Gutentag, Susan H., Michael F. Beers, Bert M. Bieler, and Philip L. Ballard. Surfactant protein B processing in human fetal lung. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L559–L566, 1998.—Surfactant protein B (SP-B) is an 8-kDa hydrophobic protein essential for surfactant and normal lung function, is produced from the intracellular processing of proSP-B. To characterize SP-B processing in human type 2 cells, we used human fetal lung in explant culture and polyclonal antibodies to human SP-B8 (Phe201–Met279), an 8-kDa hydrophobic protein essential for surfactant and normal lung function, is produced from the intracellular processing of proSP-B. To characterize SP-B processing in human type 2 cells, we used human fetal lung in explant culture and polyclonal antibodies to human SP-B8 (Phe201–Met279) and to specific epitopes within the NH2- and COOH-terminal propeptide domains (Ser145–Leu160, Gln186–Gln200, and Gly284–Ser304). Western blot analysis revealed a novel intermediate at ~9 kDa, representing mature SP-B8, with a residual NH2-terminal peptide of ~10 amino acids. Pulse-chase studies showed a precursor-product relationship between the 9- and 8-kDa forms. During differentiation of type 2 cells in explant culture, the rate of proSP-B conversion to 25-kDa intermediate remained constant, whereas the rate of 25-kDa intermediate conversion to SP-B8 increased, resulting in a net increase in tissue SP-B8. Dexamethasone did not affect the rate of proSP-B processing but markedly enhanced the rate of SP-B8 accumulation. We conclude that NH2-terminal propeptide cleavage of proSP-B is a multistep process and that more distal processing events are rate limiting and both developmentally and hormonally regulated.

alveolar type II cell; protein processing

Human (h) surfactant protein (SP) B8 is an 8-kDa (reduced) hydrophobic protein essential to the surface active properties of pulmonary surfactant (14, 28). SP-B assists in the adsorption of phospholipid to the air-liquid interface and in lowering surface tension within lung air spaces. The importance of SP-B8 in the lung is underscored by the description of inherited deficiency of SP-B, which is marked by severe respiratory distress in near-term infants, often resulting in death (9). Despite the presence of normal amounts of surfactant phospholipid, SP-B-deficient surfactant is unable to lower alveolar surface tension. SP-B deficiency is also associated with aberrant type 2 cell ultrastructure, including absent lamellar bodies and abnormal SP-C processing (9, 10, 26). These clinical findings have been confirmed experimentally in transgenic SP-B knockout mice (8). Together these observations suggest that SP-B is involved in lamellar body genesis, which in turn may be essential for full processing of SP-C and packaging of surfactant phospholipids.

Immunoreactive SP-B is expressed by type II alveolar cells and bronchiolar Clara cells. Previous work indicated that SP-B is synthesized as a 381-amino acid precursor that undergoes extensive posttranslational modification, resulting in mature 8-kDa SP-B consisting of amino acids Phe201 through Met279 (reviewed in Refs. 14 and 28). In the alveolus, SP-B exists as a dimer linked via intermolecular disulfide bonding at Cys18 of the mature protein. The processing of SP-B has been studied in freshly isolated rat type 2 cells (27), the Clara cell-like NCI-H441-4 cell line (22), and transfected cell lines such as the Chinese hamster ovary (15), AtT-20, and PC-12 pituitary cell lines (19, 20) and in human fetal lung (30). A processing scheme fitting the available data (15, 22, 27, 29–31) was suggested by Weaver et al. (29) in which the primary translation product (propreSP-B) is glycosylated and the signal peptide is cleaved, producing 42-kDa proSP-B. Cleavage of the NH2-terminal propeptide to the Phe201 is followed by complete cleavage of the COOH-terminal propeptide at Met279, releasing the mature protein, which then dimerizes.

Studies of SP-B processing have been hampered by antibody specificities and by the cell-type specificity of SP-B expression. Some antisera used in prior studies have not identified mature 8-kDa SP-B, often due to differences in antigenicity across species (30). Transfected cell lines do not fully process proSP-B, and isolated type 2 cells quickly lose their ability to express and process endogenous SP-B (15, 22, 27). The immortalized mouse cell line MLE-15 has been shown to produce SP-B8, but little information is currently available on processing in this cell line (31).

