Involvement of Napsin A in the C- and N-terminal Processing of Surfactant Protein B in Type-II Pneumocytes of the Human Lung*  

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Surfactant protein B (SP-B) is a critical component of pulmonary surfactant, and a deficiency of active SP-B results in fatal respiratory failure. SP-B is synthesized by type-II pneumocytes as a 42-kDa propeptide (proSP-B), which is posttranslationally processed to an 8-kDa surface-active protein. Napsin A is an aspartic protease expressed in type-II pneumocytes. To characterize the role of napsin A in the processing of proSP-B, we colocalized napsin A and precursors of SP-B as well as SP-B in the Golgi complex, multivesicular, composite, and lamellar bodies of type-II pneumocytes in human lungs using immunogold labeling. Furthermore, we measured aspartic protease activity in isolated lamellar bodies as well as isolated human type-II pneumocytes and studied the cleavage of proSP-B by napsin A and isolated lamellar bodies in vitro. Both, napsin A and isolated lamellar bodies cleaved proSP-B and generated three identical processing products. Processing of proSP-B by isolated lamellar bodies was completely inhibited by an aspartic protease inhibitor. Sequence analysis of proSP-B processing products revealed several cleavage sites in the N- and C-terminal propeptides as well as site in the mature peptide. Two of the four processing products generated in vitro were also detected in type-II pneumocytes. In conclusion, our results show that napsin A is involved in the N- and C-terminal processing of proSP-B in type-II pneumocytes.

The integrity and function of pulmonary surfactant are of paramount importance for lung function. Disturbance of surfactant activity leads to respiratory distress (1). The main function of pulmonary surfactant is the reduction of the surface tension at the air/liquid interface in the lung, thus preventing alveolar collapse at end-expiration. Pulmonary surfactant is a complex mixture of ~90% lipids and ~10% proteins that is synthesized, stored, secreted, and to a large extent recycled by type-II pneumocytes of the alveolar epithelium.

The hydrophobic surfactant protein B (SP-B) interacts with phospholipids and contributes to the formation of intracellular lamellar bodies, the structural rearrangement of secreted surfactant lipids into tubular myelin, as well as the subsequent rapid insertion of secreted surfactant phospholipids into the surface film (reviewed in Ref. 2). Hereditary SP-B deficiency in infants or mice leads to respiratory failure at birth (3–6). However, hereditary alveolar proteinosis in babies without any detectable mutations in the SP-B gene as well as acquired pulmonary alveolar proteinosis in children and adults are characterized by an intraalveolar accumulation of mature surfactant proteins and abnormal SP-B precursors. Furthermore, only SP-B precursors are detected in babies with congenital surfactant defects characterized by the absence of lamellar bodies in type-II pneumocytes. Therefore, insufficient processing of proSP-B due to a lack or dysfunction of one or more proteases involved in SP-B processing might be yet another undiscovered cause of surfactant dysfunction in pulmonary diseases in babies, children, and adults.

SP-B is synthesized in type-II pneumocytes as a 381 amino acid 42 kDa preproprotein. On route from its site of synthesis to the lamellar bodies, the processing to mature SP-B involves the cleavage of the signal peptide, glycosylation of the C terminus, followed by the cleavage of the N-terminal and C-terminal propeptide (7). The processing of the ~42-kDa proSP-B through ~23–25- and ~9-kDa proSP-B processing intermediates to mature 8-kDa SP-B is an at least three-step process with two distinct cleavages of the N-terminal propeptide and one of the C-terminal propeptide (8). Although various proSP-B processing steps and the site of proSP-B processing have been described (8, 9), little is known about the identity of the proteases involved in the posttranslational processing.

It has been speculated that a cathepsin D-like protease is involved in the posttranslational processing of the hydrophobic

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1 The abbreviations used are: SP-B, surfactant protein B; E64, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane; PNGase F, peptide-N-glycosidase F; immuno-EM, immunoelectron microscopy; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MS, mass spectrometry.
2 F. Brasch, unpublished observations.

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surfactant protein B (10), but cathepsin D itself was not detectable in type-II pneumocytes, and no specific activity was found in isolated lamellar bodies (11, 12). Recently, a novel aspartic protease, napsin A, was localized in type-II pneumocytes of the human lung by immunohistochemistry as well as in situ hybridization (13–15). The restricted tissue localization of napsin A in the lung suggests distinct physiological functions. Because napsin A shows 48% sequence identity with human cathepsin D and has a similar preference for cleavage between hydrophobic residues, we hypothesized that napsin A might be the cathepsin D-like protease involved in the processing of proSP-B (10, 16–18).

