Intracellular Localization of Processing Events in Human Surfactant Protein B Biosynthesis*

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Surfactant protein B (SP-B) is essential to the function of pulmonary surfactant and to alveolar type 2 cell phenotype. Human SP-B is the 79-amino acid product of extensive post-translational processing of a 381-amino acid preproprotein. Processing involves modification of the primary translation product from 39 to 42 kDa and at least 3 subsequent proteolytic cleavages to produce the mature 8-kDa SP-B. To examine the intracellular sites of SP-B processing, we carried out immunofluorescence cytochemistry and inhibitor studies on human fetal lung in explant culture and isolated type 2 cells in monolayer culture using polyclonal antibodies to human SP-B (Phe201-Met279) and specific epitopes within the N-(NFProx, Ser145-Leu160; NFlank Gln186-Gln200) and C-terminal (CFlank, Gly284-Ser304) propeptides of pro-SP-B. Fluorescence immunocytochemistry using epitope-specific antisera showed colocalization of pro-SP-B with the endoplasmic reticulum resident protein BiP. The 25-kDa intermediate was partially endo H-sensitive, colocalized with the medial Golgi resident protein MG160, and shifted into the endoplasmic reticulum in the presence of brefeldin A, which interferes with anterograde transport from endoplasmic reticulum to Golgi. The 9-kDa intermediate colocalized in part with MG160 but not with Lamp-1, a transmembrane protein resident in late endosomes and lamellar bodies. Brefeldin A induced a loss of colocalization between MG160 and NFlank, shifting NFlank immunostaining to a juxtanuclear tubular array. In pulse-chase studies, brefeldin A blocked all processing of 42-kDa pro-SP-B whereas similar studies using monensin blocked the final N-terminal processing event of 9 to 8 kDa SP-B. We conclude that: 1) the first enzymatic cleavage of pro-SP-B to the 25-kDa intermediate is in the brefeldin A-sensitive, medial Golgi; 2) cleavage of the 25-kDa intermediate to a 9-kDa form is a trans-Golgi event that is slowed but not blocked by monensin; 3) the final cleavage of 9 to 8 kDa SP-B is a monensin-sensitive, post-Golgi event occurring prior to transfer of SP-B to lamellar bodies.

Surfactant protein B is a 79-amino acid hydrophobic protein that is essential to the function of pulmonary surfactant, as illustrated by lethal SP-B deficiency in humans and the transgenic homozygous SP-B knock-out mouse (reviewed in Ref. 1). The 8-kDa protein is the result of extensive post-translational processing of a large 381-amino acid precursor within alveolar type 2 cells. Previous studies in cell lines, isolated rat type 2 cells, and human fetal lung (2–7) indicated that processing to the mature 8-kDa protein involves signal peptide cleavage and glycosylation of the C terminus, followed by cleavage of the N terminus and C terminus in succession. We have recently shown that cleavage of the N terminus occurs in two steps, leaving an approximately 10-amino acid remnant flanking mature SP-B which is removed in a final processing step that releases mature SP-B (8). The subcellular location of these processing events and the enzymes necessary for processing SP-B are poorly understood. Previous work by Voorhout and colleagues (9) utilizing immunoelectron microscopy with antisera to mature SP-B and a synthetic pro-SP-B showed pro-SP-B in the endoplasmic reticulum and mature SP-B in lamellar bodies of adult human type 2 cells. Analysis of grain density over other organelles showed intermediate grain densities over multivesicular bodies and Golgi, indicating the involvement of these organelles in SP-B transport and/or processing.

The extensive post-translational processing of SP-B is similar to the post-translational processing of the other hydrophobic surfactant protein, SP-C (10–13). The 21-kDa pro-SP-C undergoes sequential enzymatic cleavages resulting in a 3.7-kDa mature protein. Pro-SP-C is detected in endoplasmic reticulum and a 6-kDa intermediate is enriched in lamellar bodies. Inhibitors of intracellular trafficking and acidification inhibit release of mature SP-C in vitro disrupt all processing beyond the 16-kDa SP-C intermediate. Processing of SP-B and SP-C are linked, since in alveolar type 2 cells of patients with inherited SP-B deficiency SP-C is not processed beyond the 6-kDa intermediate (14, 15).