Cultured human fetal lung provides a stable model for the in vitro characterization of SP-B processing (4, 30). Although SP-B mRNA is abundant in midgestation human fetal lung, scarce immunoreactive SP-B is detected by immunohistochemistry, and only small amounts of proSP-B are noted on Western analysis until 24 wk gestation. The same tissue in explant culture develops the ability to express and process SP-B coincident with the development of lamellar bodies in type 2 cells lining presumptive air spaces. SP-B8 expression is further enhanced in the presence of dexamethasone.

In this study, we characterize the processing of endogenous hSP-B8 in air space epithelial cells of second-trimester human fetal lung in explant culture. Using antibodies to hSP-B8 and to epitopes within the NH2- and COOH-terminal propeptides of human proSP-B, we describe a novel intermediate of SP-B processing. In addition, we demonstrate that the kinetics of SP-B processing are under developmental and hor-
monal regulation, thereby providing an additional mechanism for the control of SP-B₈ expression in type 2 cells. Preliminary reports have been published previously (12, 13). 

**EXPERIMENTAL PROCEDURES**

Reagents. Express protein labeling mix was obtained from New England Nuclear (Boston, MA). Protein A-agarose was obtained from Life Technologies (Gaithersburg, MD). Dexamethasone, isobutyl methylxanthine, and 8-bromo-cAMP (8-BrcAMP) were obtained from Sigma Chemical (St. Louis, MO). All other reagents were electrophoretic grade and were purchased from Bio-Rad Laboratories (Hercules, CA). Culture medium were produced by the Cell Center Facility, University of Pennsylvania.

Explant and cell culture. Human fetal lung was obtained from second-trimester therapeutic abortions (20–23 wk 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 airways, chopped into 1-mm³ explants, and cultured in Waymouth's medium on a rocking platform as previously described (11). After overnight culture, either 10 nM dexamethasone or 10 nM dexamethasone-0.1 mM 8-BrcAMP-0.1 mM isobutyl methylxanthine were added to the medium for the remainder of the culture period. Media were changed daily, and tissue was studied on days 1, 3, and 5 of culture. Lysates prepared from isolated type 2 cells cultured for 1–5 days (1) were supplied by Drs. J. Joseph Alcorn and Carole Mendelson. In brief, second-trimester human fetal lung explants cultured for 5 days in 1 mM dibutyl-cAMP in Waymouth's medium were enzymatically digested and then incubated in DEAE-dextran to remove fibroblasts. The enriched type 2 cells were plated on plastic dishes that had been coated with the extracellular matrix of Madin-Darby canine kidney cells for Western blot analysis. Type 2 cells were cultured in Waymouth's medium supplemented with 1 mM dibutyl-cAMP for 1–5 days.

Epitope-specific antibody preparation. The hSP-B sequence (23) was analyzed for antigenicity using MacVector software (version 3.53; International Biotechnologies, New Haven, CT) as previously described (5). With the aid of a computer, 15 amino acids, an antigenic index for regions of human preproSP-B, was determined by analysis of contiguous segments for hydrophilicity (Kyte-Doolittle index), surface probability, and flexibility as determined by a-helicity. On the basis of the antigenicity index of preproSP-B, peptide sequences were chosen for production of synthetic peptides (NFPROX, Ser₁⁴⁵–Leu₁⁶⁰; NFLANK, Gln₁⁸⁶–Gln₂⁰⁰; and CFLANK, Gly₂⁸⁴–Ser₃⁰⁴; Fig. 1). Synthetic peptides were commercially prepared using the Merrifield method by either Macromolecular Resources (Fort Collins, CO) or the Nucleic Acid/Protein Core, Children's Hospital of Philadelphia. Coupling efficiency was determined as >90% at each step by ninhydrin reaction, and the resulting peptides were purified by reverse-phase high-performance liquid chromatography. Peptide purity was confirmed by mass spectroscopy and/or NH₂-terminal sequencing by Edman degradation. Synthetic peptides were conjugated to keyhole limpet hemocyanin via an NH₂-terminal cysteine residue and injected in Freund's adjuvant into New Zealand rabbits by Macromolecular Resources or HTI (Ramona, CA). hSP-B antiserum was prepared using purified hSP-B₈ isolated from patients with pulmonary alveolar proteinosis. hSP-B₈ was injected into rabbits in Freund's adjuvant, and the antiserum obtained were subsequently immunoaffinity purified using methods previously described for anti-bovine SP-B antibody (6). Rabbits were bled at biweekly intervals starting at week 4 and given two to four booster doses of peptide. Antisera were screened for reactivity against the immunizing peptide by immunodot-blot assay.

