Bone Morphogenetic Protein 1 Is an Extracellular Processing Enzyme of the Laminin 5 γ2 Chain*

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Epithelial cells maintained in culture medium containing low calcium proteolytically process laminin 5 (α3β3γ2) within the α3 and γ2 chains (1). Experiments were designed to identify the enzyme(s) responsible for the laminin 5 processing and the sites of proteolytic cleavage. To characterize the nature of laminin 5 processing, we determined the N-terminal amino acid sequences of the proteolytic fragments produced by the processing events. The results indicate that the first α3 chain cleavage (200-165 kDa α3) occurs within subdomain G4 of the G domain. The second cleavage (165-145 kDa α3) occurs within the IIia domain, 11 residues N-terminal to the start of domain II. The γ2 chain is cleaved within the second epidermal growth factor-like repeat of domain III. The sequence cleaved within the γ2 chain matches the consensus sequence for the cleavage of type I, II, and III procollagens by bone morphogenetic protein-1 (BMP-1), also known as type I procollagen C-proteinase (2). Recombinant BMP-1 cleaves γ2 in vitro, both within intact laminin 5 and at the predicted site of a recombinant γ2 short arm. α3 is also cleaved by BMP-1 in vitro, but the cleavage site is yet to be determined. These results show the α3 chain and γ2 chains to be substrates for BMP-1 in vitro. We speculate that γ2 cleavage is required for formation of the laminin 5–6 complex and that this complex is directly involved in assembly of the interhemidesmosomal basement membrane. This further suggests that BMP-1 activity facilitates basement membrane assembly, but not hemidesmosome assembly, in the laminin 5-rich dermal-epidermal junction basement membrane in vivo.

The occurrence of physiological, extracellular proteolytic processing of collagens is well documented, as is the important role that it plays in controlling the fibrillogenesis of bundled collagen fibers (3). An enzyme responsible for removal of the C-terminal procollagen propeptides of the major fibrillar collagen types I–III has been identified as BMP-1 (2).1 BMP-1 was first identified in osteogenetic fractions of mammalian bone (4–7) but was subsequently found to show substantial homology to proteins involved in morphogenetic patterning, such as the products of Drosophila genes tolloid (tld) and tlr-1 (12, 43) and of sea urchin gene products BP10 and SpaN (8, 9). Each contains an N-terminal astacin-like zinc-binding metalloendopeptidase domain (10) followed by varying numbers of epidermal growth factor-like (EGF-like) motifs and internal repeats termed CUB domains thought to be responsible for protein-protein interactions (44).

There is abundant genetic and molecular evidence that Drosophila tld mediated dorsal-ventral patterning in the fly embryo (11–13), with null phenotypes of tld showing partial transformation of the dorsal ectoderm into ventral ectoderm (14). Genetic and developmental expression studies have also indicated that the tld gene product TLD participates within the same developmental pathway as the product of the decapentaplegic gene, DPP, the fly cognate of mammalian BMP-2 and BMP-4. The latter copurify from bone extracts with BMP-1(6), thus suggesting a signaling pathway that has been conserved in flies and mammals. An intact protease domain of TLD is required for the correct functioning of DPP (15).

Actual substrates for BMP-1/TLD-like proteases had not been demonstrated prior to the identification by Kessler et al. (2) of BMP-1 as a type I procollagen C-proteinase. However, fibrillar collagens have not been detected in Drosophila, and recent evidence suggests that TLD acts in dorsal-ventral patterning of Drosophila by cleaving the product of the short gastrulation gene SOG, a secreted protein inferred to bind DPP in a latent complex (16). Similarly, the BMP-1/TLD-like proteases Xenopus Tolloid (17) and zebrafish Tolloid (18) and BMP-1 itself (31) have now been shown to have the ability to cleave Chordin, the vertebrate cognate of SOG (45). Thus, it has been suggested (31) that enzymes like BMP-1, with dual activities in processing procollagens and cleaving Chordin, may serve to orchestrate the deposition of collagenous matrix with

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1 The abbreviations used are: BMP-1, bone morphogenetic protein-1; EGF, epidermal growth factor; DME, Dulbecco’s modified Eagle’s medium; RIPA, radioimmunoprecipitation assay; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.
the liberation of growth factors like BMP-2 and BMP-4. Nevertheless, ablation of the mouse BMP1 gene by homologous recombination (19) produced homozygous mutant embryos with defects in procollagen processing and fibrillogenesis, but with an absence of early patterning abnormalities. The phenotype of these mice thus suggests that in mammals BMP-1 functions primarily in the deposition of extracellular matrix rather than in embryonic patterning.