In this report, we use epitope-specific antisera and pulse-chase labeling studies with inhibitors of protein processing to show that most human pro-SP-B processing is in post-endoplasmic reticulum but pre-lamellar body compartments. Our data extend previous observations of pro-SP-B trafficking and processing to show that early N-terminal propeptide and C-terminal propeptide processing events occur within the Golgi apparatus with processing of the small vestigial N-terminal propeptide domain as a post-Golgi event. We speculate that the N-terminal remnant is involved in trafficking SP-B toward the lamellar body. Previous reports of these data have appeared elsewhere in abstract form (16, 17).

EXPERIMENTAL PROCEDURES

Reagents—Express Protein Labeling Mix was obtained from NEN Life Science Products Inc. (Boston, MA). Protein A-agarose was obtained from Life Technologies, Inc. (Gaithersburg, MD). Dexamethasone, isobutylmethylxanthine, and 8-Br-cAMP were obtained from Sigma. Endoglycosidase H (endo H) and PNGase F were obtained from New England Biolabs (Beverly, MA). All other reagents were electroforetically pure and were purchased from either Bio-Rad or Novex (San Diego, CA). Culture media were produced by the Cell Center Facility, University of Pennsylvania.
The polyclonal antibody to BiP was supplied by StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). The MG160 polyclonal antisem was the generous gift of G. N. Mita, Division of Neopathology, University of Pennsylvania. The Lamp-1 (HA3) monoclonal antisem developed by J. T. Augustine and J. E. Hildreth was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA.

**Explant and Cell Culture—**Human fetal lung was obtained from second trimester therapeutic abortions (20–23-week estimated gestational age) under protocols approved by the Committee for Human Research, Children's Hospital of Philadelphia. Fetal lung parenchyma was dissected free of large vessels, chopped into 1-mm³ explants, and cultured in Waymouth's media on a rocking platform as described previously (18). After overnight culture, hormones (10 ng dexamethasone, 0.1 μM 8-Br-cAMP, and 0.1 μM isobutylmethylxanthine (DCI)) were added to the media for the remainder of the culture period. Media were changed daily and tissues were studied on day 5 of culture. Type 2 cells were isolated from human fetal lung explants after 4 days in culture with DCI using collagenase-trypsin digestion and differential centrifugation. Two cell suspensions were obtained under the auspices of the NICHD, National Institutes of Health, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA.

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**Human SP-B Antiserum and FITC Labeling—**Based on the antigenicity of prepro-SP-B peptides, peptide sequences were chosen for production of synthetic peptides and antisem preparation (NFPProx, Ser145-Leu160; NFlank, Gln186-Gln200; CFflank, Gly284-Gln286). The hSP-B antisem was developed using 8 kDa SP-B isolated from human alveolar proteinosis fluid (21).

**Pulse-Chase Labeling of Human Fetal Lung Explants—**Cell media was replaced with Met-Cys-free Dulbecco's modified Eagle's medium (2 ml/60-mm plate) with or without inhibitors for 2 h while incubating on a rocking platform. Inhibitors or 10 μg/ml brefeldin A (10 μg/ml) or monensin (2 μM) were added at the beginning of the starvation period and maintained throughout the pulse and chase periods. Met-Cys-free Dulbecco's modified Eagle's medium ± inhibitors was then replaced with Met-Cys-free Dulbecco's modified Eagle's medium ± inhibitors supplemented with 200 μCi/ml 35S-Express Protein Labeling Mix (2 ml/60-mm plate) which is composed of 70% methionine and 15% cysteine (NEN Life Science Products Inc.). After a 4-h pulse, the media was changed to complete Waymouth's media with DCI ± inhibitors. To ensure that inhibitor concentrations remained constant, media was changed at each subsequent time point through the 8-h time point of the 24-h chase. Samples were harvested immediately after the 8-h labeling and at regular intervals through 8 h post-labeling. Samples were washed in PBS with detergent (0.5% Triton X-100, 2 mM benzamidine HCl, and 80 mM phenylmethylsulfonyl fluoride) followed superimposition of fluorescent labeling with FITC and Cy3 fluorophores. Laser power was fixed at 75% for all image acquisition. Image output was at 1024 × 1024 pixels and photomicrographs were later embossed with a 20-μm bar unless otherwise indicated.