To investigate the potential role of napsin A in the processing of proSP-B we studied the localization of SP-B precursors, SP-B, and napsin A in human lungs by immunoelectron microscopy. We also measured napsin A activity in isolated lamellar bodies and type-II pneumocytes. We characterized the proSP-B cleavage products generated by napsin A in vitro and compared them with processing intermediates generated by isolated lamellar bodies and SP-B precursors in human type-II pneumocytes.

**MATERIALS AND METHODS**

**Human Lungs**—For the present study, we used eight non-transplanted human single donor lungs. Left and right donor lungs were separated short time before transplantation. Although one donor lung was used for transplantation, the contralateral donor lung was fixed at the time of transplantation as soon as the clinical procedure allowed. Donor lungs were used for investigation only if they could not be made available for another suitable recipient by The Eurotransplant Foundation Center, Leiden, The Netherlands. All lungs were carefully examined by two pathologists (K.-M. M. and F. B.) by light and electron microscopy. As previously reported in detail, none of the non-transplanted donor lungs used for this study showed pathological changes and transplanted patients had a favorable outcome (19, 20).

**Isolated Human Type-II Pneumocytes**—Isolated type-II pneumocytes were prepared as previously described (21). Briefly, 1 mm3 tissue explants of human fetal lung parenchyma from second trimester therapeutically aborted were cultured overnight in Waymouth’s media (protocols were approved by the Committee for Human Research, Children’s Hospital of Philadelphia). After overnight culture, 10 mm dexamethasone, 0.1 mm 8-BrcAMP, and 0.1 mm isobutylmethylxanthine (DCI) was added to the medium and explants were cultured for 6–7 days to induce type II cell differentiation. Some explants were cultured with E64 in the media for 6 h to induce type II cell differentiation. Some explants were cultured with E64 in the media for 6 h to induce type II cell differentiation. Some explants were cultured with E64 in the media for 6–7 days to inhibit cytoeine protease activity as previously described (22). Type-II pneumocytes were isolated from tissue explants by enzymatic digestion and panning on plastic culture dishes to remove fibroblasts.

**Deglycosylation of Type II Cell Homogenates by PNGase F**—Deglycosylation was performed as recommended by the supplier. Homogenized type-II pneumocytes (1 mg/ml) were incubated for 10 min at 95 °C in denaturation buffer. Then, 10,000 units of PNGase F and G7 Buffer (50 mm sodium phosphate (pH 7.5)) (NEB, Beverly, MA) containing 1% Triton X-100 were added to the medium and explants were cultured for 10 min at 95 °C, and then in 4°C for at least 36 h. The lungs were fixed by instillation of a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.2 M HEPES-buffer (pH 7.4) into the alveoli via the airways. Sections of tissue blocks were performed according to the rules of systematic uniform random sampling by superimposing a transparent grid over lung slices at random distribution (23). Tissue blocks were infiltrated with 0.5% uranyl acetate and 1:1 and 2:1 for 2 h each. The blocks were polymerized under UV-light for 45 min at room temperature. After several rinses in buffer (Buffer Kit, Dako, Denmark), the immunoreaction was demonstrated using the APAAP kit (Dako) according to the specifications of the manufacturer. Fast Red (Dako) was used as alkaline-phosphatase substrate. Finally, sections were rinsed in distilled water and counterstained with Mayer’s hematoxylin (Dako).

**Immunoelectron Microscopy (Immuno-EM): Tissue Preparation and Immunostaining**—For immunohistochemistry, two human non-transplanted single donor lungs were fixed by instillation of 4% buffered formaldehyde. Several samples from different sites were taken and subsequently routine paraffin embedding was performed. Immunostaining was performed using the alkaline-phosphatase method. Sections of 4 μm thickness were mounted on poly-l-lysine capillary slides and dried overnight at 37 °C. Paraffin sections were dewaxed with xylene, rehydrated in a graded series of alcohol, and finally washed in Tris-HCl (pH 7.6) for 10 min. The following steps were performed at room temperature with an autostainer (Dako, Denmark). To avoid nonspecific staining, sections were blocked with buffer 1 (Dako) for 5 min prior to incubation with the primary antibody at the appropriate dilution in blocking buffer (Zytomed, Berlin, Germany) for 30 min at room temperature. After several rinses in buffer (Buffer Kit, Dako), the immunoreaction was demonstrated using the APAAP kit (Dako) according to the specifications of the manufacturer. Fast Red (Dako) was used as alkaline-phosphatase substrate. Finally, sections were rinsed in distilled water and counterstained with Mayer’s hematoxylin (Dako).

