary translation product and the processed, secreted, mature SP-C peptide represent bitopic transmembrane proteins. Because of the extreme hydrophobicity of the mature SP-C molecule, it is likely that the successful export of proSP-C from early (ER and Golgi) compartments is dependent on proper ER membrane insertion (orientation), conformational folding, and exposure of targeting motifs. On the basis of our results in this study and previous work by our group and others (7, 8, 19, 27–29), we can propose a model for the role of the proSP-C peptide domains: during translation, the proSP-C primary translation product is translocated to the ER and inserted into the ER membrane as an integral membrane protein in a type II membrane orientation (NH2 in cytoplasm) that is mediated by the domain SP-C1–59. After membrane insertion, disulfide-mediated folding of the COOH terminus can occur in the oxidizing environment of the ER lumen. It is likely that membrane insertion and protein folding expose the targeting motif within the NH2 flanking sequence (Met10 to Gln23) that subsequently facilitates exit from the Golgi compartment via transport vesicles. Still membrane associated, the propeptide is remodeled by proteolytic cleavage of initially COOH, then NH2, domains during transport to multivesicular bodies and subsequent transfer to lamellar bodies. Although this model assumes that SP-C is a type II bitopic membrane protein, the exact orientation of proSP-C in intracellular membranes is unclear. By utilizing modeling studies of other bitopic transmembrane proteins, on the basis of charge differences on either side of the transmembrane α-helix (window = 15 residues; C-N = −1), the NH2 terminus of proSP-C is predicted to be within the cytoplasm (16). This prediction has been supported by an in vitro study that used protease protection assays in model vesicular systems (18). However, using slightly different methodology, another group has found a type III orientation (COOH in the cytoplasm) (29). Therefore, additional studies using in situ techniques will likely be required, and the determination of precise orientation of proSP-C will also promote further targeting studies that can test the ability of the NH2 flanking sequence (Met10 to Gln23) for the targeting of other heterologous transmembrane protein segments.

In conclusion, SP-C, like SP-B, is expressed as a larger proprotein that undergoes extensive posttranslational processing before it achieves a mature, functional form. Although both proteins contain motifs critical for proper folding and appear to rely on NH2 flanking domains to effect targeting to the secretory pathway of type II cells, our results with heterologous fusion proteins demonstrate that SP-C differs from
SP-C, in that it contains a noncleavable internal signal peptide sequence (mature SP-C). Furthermore, although complete processing appears to be cell specific, recognition of the proSP-C targeting motifs appears to be ubiquitous, inasmuch as a neuroendocrine cell line and an alveolar epithelial cell line are capable of recognizing these domains for the direction of fusion proteins.

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REFERENCES