**Immunoprecipitation—**Radiolabeled lung homogenates were immunoprecipitated by modification of our previous method (8). Immunoprecipitations were performed on samples containing 10⁶ trichloroacetic acid precipitable counts/min unless otherwise specified, using 3 μl of anti-human SP-B antibody (hSP-B) or preimmune rabbit serum. After the first immunoprecipitation, protein A-agarose beads were washed and the immunoprecipitated proteins were solubilized in 40 μl of gel sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.72 mM 2-mercaptoethanol, 10% glycerol, 0.0075% bromophenol blue). A 5-μl aliquot was taken for scintillation counting and a 30-μl aliquot was subjected to SDS-PAGE in 16.5% polyacrylamide gels using a Tris-Tricine buffer system as described previously (5). Electrophoresed samples were transferred to polyvinylidene difluoride (Bio-Rad) at 20 mA/cm² for 13–16 h. After transfer to membranes, blots were visualized using the Storm PhosphorImager system (Molecular Dynamics, Sunnyvale, CA), analyzed using Imagequant software and later subjected to autoradiography.

**Localization of SP-B Processing**

**Pulse-Chase Labeling of Human Fetal Lung Explants—**Culture media was replaced with Met-Cys-free Dulbecco's modified Eagle's medium (2 ml/60-mm plate) with or without inhibitors for 2 h while incubating on a rocking platform. Inhibitors or brefeldin A (10 μg/ml) or monensin (2 μM) were added at the beginning of the starvation period and maintained throughout the pulse and chase periods. Met-Cys-free Dulbecco's modified Eagle's medium ± inhibitors was then replaced with Met-Cys-free Dulbecco's modified Eagle's medium ± inhibitors supplemented with 200 μCi/ml 35S-Express Protein Labeling Mix (2 ml/60-mm plate) which is composed of 70% methionine and 15% cysteine (NEN Life Science Products Inc.). After a 4-h pulse, the media was changed to complete Waymouth's media with DCI ± inhibitors. To ensure that inhibitor concentrations remained constant, media was changed at each subsequent time point through the 8-h time point of the 24-h chase. Samples were harvested immediately after the 8-h labeling and at regular intervals through 8 h post-labeling. Samples were washed in PBS with detergent (0.5% Triton X-100, 2 mM benzamidine HCl, and 80 mM phenylmethylsulfonyl fluoride) followed superimposition of fluorescent labeling with FITC and Cy3 fluorophores. Laser power was fixed at 75% for all image acquisition. Image output was at 1024 × 1024 pixels and photomicrographs were later embossed with a 20-μm bar unless otherwise indicated.

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**Endoglycosidase H and PNGase F—**After immunoprecipitation from hormone-treated human fetal lung pulse-chase labeled for 4 h, triplicate samples still complexed to protein A-agarose beads were treated with either endo H or PNGase F. Control samples were incubated in wash buffer. Endo H-treated samples were resuspended in 1 × G5 buffer with 2000 units of endo H while PNGase F-treated samples were resuspended in 1 × G7 buffer, 1% Nonidet P-40 with 4000 units of PNGase F. All samples were incubated at 37 °C for 2 h followed by a final wash before solubilizing the beads in NuPAGE SDS sample buffer with DTT as the reducing agent. For superior band resolution, these samples were separated using a 10% NuPAGE Bis-Tris gel with MES SDS Running Buffer as per the manufacturer's protocol (Novex, San Diego, CA), including transfer to Duralose membrane (Stratagene, La Jolla, CA) for PhosphorImager analysis.

**RESULTS**

**Subcellular Localization of Pro-SP-B Peptides Using Immunofluorescence with Epitope-specific Antisera**—To localize SP-B precursor and intermediate forms within type 2 cells of human fetal lung, we used epitope-specific antisera developed against
antigenic sequences within the N- and C-terminal propeptides. Fluorescence cytochemistry of type 2 cells isolated from cultured, hormone-treated human fetal lung showed distinct immunostaining patterns for each of the antisera (Fig. 2). The NFPProx antiserum immunostaining occupied a perinuclear pattern. The CFlank antiserum exhibited a vesicular pattern of fluorescence throughout the cell. The NFFlank antiserum also showed a vesicular pattern but in close approximation to lamellar bodies which have a perinuclear distribution by phase-contrast microscopy. By comparison, the antiserum to mature SP-B (hSP-B) intensely labeled lamellar bodies and no other structures presumably due to the high concentration of SP-B within lamellar bodies. The specificity of each antiserum was confirmed by the ability of synthetic peptide to block the signal in immunohistochemistry (data not shown).