Western blot analysis. One-dimensional SDS-PAGE was performed in 16.5% polyacrylamide gels using a Tris-tricine buffer system as previously described (3). All samples were prepared under reducing conditions unless otherwise indicated. Electrophoresed samples were transferred to Duralose (Stratagene, La Jolla, CA) or polyvinylidine fluoride (Bio-Rad) at 20 mA/cm² for 13–16 h for subsequent immunoblotting or autoradiography. Explant or cell samples were sonicated, and total protein was quantified by the method of Bradford (7). Immunoblotting was performed using a horse-radish peroxidase system (Bio-Rad), and bands were visualized by enhanced chemiluminescence (ECL) using the Renaissance ECL kit (NEN) as described previously (4). Primary antibody concentrations were 1:5,000 to 1:10,000, and secondary antibody was used at a dilution of 1:10,000. In some cases, blots were stripped free of antibody by incubation in 2% SDS-0.06 M Tris·Cl (pH 6.5)-0.72 M 2-mercaptoethanol for 20 min at 50°C and then reprobed with an additional primary antibody. The specificity of epitope-specific antibodies for SP-B intermediates was confirmed by preabsorption of epitope-specific antibodies with the immunizing peptide (1 mM peptide with 2 × 10⁻⁵ mM antibody) at 4°C overnight followed by Western blotting.

Pulse-chase labeling. Explants in culture were starved by replacing Waymouth's medium with Met-Cys-free Dulbecco's modified Eagle's medium (DMEM; 2 ml/60-mm plate) for 2 h while incubating in 95% air-5% CO² on a rocking platform. This medium was then replaced with Met-Cys-free DMEM supplemented with 200 µCi/ml of express protein labeling mix (2 ml/60-mm plate), which is composed of 70% methionine and 15% cysteine. After 1 h, the tissue was washed and placed in complete Waymouth's medium. Duplicate samples were harvested at 0 h, and single samples were harvested at 1, 2, and 4 h of chase unless otherwise specified. Samples were washed in phosphate-buffered saline (PBS) with protease inhibitors (10 mM N-ethylmaleimide, 2 mM benzamidine hydrochloride, and 80 mM phenylmethylsulfonyl fluoride) and then sonicated in 500 µl of 1% SDS with protease inhibitors.

Immunoprecipitation. Radiolabeled lung homogenates and cell lysates were double immunoprecipitated by modification of the method described by Hawgood et al. (15). Total protein and total TCA-precipitable counts were determined from duplicate 10-µl samples of labeled homogenate. Immunoprecipitation was performed on samples containing 10⁶ incorpo-
Epitope-specific antisera to human proSP-B identify a novel intermediate of SP-B processing. Purified, synthetic peptides (NFPROX from Ser145–Leu160, NFLANK from Gin186–Gin200, and CFLANK from Gin288–Ser304; Fig. 1) were used to generate polyclonal antisera in rabbits. Each peptide elicited an antigenic response as determined by immunodot blotting using the immunizing peptide (data not shown). By Western analysis, each peptide-specific antiserum was specific for its immunizing peptide (20 amino acids). Conversely, immunization of rabbits with the predicted size of the cleaved propeptide fragment from the NH2 terminus of proSP-B was confirmed by competitive preabsorption in which each antiserum was preincubated with excess peptide before immunoblotting. Peptide competition demonstrated that each epitope-specific antiserum was specific for its immunizing antigen, with no cross-reactivity to bovine serum albumin (BSA), SP-A, SP-C, or mature SP-B8.

RESULTS

Western analysis of preculture and cultured human fetal lung treated with dexamethasone, cAMP, and isobutyl methylxanthine for 5 days to maximally induce type 2 cell differentiation is shown in Fig. 2. The hSP-B antibody identified proSP-B at 40–42 kDa, often resolved as a doublet due to glycosylation of the COOH-terminal propeptide, and a prominent intermediate with a relative molecular mass of 25 kDa (Fig. 2). The hSP-B antibody also identified mature SP-B at 8 kDa under reducing conditions. In addition, a previously unrecognized band was detected at 9 kDa, separate and distinct from mature SP-B8.