Proteolytic cleavage of laminin was first noted by Ehrig et al. (20) for the laminin α2 chain. The cleavage occurs within the G domain of the chain, but the excised fragment remains covalently associated with the precursor molecule through a disulfide bond(s) (20). Proteolytic processing of laminin 5 was first identified by Rousselle et al. (21) and subsequently characterized by Marinkovich et al. (1). Laminin 5 is a major component of the anchoring filaments within the dermal-epidermal junctional basement membrane of skin and plays an important role in stabilizing the attachment of epithelial hemidesmosomes to the basement membrane. Null mutations in the LAMA3, LAMB3, or LAMC2 genes encoding the laminin 5 chains result in the lethal blistering condition Herlitz's junctional epidermolysis bullosa (22). Cultured keratinocytes synthesize an intracellular precursor of the tissue form that is composed of a laminin α3 (200 kDa), a β3 (140 kDa), and a γ2 (155 kDa) chain, but they do not synthesize significant amounts of other forms predicted (23, 24) to arise via alternative splicing or via use of alternative promoters (1). Laminin 5 extracted from tissues is composed of α3 (165 or 145 kDa), β3 (140 kDa), and γ2 (105 kDa) chains. Approximately one-half of the laminin 5 extracted from tissue is covalently associated by disulfide bonds with laminin 6 (α3β1γ1) in skin and with laminin 6 and laminin 7 (α3β2γ1) in amnion (25). These associations may be mediated by cysteiny1 residues predicted to be unpaired within the fourth EGF-like repeat of domain IIIα of α3 and within domain VI of β3. Recent evidence (26) suggests that monomeric laminin 5, but not complexed laminin 5, directly mediates the interactions of hemidesmosomal integrin α6β4 with the anchoring fibril protein type VII collagen. However, the role of the laminin 5–6/7 complexes are unclear. Monomeric laminin 5 is unable to interact with other known components of the basement membrane, because the processed molecule lacks the required VI domains of the short arms and the nidogen/entactin-binding domain identified within the γ1 chain (27, 28).

However, complex formation of laminin 5 with laminins 6 or 7 provides a binding site for nidogen/entactin because of the presence of the γ1 chain. The complex, but not the monomer, is therefore predicted to be competent to participate in basement membrane assembly. Proteolytic processing within the short arms of the laminin α3 and γ2 chains is hypothesized to be required for complex formation, because the proteolytic events remove substantial portions of the γ2 short arm, thereby allowing access of the unpaired cysteiny1 residue within the β3 domain VI to the unpaired cysteiny1 residue within the IIIα domain of the α3 chain. The present studies support the above hypothesis by identifying the cleavage sites resulting from laminin 5 processing and identifying one of the proteolytic enzymes involved.

MATERIALS AND METHODS

Keratinocyte Culture

Human keratinocytes were cultured from newborn foreskins in serum-free keratinocyte growth medium (Life Technologies, Inc.). Dissociated third passage keratinocytes were used for experiments.

Cell Labeling

Dissociated third passage keratinocytes were cultured in DMEM containing 10% fetal bovine serum overnight. Cells were briefly washed and then incubated for 30 min or 1 h with methionine- and cysteine-deficient DMEM. Labeling was performed in deficient medium containing 250 µCi/ml each of [35S]methionine and [35S]cysteine (Amersham Pharmacia Biotech) for 10 min in pulse-chase experiments and in the deficient medium containing 50 µCi/ml each of [35S]methionine and [35S]cysteine (27) for 24 h in other experiments under standard culture conditions. Labeled medium was removed from culture plates and centrifuged at 2,000 rpm. Cell layers were washed once with nonradioactive culture medium and then harvested with a cell scraper and ice-cold radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 250 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, 2 mM 1-methionine, 2 mM 1-cysteine, 0.3% Nonidet P-40, 0.05% Triton X-100, 0.3% sodium deoxycholate, 0.1% bovine serum albumin) containing 0.1% SDS. All subsequent steps were performed at 4°C. Labeled cell material was solubilized in a Dounce homogenizer and clarified at 160,000 × g (× min). To examine the effects of the protease inhibitors on laminin 5 processing, keratinocytes were cultured in DMEM containing 10% fetal bovine serum on membrane supports (0.4 µm, Transwell Insert) overnight. Cells were briefly washed and then incubated for 1 h with methionine and cysteine-deficient DMEM. Labeling was performed in each deficient medium containing 100 µCi/ml each of [35S]methionine and [35S]cysteine for 1 h. Labeled cells were preincubated in DMEM containing 0.1% bovine serum albumin with or without protease inhibitors for 30 min, and conditioned media were removed. Fresh DMEM containing 0.1% bovine serum albumin with or without protease inhibitors was added, and 3 h later the conditioned media were collected from lower chambers and clarified at 160,000 × g × min.