Double immunofluorescence labeling was used to identify the subcellular location of staining with the epitope-specific antisera using known markers of subcellular organelles in type 2 cells. Contaminating fibroblasts were occasionally present but were recognizable by absence of staining with the FITC-labeled epitope-specific antiserum despite positive staining for subcellular organelles. Confocal images were obtained using the following laser parameters for FITC-labeled primary SP-B antisera: NFPProx voltage mean 808 (range 781–840), offset −15 to −17; CFlank voltage mean 844 (range 812–877), offset −7 to −26; NFFlank voltage mean 775 (range 754–789), offset −19 to −24; hSP-B voltage mean 726 (range 705–740), offset −16 to −19. By comparison, the parameters for Cy3-labeled secondary IgG were voltage mean 807 (range 691–891), offset −4 to −22. Pinhole settings, which were identical for both FITC and Cy3 images due to simultaneous scanning, were: NFPProx/Cy3 mean 169 (range 157–181), CFlank/Cy3 mean 179 (range 146–207), NFFlank/Cy3 mean 173 (range 141–191), hSP-B/Cy3 mean 163 (range 129–194). Overall, the hSP-B antiserum yielded more intense staining as reflected by the lower voltage and pinhole settings required for optimal image acquisition. Conversely, the epitope-specific antiserum required higher acquisition settings to achieve equivalent image quality.

Fig. 3 illustrates the results of double immunofluorescence cytochemistry of isolated type 2 cells using monoclonal antibody to BiP (as a marker of endoplasmic reticulum and identified by Cy3-labeled secondary antibody) and SP-B epitope-specific antiserum directly conjugated to FITC. Colocalization of NFPProx immunostaining with BiP in the endoplasmic reticulum was evident in both merged (yellow; Fig. 3a) and unmerged images (Fig. 3b). There was minimal colocalization of CFlank and NFFlank antiserum with BiP, whereas the steady-state pool of pro-SP-B in the endoplasmic reticulum was preferentially identified by NFPProx. There was minimal colocalization of BiP with hSP-B antiserum.

Fig. 4 illustrates double immunofluorescence photomicrographs of isolated type 2 cells using polyclonal antibody to MG160, identified by Cy3-labeled secondary antibody, and FITC-conjugated epitope-specific antiserum. MG160 is a medial Golgi resident transmembrane sialoglycoprotein which is found as a component of the Golgi apparatus of most cells (22, 23). By confocal fluorescence microscopy, NFPProx did not colocalize with MG160. CFlank and NFFlank immunostaining colocalized intensely with MG160 in a tubular network lying in close proximity to the nucleus which is characteristic of the medial Golgi. Although this region was also close to lamellar bodies, there was no colocalization of hSP-B with MG160.

Fig. 5 shows double immunofluorescence images of isolated type 2 cells labeled using Lamp-1 monoclonal antibody, identified by Cy3-labeled secondary antibody, and FITC-conjugated epitope-specific antiserum. Lamp-1 is a transmembrane protein which localizes to late endosomes, lysosomes and, in alveolar type 2 cells, lamellar bodies (24). Neither NFPProx nor CFlank antiserum colocalized with Lamp-1. NFFlank immunostaining highlighted vesicles adjacent to lamellar bodies (as seen in Fig. 2) which were Lamp-1-negative. By contrast, the hSP-B antiserum localized to dense regions within Lamp-1-positive lamellar bodies (Fig. 5, inset). Taken together, these immunofluorescence data suggest that at steady state pro-SP-B is endoplasmic reticulum resident and the 25-kDa intermediate, which is identified by both CFlank and NFFlank, is distributed...
within the Golgi. In contrast, mature SP-B is concentrated within the lamellar body.