Each epitope-specific antibody identified proSP-B, although recognition of the proSP-B doublet by NFPROX was less intense compared with NFLANK and CFLANK (Fig. 2). The 25-kDa SP-B intermediate was identified by CFLANK and NFLANK antibodies but not by NFPROX antibody. Furthermore, NFLANK antibody identified the same 9-kDa intermediate detected with the hSP-B antibody, whereas the other epitope-specific antibodies did not. The size of the ~19-kDa band identified by NFPROX antibody is consistent with the predicted size of the cleaved propeptide fragment from the NH2 terminus of proSP-B. Antiserum specificity for SP-B proteins was confirmed by competitive preabsorption in which each antiserum was preincubated with excess peptide before immunoblotting. Peptide competition demonstrated that each epitope-specific antiserum was specific for its immunizing antigen, with no cross-reactivity to bovine serum albumin (BSA), SP-A, SP-C, or mature SP-B8. To further characterize NH2-terminal propeptide processing, human fetal lung preparations were cultured for 5 days with dexamethasone, cAMP, and IBMX) were electrophoresed and transferred to Duralose membranes. Blots were incubated with polyclonal rabbit antisera to human proSP-B (hSP-B), NFLANK, NFPROX, and CFLANK peptides at a 1:5,000 dilution for Western analysis. Lanes exhibit the complete banding patterns for each antiserum. In cultured tissue, hSP-B antibody identified proSP-B at 42 kDa, intermediates at 25 and 9 kDa, and mature SP-B at 8 kDa. NFLANK identified proSP-B and intermediates at 25 and 9 kDa, whereas NFPROX identified proSP-B weakly and an additional peptide at ~19 kDa, representing the cleaved NH2-terminal propeptide. CFLANK identified proSP-B and the 25-kDa intermediate. In preculture tissues, these bands were not detected except for weak staining of proSP-B at 42 kDa. Nonspecific bands found in both preculture and cultured lung samples were noted (*), and these nonspecific bands did not compete away after preincubation of specific antisera with immunizing peptide (data not shown).
both contain the complete sequence of the CFLANK immunizing peptide (data not shown).

To exclude the possibility that the 9-kDa SP-B was an artifact of explant culture, Western blot analysis (Fig. 4), performed on 1) normal lung tissue obtained from adults at lung biopsy and from infant lung transplant donors, 2) infants with chronic lung disease who underwent lung transplantation, and 3) type 2 cells prepared from second-trimester human fetal lung explants and cultured on matrix for 3 days with dibutyryl-cAMP and dexamethasone. In each sample, the NFLANK antibody identified the 25-kDa SP-B intermediate, indicating that residual epitopes N-flanking to Phe201 were preserved in the 25-kDa intermediate. The 9-kDa intermediate was present in low amounts in cultured human fetal lung and bronchoalveolar lavage samples but was not visualized in other samples (data not shown).

Human proSP-B processing is induced by lung maturation. Beers et al. (4) and others (30) have previously shown that 8-kDa SP-B is present in very low amounts before 24 wk of gestation in human fetal lung and that explant culture induces the accumulation of SP-B8. In the present study, 20- to 23-wk human fetal lungs were used for explant culture because they typically exhibited no SP-B proteins smaller than 42 kDa. Western analysis with enhanced chemiluminescence demonstrated proSP-B in second-trimester human fetal lung as early as 19 wk of gestation, with no evidence of SP-B processing until the 25-kDa intermediate was demonstrated at 24 wk (Fig. 5A). The 20- to 23-wk human fetal lung used for pulse-chase labeling exhibited only 25-kDa intermediate after 1 day in culture. SP-B8 was evident by 3 days in culture, increasing further over 5 days of culture as previously shown (Fig. 5B).

Because of limitations in drawing conclusions on processing kinetics from Western blots, we examined SP-B processing by pulse-chase with [35S]Met/Cys labeling in human fetal lung cultured for 1–5 days to determine whether the enhanced processing observed by Western blot analysis was due solely to the increased availability of proproSP-B via transcriptional induction. Figure 6 shows a representative pulse-chase experiment after immunoprecipitation with the hSP-B antibody. On day 1, proSP-B was rapidly synthesized over the 1-h pulse and was partially processed to a
25-kDa intermediate (Fig. 6), which occurred between 30 and 60 min of pulse (data not shown). As predicted by Western blotting, no further processing occurred, and these proteins decayed over the 4-h chase period. At 3 and 5 days in culture, proSP-B was also rapidly synthesized. Day 3 explants processed proSP-B to the 25-kDa intermediate, and after 4 h of chase, little SP-B was detected, whereas after 5 days in culture, explants processed newly synthesized proSP-B to mature SP-B. The 9-kDa band was easily visualized in day 5 samples as was a nonspecific band at ~14 kDa frequently seen on Western blot using the hSP-B antibody.