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quickly 3 times with distilled water, and then dried overnight at 40 °C. 2) Double labeling was performed for SP-B and napsin A. The labeling steps were performed as described above. Both the primary specific polyclonal antibody against napsin A (anti-napsin A) and the monoclonal antibody against SP-B (anti-SP-B) were diluted in blocking buffer. The immunoreactivity was visualized by incubation with a secondary 5-nm gold-coupled antibody against rabbit IgG and 15-nm gold-coupled antibody against mouse IgG (both diluted to 1:30) and Type-II pneumocytes. As an internal positive control, the alveolar macrophages as well as the lung homogenates by upward flotation on a discontinuous sucrose gradient by modification of the method of Duck-Chong (24). Briefly, the heart, trachea, and large bronchi were removed and the lungs were chopped into small pieces. The lung tissue was homogenized and density gradients of seven consecutive layers of 0.8 M to 0.2 M sucrose were layered over the homogenate in centrifuge tubes. The tubes were first centrifuged at 1,000 rpm and 7 °C to sediment cellular debris and subsequently, without stopping, the speed was increased to 80,000 × g for 180 min. The lamellar body-rich layer was clearly detectable in the upper third of the tubes.

**Enzyme Assay for Napsin A**—The determination of napsin A activity was previously described in detail (25). Briefly, the napsin A activity was monitored by the cleavage of the fluorescence energy transfer-based substrate DS3 (K(Dabsyl)-PQFFTEQ Lucifer yellow) over a 10 min time period using excitation at 390 nm and emission at 538 nm. The reaction was performed in reaction buffer containing 0.1 M sodium acetate buffer, 20 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (serine protease inhibitor), and 5 μM trans-epoxysuccinyl-l-leucylamide-(4-guanidino)butane (E64). Normally, 39 μl of reaction buffer was mixed with 1 μl of isolated lamellar bodies, and the reaction was initiated by the addition of 10 μl of substrate. As a result of enzyme activity, an increase in the fluorescence signal should be observed.

**Expression and Purification of Human Napsin A**—Expression and purification of napsin A was performed as previously described (25). Briefly, the human kidney 293T (Invitrogen) cells were transfected with an expression vector containing human napsin A cDNA. Napsin A was purified from the cell extract using the biotinylated propeptide ligands EK191 (Biotin-C42-TLIRILHRVPQGPRRLN) and EK193 (Biotin-C42-(β-Ala)-TLIRILHRVPQGPRRLN). The fractions containing napsin A were pooled and stored in buffer (0.1% formic acid, 0.1% Nonidet P40, 10% glycerol, pH 3.75) at 4 °C.

**In Vitro Processing of Recombinant proSP-B with Recombinant Napsin A or Isolated Lamellar Bodies**—Recombinant proSP-B was incubated with recombinant napsin A in sodium-acetate buffer, pH 5.5, containing 0.01% Triton X-100 and 10 mM EDTA for 16 h at 37 °C. In control reactions, recombinant proSP-B was incubated without napsin A in the absence of propeptide. After incubation with napsin A and 100 μM pepstatin. Furthermore, recombinant proSP-B was incubated with isolated lamellar bodies alone as well as in the presence of E64 (5 μM, Sigma), diisopropyl-fluorophosphate (DFP, 10 μM, Sigma), and pepstatin (100 μM, Sigma). After incubation, the reaction mixture was separated by gel electrophoresis as described in the following paragraph.

**Western Blot Analysis of proSP-B Processing Products Generated in Vitro and Type-II Pneumocytes**—Aliquots of the reaction mixture and homogenized type-II pneumocytes before and after deglycosylation by PNGase F were separated using 4–12% NuPage® Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences) according to the standard procedure of the manufacturer. The membranes were blocked by using blocking buffer (Bio-Rad) for 1 h at room temperature and incubated with one of the following antisera: anti-SP-B, anti-CproSP-B, or anti-CFPlankn-SP-B. The immunoreaction was detected using polyclonal antisera against rabbit IgG conjugated to horseradish peroxidase (1:20,000, Dianova, Hamburg, Germany). The immunoreaction was visualized using the Western blot enhancement module and the Opti-4CN Substrate Kit (Bio-Rad).