**Initial Cleavage of the N Terminus of Pro-SP-B Occurs in the Medial Golgi**—To examine ER to Golgi transport of SP-B precursors, we carried out pulse-chase labeling of cultured human fetal lung in the presence or absence of brefeldin A, an inhibitor of anterograde trafficking between endoplasmic reticulum and Golgi (25). We showed previously that processing from pro-SP-B through 25- and 9-kDa intermediates to mature 8-kDa SP-B occurs within 1–2 h postlabeling in hormone-treated human fetal lung explants (8) (Fig. 6). In the presence of brefeldin A, processing of pro-SP-B was blocked with no accumulation of 25 kDa or more distal intermediates over 8 h of chase (Fig. 6). This suggests that the initial N-terminal cleavage of pro-SP-B occurs in a brefeldin A-sensitive cis or medial Golgi compartment.

We also performed endoglycosidase digestions of SP-B intermediates isolated by immunoprecipitation after a 4-h pulse-chase. The 9- and 8-kDa SP-B proteins are not glycosylated and do not shift apparent M, after treatment with either PNGase F or endo H (Fig. 7). PNGase F, which cleaves all carbohydrates, reduces the 42-kDa pro-SP-B to ~39 kDa and the 25-kDa intermediate to ~21 kDa. After endo H treatment, both pro-SP-B and the 25-kDa intermediate appear to be partially endo H-sensitive (39- and 21-kDa bands, respectively) and endo H-resistant (42- and 25-kDa bands, respectively). The majority of pro-SP-B is endo H-sensitive but a small fraction are endo H-resistant. Conversely, a small amount of the 25-kDa intermediate is endo H-sensitive and the bulk of this intermediate is endo H-resistant. Together with the brefeldin A studies, these data place the initial N-terminal cleavage of pro-SP-B to 25-kDa intermediate in the medial Golgi.

**C-terminal Propeptide Cleavage of the 25-kDa SP-B Intermediate Occurs in the Trans-Golgi**—To discriminate the location of the first N-terminal cleavage from the subsequent cleavage of the C terminus, we examined the steady state distribution of SP-B intermediates in the presence of brefeldin A using double immunofluorescence staining of isolated type 2 cells. In Fig. 8, brefeldin A-treated cells were fixed and double stained using antibodies to BiP and NFProx and to a lesser extent for CFlank and NFlank (panel A, merged images; panel B, unmerged images for NFProx and BiP). There was no colocalization between BiP and hSP-B. Images are representative of triplicate experiments; immunostaining patterns shown were characteristic of ~75% of cells per slide. Bar, 20 μm.

**Cleavage of the Vestigial N Terminus Occurs in a Post-Golgi but Prelamellar Body Compartment**—To localize late process-
staining is not found within lamellar bodies, this places the terminal cleavage event in a post-Golgi but pre-lamellar body compartment.

**DISCUSSION**

The processing of pro-SP-B to mature SP-B in alveolar type 2 cells requires a series of post-translational modifications and proteolytic cleavages. Through the course of SP-B processing, the mature protein must be transported to the lamellar body where it is concentrated with SP-C and surfactant-specific phospholipids. The mechanisms controlling the process of lamellar body formation and the aggregation of these diverse surfactant components are poorly understood. To elucidate these events, it became important to examine the intracellular localization of SP-B processing. Previous studies by others using immunoelectron microscopy localized the primary translation product, pro-SP-B, to the endoplasmic reticulum with mature SP-B concentrated in lamellar bodies. The present study extends these observations with new data demonstrating that the initial proteolytic cleavage of the N-terminal propeptide is in the brefeldin A-sensitive, medial Golgi with a subsequent C-terminal cleavage in the trans-Golgi and a final N-terminal cleavage event in a post-Golgi but pre-lamellar body compartment as depicted in Fig. 10.

We used type 2 cells isolated from hormone-treated human fetal lung explants for immunofluorescence studies. Recent advances in type 2 cell culture allow maintenance of type 2 cell phenotype for extended periods (19, 20). Although isolated type 2 cells lose their basal-apical orientation, the lamellar bodies have a characteristic perinuclear distribution which permits detailed examination of subcellular structures in close proximity to lamellar bodies. We have recently used these cells in pulse-chase labeling studies and have found no significant differences in SP-B processing over 4 h compared with similar studies in human fetal lung explants. This culture technique provides a useful system for studies characterizing lamellar body genesis and secretion as well as in the surfactant protein processing. In the present study the combination of type 2 cell immunofluorescence cytochemistry with pulse-chase studies of human fetal lung explants facilitated correlation of steady state pools of SP-B intermediates with the effects of inhibitors and endoglycosidases on dynamic SP-B processing.