To determine human proSP-B processing kinetics, we analyzed by densitometry the autoradiograms from all pulse-chase experiments (n = 2–6) using methods described previously (3, 15). In short, the densitometric units within each band were first corrected for the number of Cys and Met residues; for example, results from 8-kDa bands were multiplied by 35/10 (total Met/Cys in proSP-B/total Met/Cys in 8-kDa SP-B) to adjust for counts cleaved from NH2 and COOH termini. The total densitometric units after the 1-h pulse were determined by adding the corrected densitometric units in all 35S-labeled SP-B protein forms; this is referred to as the initial incorporated label postpulse. Corrected densitometric units for each SP-B form at all subsequent time points were expressed as the percent of initial incorporated label. Figure 7A illustrates graphically the results of densitometric analysis of the kinetics of SP-B processing in cultured human fetal lung focusing on the loss of proSP-B and appearance of SP-B8. Accumulation of mature SP-B increased with time in culture with rates of 0, 4, and 9%/h after 1, 3, and 5 days, respectively. By comparison, the rate of processing of 42-kDa SP-B to the 25-kDa intermediate did not change significantly over 5 days of culture.

Human proSP-B processing is hormonally regulated. To further characterize the previous observation by Western analysis that glucocorticoid treatment of human fetal lung explants increased SP-B content (4, 11, 30), pulse-chase experiments were performed on tissues cultured in 10 nM dexamethasone for 3–5 days (n = 4–6). As previously described, Western analysis showed that dexamethasone induced precocious appearance of proSP-B, SP-B intermediates, and SP-B8 (Fig. 5B, lanes 4 and 6) by day 3 of explant culture. Dexamethasone treatment markedly enhanced processing at this time point, resulting in an increased rate of accumulation of labeled SP-B8 in pulse-chase experiments (Fig. 7B). On day 3 of culture, the rate of SP-B8 accumulation was approximately fourfold faster than control when tissue was cultured in the presence of dexamethasone, and on day 5 there was a twofold increase. By contrast, there was no effect of dexamethasone on the rate that proSP-B disappeared.

**Fig. 6.** Effect of time in culture on processing of SP-B in human fetal lung explants. Human fetal lung explants were pulse-chase labeled with [35S]Met/Cys on days 1 (n = 2), 3 (n = 6), and 5 (n = 4) of culture. Explants were collected at 0, 1, 2, and 4 h of chase and immunoprecipitated 2 times using human SP-B antibody or NIS. Representative autoradiograms after Tris-tricine SDS-PAGE are shown. Over time in culture, fetal lung explants exhibited increases in both proSP-B and processing to mature 8-kDa SP-B. ProSP-B processing to the 25-kDa intermediate on day 1 was not associated with the appearance of more distal intermediates or SP-B8, suggesting that these proteins were being degraded in the absence of further processing.

**Fig. 7.** ProSP-B processing kinetics are developmentally and hormonally regulated. Pulse-chase immunoprecipitation blots (e.g., Fig. 6) were analyzed by densitometry. After calculating the total densitometric units postpulse immunoprecipitated by the hSP-B antibody, we determined the %densitometric units in the 42-kDa proSP-B and 8-kDa mature SP-B bands at each subsequent time point, correcting for the number of Cys and Met residues in each peptide. Rates of disappearance of proSP-B and appearance of SP-B8 were determined from regression analysis of curves plotting %densitometric units vs. time. Bars represent the mean rate ± SE from 2–6 separate experiments and were examined for significance using 1- or 2-tailed paired Student’s t-test. A: effect of time in explant culture. Rate of proSP-B disappearance did not change significantly over 5 days of culture. By contrast, the rate of accumulation of SP-B8 increased over 5 days in culture. B: effect of glucocorticoids. Rate of proSP-B disappearance was not affected by the presence of 10 nM dexamethasone. By contrast, glucocorticoid treatment increased the rate of SP-B8 accumulation 4- and 2.5-fold on days 3 and 5 of treatment, respectively. NS, not significant. *P < 0.05.
DISCUSSION

In this study, we examined the posttranslational processing of human proSP-B using cultured fetal lung tissue, high-resolution Tris-tricine gel electrophoresis, and antisera specific for hSP-B<sub>8</sub> and epitopes of proSP-B.