Antibodies

The following antibodies were used in these studies: polyclonal rabbit anti-laminin 5 (1); monoclonal anti-laminin α3, BM-165, and BM-4 (21); and polyclonal rabbit anti-EHS tumor laminin (Sigma). Anti-BMP-1: A BMP-1 fusion protein was produced as described previously (2) from a 1040-base pair Apal–HinCII CDNA fragment subcloned into expression vector pRSBET (Invitrogen). The fusion protein, comprising human BMP-1 residues 197–543 (29) fused to a polyhistidine domain, was purified on nickel-Sepharose (Invitrogen, Carlsbad, CA) followed by SDSE-PAGE. The recombinant BMP-1 band was visualized with Coomassie Blue, excised, equilibrated with PBS, emulsified with an equal volume of Freund’s incomplete adjuvant, and injected subcutaneously into a New Zealand White rabbit. The rabbit was boosted twice at 4-week intervals with 150 µg of fusion protein per boost, prepared as just described but using Freund’s incomplete adjuvant. For affinity purification, anti-serum was diluted 1:10 in 10 mM Tris (pH 7.5) and passed through a column of the purified fusion protein coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). After washing with column buffer volumes of 500 ml Tris (pH 7.5), bound antibody was eluted with 10 bed volumes of 100 mM glycine (pH 2.5). Antibody-containing pools were dialyzed against PBS with 0.2% sodium azide and then stored at −70°C. Specificity of the affinity-purified antibody was ascertained by immunoblotting of medium samples from cultures of MG-63 osteosarcoma cells, as described (30), in which antibodies detected only 88- and 130-kDa bands corresponding to BMP-1 and mTLD (an alternatively spliced form of BMP-1), respectively.

Radioimmunoprecipitation

For each sample, 10 µl of polyclonal rabbit anti-laminin 5, polyclonal anti-EHS tumor laminin (Sigma), or monoclonal anti-laminin 5 antibodies were added to 40 µl of protein G-Sepharose (Amersham Pharmacia Biotech). Protein G-Sepharose alone was used as the control. The mixtures were incubated at room temperature for at least 1 h with mild agitation. The antibody–protein complexes were pelleted by centrifugation at 6550 × g–min, washed once with RIPA buffer, and centrifuged again before use.

Preclearing of each sample was accomplished by adding aliquots of labeled cell or medium supernatants to the centrifuged pellets of gelatin-Sepharose (0.1 ml of gel suspension/1 ml of sample). Each sample was vortexed briefly and then left on a rocking platform overnight. The mixture was then centrifuged at 160,000 × g–min, and the supernatant was combined with a centrifuged pellet of protein G-Sepharose precomplexed with specific antibody. These were then incubated for 18 h on a rocking platform and then centrifuged at 2550 g–min. The supernatant was then removed, and the pellet was washed with RIPA buffer containing 0.1% SDS, briefly vortexed, and then recentrifuged. After five washes, the pellets were mixed with sample buffer for SDS-polyacryl-
amide gel electrophoresis, heated to 95 °C for 3 min, and centrifuged, and the supernatant solution was analyzed by SDS-PAGE.

**Heparin-Sepharose Affinity Chromatography**

Keratinocytes were labeled in methionine- and cysteine-deficient DMEM containing 100 μCi/ml each of [35S]methionine and [35S]cysteine for 1 h under standard culture conditions. Labeled cells were cultured in DMEM containing 0.1% bovine serum albumin, and 4 h later the conditioned medium was collected and clarified (160,000 g-min). The conditioned medium was precleared with gelatin-Sepharose as described above and applied to a heparin-Sepharose affinity column (1 ml, Hitrap Heparin; Amersham Pharmacia Biotech). The bound materials were eluted with a step NaCl gradient of 0.15, 0.2, 0.3, 0.45, 0.6, and 1.5 M NaCl in PBS, and radioactivities of 1 ml fractions were determined. Laminin 5 was immunoprecipitated from each peak fraction with a mixture of monoclonal anti-laminin α3 (BM-165) and anti-laminin β3 (6F12) antibodies. The precipitation products from each of the indicated fractions were evaluated by SDS-5% PAGE followed by disulfide bond reduction and visualized by autoradiography.