Pro-SP-B has a complex tertiary structure inferred from its...
amino acid sequence homology to NK-lysin and prosaposin (27, 28). Given the proposed structure of pro-SP-B, our epitope-specific antisera were designed to recognize peptides within intervening segments between tight α-helical saposin-consensus regions. Our immunofluorescence studies relied on the ability of these antisera to discriminate SP-B intermediates. We have shown previously by Western immunoblotting that these antisera appropriately recognize the relevant SP-B intermediates containing the immunizing peptide sequences and are successfully competed by preincubating each antiserum with its immunizing peptide (8). In using the epitope-specific antisera for immunolocalization studies, we assumed that all epitopes would be equally exposed and available for antibody recognition based upon our prior Western blotting experiments. Based upon this assumption, all of the SP-B antisera would recognize pro-SP-B in the ER and continue to identify intermediates until the epitope was lost in post-translational processing events. Instead, our immunofluorescence images showed variations in the intensity of fluorescence with each

![Fig. 8. Brefeldin A induces a redistribution of CFlank immunostaining. Isolated alveolar type 2 cells were incubated with or without brefeldin A (10 μg/ml) for 30 min prior to fixation and immunostaining for SP-B intermediates using the SP-B epitope-specific antisera as indicated (green) and BiP (red, panel A), or MG160 (red, panel B). Immunostaining of control cells was no different from Figs. 3 and 4. In the presence of brefeldin A, there was no change in the immunostaining patterns of hSP-B, or NFPProx and BiP, which both continue to colocalize (yellow). CFlank and MG160 lost the tubular appearance seen in control cells. CFlank colocalized with BiP and MG160 colocalized with NFPProx after brefeldin A treatment. NFlank immunostaining shifted after brefeldin A but had a juxtanuclear tubular pattern, losing all colocalization with MG160. Images are representative of triplicate experiments; immunostaining patterns shown were characteristic of ~75% of cells per slide. Bar, 20 μm.](http://www.jbc.org/)

![Fig. 9. Monensin slows pro-SP-B processing and prevents the final N-terminal processing event. Cultured hormone-treated human fetal lung explants were pulse-labeled with [35S]Met-Cys for 1 h and chased in cold complete medium for up to 8 h in the presence or absence of monensin (2 mM). Samples collected at the indicated time points were immunoprecipitated with the hSP-B antisera and analyzed by Tris-Tricine SDS-PAGE. A, PhosphorImager results of untreated control samples showed the 8-kDa mature SP-B by 1–2 h post-pulse whereas monensin treatment resulted in delayed appearance of 25- and 9-kDa intermediates and no 8-kDa SP-B at 8 h. B, control samples from 4 and 8 h and monensin-treated sample from 8 h were immunoprecipitated and electrophoresed together on the same gel to illustrate the lack of processing beyond 9 kDa in the presence of monensin (C, control; M, monensin).](http://www.jbc.org/)

![Fig. 10. Model of intracellular localization of SP-B Processing. Prepro-SP-B (40 kDa) is modified by glycosylation and signal peptide cleavage resulting in 42-kDa pro-SP-B. These events occur within the endoplasmic reticulum with prepro-SP-B and pro-SP-B, as indicated by NFPProx immunostaining, colocalizing with BiP. The initial proteolytic cleavage of the N terminus occurs within the medial Golgi since brefeldin A prevents all pro-SP-B processing and shifts CFlank immunostaining. These findings along with partial sensitivity to endo H indicate that both N terminus cleavage and oligosaccharide modification by mannosidase II occur in medial Golgi compartments. Cleavage of the C terminus, which is not monensin-sensitive, occurs in the trans-Golgi leaving a steady state pool of 9-kDa intermediates in the trans-Golgi and/or a post-Golgi compartment. In the presence of brefeldin, this pool loses colocalization with MG160 and takes on a juxtanuclear tubular appearance characteristic of trans-Golgi proteins. The final N-terminal cleavage occurs in a monensin-sensitive post-Golgi compartment, possibly the multivesicular body, resulting in only the mature form of SP-B in the lamellar body.](http://www.jbc.org/)
Localization of SP-B Processing