Using this approach, we have demonstrated three major new findings. First, processing of the NH<sub>2</sub>-terminal propeptide of human proSP-B is multistep, generating a previously unidentified 9-kDa intermediate that is cleaved of a small peptide fragment as the terminal event releasing mature SP-B<sub>8</sub>. Second, the posttranslational processing of proSP-B is developmentally and hormonally regulated in differentiating type 2 cells of second-trimester human fetal lung. Third, proSP-B processing to mature SP-B is enhanced in the presence of glucocorticoid. These new findings suggest an important role for posttranslational regulatory mechanisms in the expression of mature SP-B by type 2 alveolar cells.

We have characterized a novel intermediate in SP-B processing, thereby modifying the current schema developed by others (15, 22, 29). Previously, it was concluded that the NH<sub>2</sub>-terminal propeptide was completely cleaved from proSP-B at Phe<sup>201</sup>, representing the first amino acid residue of mature SP-B<sub>8</sub>. To further extend these observations, we employed the human fetal lung explant system to provide a stable population of differentiated type 2 cells and epitope-specific antibodies to unique sequences in the NH<sub>2</sub>- and COOH-terminal propeptides of SP-B. The proposed secondary structure of proSP-B is similar to that of the saposins, consisting of four α-helices stabilized by a regular pattern of intramolecular disulfide bonds (15), and each of the synthetic peptides, NFLANK, NFPROX, and CFLANK, lies within the intervening hydrophilic segments.

The processing scheme described by Weaver et al. (29) predicted that the NFPROX and NFLANK epitope-specific antisera would identify proSP-B as well as a cleaved NH<sub>2</sub>-terminal propeptide of ~17–19 kDa. Instead, Western blotting with NFLANK identified SP-B intermediates of 25 and 9 kDa, which were also identified by the hSP-B antibody. The novel 9-kDa intermediate was distinct from 8-kDa mature SP-B on Western analysis of the high-resolution tricine gels using the hSP-B antibody. The NFLANK synthetic peptide sequence is not contained within SP-B<sub>8</sub> and the NFLANK antiserum did not identify SP-B<sub>8</sub>. NFLANK synthetic peptide sequence is distinct from sequences previously used to generate epitope-specific antibodies (22, 29). The 9-kDa band can not represent a cleavage product because it was also recognized by the antibody to mature 8-kDa SP-B. We therefore concluded that the 9- and 25-kDa SP-B intermediates must contain a small residual NH<sub>2</sub>-terminal propeptide fragment. This additional NH<sub>2</sub>-terminal cleavage was not an artifact of the explant culture system. The residual NH<sub>2</sub>-terminal peptide was demonstrated to be part of the 25-kDa SP-B intermediate by Western analysis of a variety of human lung samples using the NFLANK antiserum.

Pulse-chase studies confirmed that there was a precursor-product relationship between 9- and 8-kDa SP-B.

In addition to characterizing a novel intermediate of SP-B processing, we showed that the later events in SP-B processing are developmentally and hormonally regulated. SP-B mRNA is detected in human fetal lung as early as 12 wk of gestation, and content increases through the second trimester to ~50% of adult levels by 24 wk of gestation due to increasing transcription rate (18, 25). ProSP-B levels parallel SP-B mRNA levels during midgestation, yet SP-B<sub>8</sub> is not consistently detectable until ~24 wk of gestation and then only at ~2% of the adult levels (4). In the present study, we also showed that SP-B intermediates were not consistently detected until ~24 wk of gestation, a time when epithelial cells lining presumptive air spaces begin to differentiate into type 2 cells containing lamellar bodies (21). Together these findings indicate that control over mature SP-B<sub>8</sub> levels during development in vivo is posttranslational.