**Mass Spectrometric Determination of Napsin A Cleavage Sites within proSP-B**—The precise determination of napsin A cleavage sites within proSP-B was performed using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF-MS). Recombinant proSP-B was incubated with napsin A, and 0.5 μl aliquots were taken at indicated time points. The samples were subsequently co-crystallised with 0.5 μl of sinapinic acid (saturated in 70% acetonitrile) on a SCOUT 384-MALDI-Target. The mass spectrometry was performed on a MALDI-TOF-MS (Reflex III, Bruker Daltonics) in reflector mode with external calibration. Annotation of the tryptic fragments was done using the BioTools 2.0 software (Bruker Daltonics).
In each experiment, the processing of proSP-B could be blocked by the addition of pepstatin (Fig. 3, a and b, lane 2).

**Mass Spectrometric Determination of Napsin A Cleavage Sites within proSP-B**—Next, we characterized the corresponding proSP-B cleavage products using mass spectrometry. For this, we incubated recombinant napsin A together with proSP-B and determined the molecular weights of the cleavage products using MALDI-TOF. They corresponded to the major peaks in the spectrogram at 19.12, 15.78, and 8.24 kDa (Fig. 4a). Additional C-terminal digestion with carboxypeptidase C allowed the exact determination of the cleavage sites by mass spectrometric comparison of the resulting degradation products (depicted in Fig. 4b).

**Processing of proSP-B by Isolated Lamellar Bodies**—To evaluate the relevance of napsin A for the intracellular proSP-B processing, we incubated proSP-B with isolated lamellar bodies. The processing products generated by isolated lamellar bodies migrated at the same size and showed the same antigenic characteristics as the cleavage products generated by napsin A (Fig. 3c, lane 1). To further investigate whether cleavage of proSP-B *in vitro* by isolated lamellar bodies is mediated by an aspartic or cysteine protease (22), we added either E64 (Fig. 3c, lane 2), an inhibitor of cysteine proteases, or pepstatin (Fig. 3c, lane 3), an inhibitor of aspartic proteases. Only pepstatin inhibited the processing of proSP-B by lamellar bodies (Fig. 3c, lane 3).

Further experiments were designed to strengthen the assumption that breakdown fragments of proSP-B generated by napsin A or lamellar bodies were identical. Due to the high complexity of the lamellar body protein content a direct MALDI-TOF-MS analysis corresponding to the degradation experiments using recombinant napsin A was impossible.
Therefore, we performed a peptide-mass-fingerprint analysis of the breakdown fragments of proSP-B generated by recombinant napsin A and lamellar bodies, respectively. Both reaction mixtures containing proSP-B cleavage products were separated by SDS-PAGE and gel areas corresponding to SP-B-immunoreactive bands of Western blots performed in parallel were excised. To prove whether the degradation products were similar an "in-gel" limiting proteolysis using trypsin was performed. Generated tryptic fragments were isolated and the molecular mass was determined by MALDI-TOF-MS. As summarized in Table I, the fingerprints of all three fragments are identical for napsin A and lamellar body mediated degradation. Although the sequences of the corresponding processing products are not completely covered by the tryptic fragments found, all of them included the core sequences of the proSP-B cleavage products described above. Noteworthy, all proSP-B protein fragments generated either by napsin A or isolated lamellar bodies were identical (Table I).

ProSP-B Processing Intermediates in Primary Type-II Pneumocytes—To compare proSP-B processing products generated in vitro with SP-B precursors in intact cells we isolated fetal human type-II pneumocytes. In type-II pneumocytes, anti-CproSP-B and anti-CFlankSP-B antibodies identified two SP-B precursors with molecular weights of 42 and 23 kDa (Fig. 3, lanes 1 and 2 (T2)). After deglycosylation by PNGase F, ~42 kDa proSP-B shifted to ~39 kDa and the ~23 kDa intermediate to ~19 and ~18 kDa (Fig. 3d, lanes 3 and 4). The characteristics of the deglycosylated ~19 kDa processing intermediate corresponded well to that of the respective cleavage products generated by napsin A or isolated lamellar bodies.