antiserum, as reflected in both the images and the laser settings used to generate the images. NFProx antiserum, which recognizes both pro-SP-B and the excised N terminus by Western blotting, colocalized predominantly with the luminal endoplasmic reticulum marker BiP (29, 30). This places pro-SP-B predominantly in the endoplasmic reticulum, which is in agreement with previous work by Voorhout and colleagues (9). However, CFlank, NFFlank, and hSP-B antisera, which identify pro-SP-B by immunoblotting, showed little colocalization with BiP. These observations can be explained by variation in the affinities of the antisera for their epitopes, in the FITC labeling of the primary antisera, in the relative concentration of SP-B proteins within the organelles, or by altered accessibility of the epitopes within the SP-B proteins. Immunostaining procedures were optimized for each antiserum to attempt to control for variability in antiserum affinity and FITC labeling and our methodologies could not evaluate whether epitopes were accessible. However, the relative amounts of pro-SP-B, intermediates and mature SP-B protein are not constants between organelles and more likely explains some of the immunostaining variability. Previously, we showed that pro-SP-B is rapidly processed to the 25-kDa intermediate in human fetal lung explants with differentiated type 2 cells (8). The accumulation of mature SP-B protein occurs more slowly. Therefore at steady state, there is relatively less 42-kDa pro-SP-B within the type 2 cell than 25-kDa intermediate. Immunostaining using the CFlank and NFFlank antisera would favor detection of the larger pool of 25-kDa intermediate over the smaller pool of pro-SP-B. Likewise, mature SP-B accumulates and is concentrated within lamellar bodies, achieving a much higher steady state pool size than any of the intermediates. As a result, immunostaining using the hSP-B antisera favors detection of the lamellar body pool of mature SP-B over the smaller intermediate pools.

Our previous studies also showed that the N-terminal propeptide is cleaved in two steps, exposing first a small vesicular propeptide that is later cleaved in the final event liberating SP-B (8). We have now shown that the initial N-terminal cleavage is a medial Golgi event. Brefeldin A, a small hydrophobic molecule that disrupts budding vesicles and induces collapse of the cis/medial Golgi where it redistributes to the endoplasmic reticulum (recently reviewed in Ref. 25), prevents all proteolytic processing of pro-SP-B. Monensin, which acts primarily on later Golgi compartments (26), does not prevent this first proteolytic cleavage event. In addition, brefeldin shifted CFlank immunostaining out of a Golgi pattern to colocalize with the ER-resident BiP. This method has been used by others to examine the trafficking of proteins to regions of the Golgi (31, 32), including MG160 which characteristically disperses after brefeldin exposure (33). Finally, the only explanation for an endo H sensitive pool of both 42-kDa pro-SP-B and the 25-kDa intermediate is that the first N-terminal cleavage occurs in the medial Golgi while the oligosaccharide modification is still endo H-sensitive. Mannosidase II, which removes mannose residues from the oligosaccharide rendering the N-acetylgalactosamine residues endo H-resistant, typically localizes to the cis/medial Golgi depending on cell type although overlap into the trans-Golgi has been described (34–36). Thus, endo H-sensitive pro-SP-B is rapidly transported from the ER to the medial Golgi where it becomes endo H-resistant concomitantly with initial N-terminal cleavage. The failure of the NFProx antisera to colocalize with MG160 at steady state indicates that the relative size of the ER pool of pro-SP-B is much greater than the pool passing through the Golgi.

The next step in pro-SP-B processing is C-terminal propeptide cleavage. Our results indicate that this occurs in a late Golgi compartment, most likely the trans-Golgi. This cleavage was not inhibited by monensin. The immunostaining results for CFlank and NFFlank in the presence of brefeldin show that the steady state pools of the 25-kDa and 9-kDa intermediates are in separate compartments. Although both CFlank and NFFlank antisera identify the 25-kDa intermediate, our previous immunoblotting studies showed that CFlank does not identify a C-terminal propeptide fragment which would potentially confound interpretation of these studies (8). Furthermore, NFFlank, but not CFlank, antisera identifies the 9-kDa intermediate. As mentioned above, CFlank immunostaining showed a shift to endoplasmic reticulum and colocalization with BiP after brefeldin while the change in NFFlank immunostaining was more characteristic of other trans-Golgi resident proteins. Thus the CFlank antisera identifies primarily the 25-kDa intermediate moving within cis and medial Golgi compartments while the NFFlank antisera identifies a trans-Golgi pool of 9-kDa SP-B intermediate, with the cleavage event occurring in the trans-Golgi.