Explant culture of human fetal lung accelerates type 2 cell differentiation and increases both SP-B mRNA and SP-B<sub>8</sub> levels (4, 11). Prior studies by others (30) using human fetal lung in explant culture did not address the kinetics of SP-B processing, in part due to the limitations of the antisera used, which did not immunoprecipitate 8-kDa SP-B. Our current results show that the induction of SP-B<sub>8</sub> during culture is not due solely to the increased availability of proSP-B resulting from enhanced SP-B gene transcription. There was clearly an increase in the immunoprecipitated SP-B products both with time in culture and in the presence of glucocorticoid, reflecting increased transcriptional activity of the SP-B gene. Moreover, the distribution of products changed with time and hormones, indicating an independent effect on processing. After 1 day in culture, labeled proSP-B was only processed to the 25-kDa intermediate, and both labeled proteins...
decayed during the remainder of the chase period, suggesting a reclamation of these proteins within undifferentiated epithelial cells. Despite adequate precipitated counts, no mature product was detected. This was consistent with the absence of 8-kDa SP-B on day 1 by Western blotting. By 3 days in explant culture, we observed labeled SP-B by pulse-chase studies corresponded to increasing amounts of 8-kDa SP-B found on Western analysis over the 5-day culture period. Not unexpectedly, the rates of both proSP-B disappearance and 8-kDa SP-B accumulation in our experiments using human fetal lung explants are very similar to the rates previously reported by others using type 2 cells isolated from adult rat lung (15). Thus the key step(s) regulating the production of SP-B during type 2 cell differentiation ex vivo are distal to the 25-kDa SP-B intermediate. This is not unexpected since the initial NH2-terminal processing of proSP-B occurs in a variety of cell types (15, 22, 29), whereas further processing to SP-B8 is type 2 cell specific.

Glucocorticoids precociously induce fetal lung maturation (reviewed in Ref. 2). In doing so, glucocorticoids enhance expression of SP-B8 (4), in part by increasing gene transcription rate and RNA stability. Our current results show that glucocorticoids also influence the posttranslational processing of proSP-B. On days 3 and 5 of explant culture, the rate of labeled SP-B8 appearance was increased two- to fourfold in the presence of glucocorticoid, whereas there was no significant change in the rate of disappearance of proSP-B. Together, our data and results of previous studies imply that there are mechanisms at work within the epithelial cells of the immature fetal lung preventing the posttranslational processing of proSP-B. Differentiation of type 2 cells therefore involves enhanced SP-B gene transcription, increased mRNA stability, and maturation of posttranslational processing events, and all are glucocorticoid responsive.

Possible mechanisms for posttranslational control of SP-B processing may include regulation of chaperone proteins required for the egress of intermediates from the endoplasmic reticulum and/or Golgi, vesicular transport of intermediates between organelles, and specific proteases required for SP-B processing. There is considerable evidence in the literature for regulation of the movement of newly synthesized proteins between processing compartments. The 78-kDa glucose regulated protein GRP-78, a chaperone necessary for translocation of nascent polypeptides into the endoplasmic reticulum, folding of nascent polypeptides, and correction of misfolded polypeptides (reviewed in Ref. 17), is transcriptionally as well as translationally regulated to respond to changes in total cell protein synthesis (24). Similarly, enzymes required for posttranslational processing events are often under transcriptional and/or posttranslational regulation, providing an additional layer of control in the expression of highly processed proteins (reviewed in Ref. 16). The cell-type specific, regulated processing of complex prohormones has been described for proteins such as Met-enkephalin, arginine vasopressin, insulin, glucagon, and ACTH. Many of the enzymes that participate in processing these prohormones are themselves expressed in specific cell types and are often hormonally regulated. For example, the subtilisin-related enzymes PC1/3 and PC2 that are involved in prohormone processing are expressed in specialized secretory granules of endocrine and neural tissues. These enzymes are regulated by glucose, thyroid hormone, and corticosteroids in a cell-type specific manner. It is likely that the enzymes involved in proSP-B processing will have similar characteristics.

In summary, we have shown that NH2-terminal propeptide processing of proSP-B includes the preservation of a small peptide N-flanking the mature SP-B sequence, resulting in a novel 9-kDa intermediate of SP-B processing as depicted in Fig. 8. Furthermore, we have shown that the type 2 cell-specific distal processing events, specifically cleavage of the COOH-terminal and preserved N-flanking peptides, are both developmentally and hormonally regulated, indicating the physiological importance of posttranslational regulation in SP-B expression.

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