Because we had already shown that a cysteine protease was involved in the final remodelling of SP-B precursors (22), we hypothesized that, due to an imbalance of proteases, additional proSP-B processing intermediates were generated by napsin A that might accumulate in type-II pneumocytes after E64 treatment. Therefore, we incubated isolated type-II pneumocytes for 6–7 days with E64. By means of Western-blotting, additional ~9 and ~10 kDa SP-B precursors were identified by anti-SP-B (Fig. 5a, lanes 3 and 4) and anti-CFlankSP-B (Fig. 5b, lanes 3 and 4). The characteristics of the ~9 kDa precursor of SP-B

!![Image](https://example.com/image.png)
corresponded well to that of the ~9 kDa cleavage product generated by napsin A or isolated lamellar bodies.

**DISCUSSION**

SP-B plays a crucial role in surfactant function. Because SP-B is synthesized as a proprotein, proper proteolytic processing is a prerequisite for normal activity. A lack of mature SP-B but not precursors of SP-B in babies with absence of lamellar bodies and the intraalveolar accumulation of precursors of SP-B in hereditary and acquired pulmonary alveolar proteinosis is associated with a severe pulmonary dysfunction. The molecular mechanisms leading to a failure of normal processing of proSP-B are still obscure. A lack or deficiency of the proteases involved in the processing might be a possible explanation.

In earlier studies, a cathepsin D-like protease was linked to the processing of proSP-B. However, cathepsin D itself was not detectable in type-II pneumocytes neither by immuno-EM nor by immunohistochemistry, and no specific activity was found in isolated lamellar bodies. Therefore, we hypothesized that the novel aspartic protease napsin A, which was localized in type-II pneumocytes of the human lung might be the aspartic protease involved.

Because napsin A is a lysosomal enzyme and SP-B a secretory protein, we first localized napsin A, precursors of SP-B, and SP-B in human lungs at the ultrastructural level by immuno-EM. Our polyclonal antisera against the C-terminal propeptide of proSP-B localized corresponding precursors of SP-B in the endoplasmatic reticulum and Golgi vesicles, but only in a few multivesicular bodies. Furthermore, in line with previous immuno-EM studies we identified SP-B in multivesicular, composite, and lamellar bodies within type-II pneumocytes in human lungs, but not in Golgi vesicles. Napsin A was found to be colocalized with precursors of SP-B or mature SP-B in all compartments between Golgi complex and lamellar bodies. The strongest labeling for SP-B and napsin A was found in lamellar bodies, which are the intracellular compartments for the storage of surfactant lipids and proteins. These data are in good correlation with previous autoradiographic and immuno-EM studies demonstrating that SP-B is transported from the Golgi complex via the multivesicular and composite bodies to the lamellar bodies and that the posttranslational processing of proSP-B occurs in compartments between the Golgi complex and lamellar bodies.

Corroborating these data, we found that napsin A is proteolytically active in type-II pneumocytes and, more specifically, in isolated lamellar bodies. Previously, it had been described that the pH optimum of napsin A fits well with the acidic pH in multivesicular and lamellar bodies.

To further study the processing of proSP-B by napsin A, we generated recombinant human proSP-B as well as human napsin A, isolated lamellar bodies, and performed *in vitro* processing experiments. We cannot be sure that the structure of the refolded protein is identical to the native form of proSP-B but the recombinant protein was water-soluble, had no interchain disulfide bonds, and contained a high alpha-helical content, properties predicted for native proSP-B. In *in vitro* degradation assays showed that napsin A generated three major processing

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3 S. Hawgood, unpublished observations.
products from recombinant proSP-B. The fragments were identical to processing intermediates generated from proSP-B by an aspartic protease in isolated lamellar bodies.

In addition to degradation experiments using recombinant enzyme or purified cell fractions, we analyzed the proSP-B processing intermediates in isolated human type-II pneumocytes. Because the ~42 kDa proSP-B and the first ~23 kDa precursor of SP-B are glycosylated at the C-terminal propep-
Tide in vivo (8), we performed a deglycosylation with PGNase F.

The characteristics of the deglycosylated 19 kDa precursor of SP-B corresponded well to that of the respective cleavage product characterized after in vitro degradation. Furthermore, after inhibition of cellular cysteine proteases an additional 9 kDa processing product was detected in type-II pneumocytes, which corresponds well to the 8.24-kDa fragment detected after in vitro degradation. However, based on the antigenic characteristics this aberrant processing product is different from the 9 kDa precursor of SP-B found in type-II pneumocytes under physiological conditions (8).