The precise location of the final N-terminal cleavage event remains unclear. This step appears to be inhibited by monensin, placing it in a pH-sensitive, post-Golgi compartment. NFFlank immunostaining was not seen within lamellar bodies, nor did it colocalize in Lamp-1-positive vesicles as with the hSP-B antisera. Instead, NFFlank-positive small vesicles were found in close proximity to Lamp-1-positive vesicles. Lamp-1 has been identified in the membranes of the small vesicles within multivesicular bodies of type 2 cells (24). Immunoelectron microscopy studies will be required to determine whether NFFlank and Lamp-1 antisera identify distinct populations of vesicles within multivesicular bodies.

The complexity of pro-SP-B processing is reminiscent of prohormone processing, in which inactive prohormones are sequentially modified and cleaved to release active forms at their site of action (reviewed in Ref. 37), and of post-translational processing of surfactant protein C (38). SP-C is also synthesized as a larger proprotein that is sequentially processed to a hydrophobic mature protein. Inhibitor studies of surfactant protein C processing showed that both brefeldin and monensin blocked most pro-SP-C processing, indicating that post-Golgi compartments (i.e. multivesicular body and/or lamellar body) are major loci for SP-C processing (11, 13). By contrast, our inhibitor studies indicated that most pro-SP-B processing occurs in a pre-lamellar body compartment.

The biologic role of the complexity of SP-B processing has been elusive. The structural and functional parallels between SP-B and saposins have been pointed out by others (27, 39). Both SP-B and saposins A, B, C, and D arise from large precursors. All 4 saposins arise from a single precursor protein (prosaposin ABCD) and are liberated through a complex series of enzymatic cleavages (40). Each of the monosaposins has a unique function in sphingolipid hydrolysis. Despite sequence homology between the monosaposins and the N- and C-terminal propeptides of pro-SP-B, to date no unique cellular functions have been attributed to the cleaved SP-B propeptides. Subcellular localization studies have shown the saposins only in lysosomes with processing kinetics that are more rapid than we have observed for SP-B (41, 42). Recently, the dermatologic manifestations of the prosaposin knock-out mouse have been characterized and show interesting functional parallels with the homozgyous SP-B −/− knock-out mouse. Keratinocytes, like alveolar type 2 cells, develop lamellar bodies which are extruded into the extracellular space where the monosaposins modify ceramides to form a water-tight barrier. The homozygous prosaposin knock-out mice have a thinned epidermis that on electron microscopy appears disordered. The keratinocytes
have abnormal lamellar bodies reminiscent of the abnormal lamellar bodies of alveolar type 2 cells of the homozygous SP-B knock-out mouse and human infants with inherited SP-B deficiency.

The many parallels between saposin and SP-B suggest post-translational processing complexity is necessary to maintain these proteins in an inactive state until reaching their sites of action. In addition, it is possible that processing uncovers trafficking motifs that facilitate the movement of these proteins to their final destination. There are no known trafficking motifs within the amino acid sequences of either prosaposin or pro-SP-B. However, the small N-terminal peptide cleaved in the final step of SP-B processing is a potential candidate. Studies in transgenic homozygous SP-B--/-- mice involving the knocking-in of various SP-B constructs have shown that the N terminus is essential for successful rescue of this lethal phenotype (5, 6, 43). In these studies the complete N terminus, including the vestigial N-terminal epitope retained after the initial pro-SP-B cleavage, was eliminated. Our data show that this epitope was retained through a post-Golgi, pre-lamellar body compartment which suggests a role for this epitope in trafficking SP-B toward lamellar bodies. Additional studies will be required to determine whether this peptide is both necessary and sufficient for trafficking SP-B to similar compartments within type 2 cells and other cell types with specialized secretory functions.

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