Using mass spectrometry, we characterized the napsin A cleavage sites within the proSP-B protein. Two of the processing products contained the “mature” SP-B peptide and parts of the C- as well as N-terminal propeptide. In vivo, the proteolytic cleavage of the major part of the N-terminal propeptide has been shown to be the first proSP-B processing step (8, 10, 28).

In good correlation with the predicted cleavage site between amino acids Lys160 and Gln186 in the N-terminal propeptide in vivo (8), an N-terminal cleavage site was identified between amino acids Val237 and Arg252 in the 19.12 kDa proSP-B processing product generated by napsin A and isolated lamellar bodies in vitro. In addition, mass spectrometric peptide mapping also revealed a still unknown cleavage site in the C-terminal propeptide between amino acids W354 and D355 indicating that the first processing step of proSP-B (42 kDa proSP-B) is not only characterized by an N- but also by a still unknown C-terminal cleavage. Furthermore, a 15.78 kDa and a 8.24 kDa processing product of proSP-B were identified after processing

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**Table I**

Tryptic fragments of proSP-B cleavage products generated by human recombinant napsin A or isolated lamellar bodies

| Mass (Da) | Sequence | Mass (Da) | Sequence |
|-----------|----------|-----------|----------|
| ~19 kDa proSP-B processing product | 1996.330 Ile218-Arg236 | 1996.330 Ile218-Arg236 |
| | 1742.234 Val237-Arg252 | 1742.234 Val237-Arg252 |
| | 1363.093 Tyr253-Arg264 | 1363.093 Tyr253-Arg264 |
| | 1016.765 Met265-Arg272 | 1016.765 Met265-Arg272 |
| | 1042.755 Ser287-Arg295 | 1042.755 Ser287-Arg295 |
| | 1906.402 Gln377-Arg382 | 1906.402 Gln377-Arg382 |
| ~16 kDa proSP-B processing product | 1742.234 Val237-Arg252 | 1742.234 Val237-Arg252 |
| | 1363.093 Tyr253-Arg264 | 1363.093 Tyr253-Arg264 |
| | 1016.765 Met265-Arg272 | 1016.765 Met265-Arg272 |
| | 1042.755 Ser287-Arg295 | 1042.755 Ser287-Arg295 |
| ~9 kDa proSP-B processing product | 1363.093 Tyr253-Arg264 | 1363.093 Tyr253-Arg264 |
| | 1016.765 Met265-Arg272 | 1016.765 Met265-Arg272 |
| | 1042.755 Ser287-Arg295 | 1042.755 Ser287-Arg295 |

Fig. 5. Characterization of proSP-B processing intermediates accumulating in type-II pneumocytes (T2) after inhibition of cystein proteases. After inhibition of cathepsin H activity in vivo, anti-SP-B (a) as well as anti-CFlankSP-B (b) detected two ~9 and ~10 kDa precursors of SP-B in type-II pneumocytes (a and b, lanes 3 and 4), which were not identified in controls (a and b, lanes 1 and 2).
in vitro which were not detected in type II pneumocytes under physiological conditions. Surprisingly, the 8.24 kDa processing product resulted from a cleavage within the sequence of mature SP-B peptide and the C-terminal propeptide. Probably, this renders the resulting peptide functionally inactive. These processing products may result from overdigestion due to the conditions in vitro or may reflect misfolding of mature SP-B peptide in the recombinant substrate. However, because both napsin A and isolated lamellar bodies generated the 8.24 kDa processing product and a similar processing intermediate accumulated in type-II pneumocytes after inhibition of cellular cysteine proteases, an aberrant processing of proSP-B due to an imbalance of proteases might be more likely. These data strongly support the important role of napsin A in the cellular processing of proSP-B. Furthermore, they show that, in addition to napsin A, other enzymes might be involved in matura-
tion of SP-B. Insufficient activity of one of the proteases involved in the processing of proSP-B and proSP-C could result in an intracellular or intraalveolar accumulation of aberrant and functional inactive cleavage products (22).

In conclusion, our results indicate that napsin A is involved in the processing of proSP-B in type-II pneumocytes in the human lung. We provide six lines of evidence for the involvement of napsin A in the N- and C-terminal processing of proSP-B. 1) By immuno-EM, we found a colocalization of napsin A and precursors of SP-B as well as SP-B in the same cellular compartments in type-II pneumocytes. 2) Isolated lamellar bodies and type-II pneumocytes contained napsin A cellular compartments in type-II pneumocytes. 3) Isolated napsin A and precursors of SP-B as well as SP-B in the same processing products may result from overdigestion due to the con-

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