**Preparation of Recombinant Proteins**

rBMP-1—To produce rBMP-1, a 2199-base pair Spal-BglI cDNA fragment containing the full-length human BMP-1 coding sequence from clone K71 was ligated into vector pBacPAK9 (CLONTECH, Palo Alto, CA) and recombined with BsaI-361-digested BacPAK6 (CLONTECH) viral DNA by co-transfection into Sf21 insect cells. After 60–72 h, resultant virus was plaque-purified and amplified for 4 days on Sf21 monolayers in 10% fetal calf serum and Grace's medium (Sigma). Virus stocks were then replated in Sf21 suspension cultures in serum-free SF-900 (Life Technologies, Inc.). After 6 days, fresh cells were infected with reamplified virus and grown in fresh SF-21–900 medium, which was harvested for recombinant protein 4 days after infection. Sf21 cells infected with parental BacPAK viral stock, in which the polyhedrin promoter drives LacZ expression, were used to provide control media.

Conditioned media from insect cells expressing recombinant proteins BMP-1 and β-galactosidase as control were dialyzed against enzyme assay buffer, 50 mM Tris-HCl, 0.5 mM NaCl, 0.1% soy bean trypsin inhibitor, and 5 mM CaCl2 (pH 7.5) and then concentrated above 5-fold with Centricon filter units (Amicon, Beverly, MA). Medium was stored at −80 °C.

r γ2 Short Arm—The short arm of γ2 was cloned by reverse transcription-PCR. 1 μg of total RNA from cultured keratinocytes was reverse transcribed (CLONTECH). PCR was performed following the manufacturer's instructions (PfuTurbo DNA Polymerase; Stratagene) using the following primers on keratinocyte cDNA: sense primer 5′-dGAGCTAGCAACCTCCAGGAGGGAAGTCTGTGAT; antisense primer 5′-dCCTGGATCTCCATTCTCTCTAAGCTGCTG (Genbank accession number: NM_005562). The PCR product was agarose gel purified (Qiagen) and subcloned (rapid DNA ligation kit; Roche Diagnostics GmbH) into a modified PCEP-4 (gift from Ernst Poeschl) expression vector with the following restriction enzymes: Nhel and BamHI (Promega). For convenience, a six-histidine tag followed by a stop codon served, cultured cells were pulse-labeled for 10 min with [35S]methionine and [35S]cysteine and subsequently chased for 10, 30, and 60 min and 3 h. The laminin 5 present within the cellular compartment at each time point was evaluated by immunoprecipitation using polyclonal anti-laminin antibodies or monoclonal anti-α3 antibody BM-165. The results shown in Fig. 1 indicate that even for a 3-h chase, the intracellular laminin 5 was done using polyclonal anti-laminin 5 and for BMP-1 using polyclonal anti-recombinant BMP-1 (see above) according to the protocols previously described (21).

**RESULTS**

**Laminin Processing in Keratinocyte Cultures**—To further characterize the biosynthetic processing of laminin 5 in normal human keratinocytes, it was necessary to obtain larger amounts of cell culture derived materials. Therefore, cell culture conditions were adjusted for maximal laminin 5 production. The results of these studies indicated that culture of third passage neonatal foreskin keratinocytes in DMEM plus 10% fetal bovine serum produced sufficient materials for the studies described below.

To be certain that the new culture conditions produced the same proteolytic processing patterns as those previously observed, cultured cells were pulse-labeled for 10 min with [35S]methionine and [35S]cysteine and subsequently chased for 10, 30, and 60 min and 3 h. The laminin 5 present within the cellular compartment at each time point was evaluated by immunoprecipitation using polyclonal anti-laminin antibodies or monoclonal anti-α3 antibody BM-165. The results shown in Fig. 1 indicate that even for a 3-h chase, the intracellular laminin 5 appeared to be identical to the material seen at 0 h following labeling, indicating that processing did not occur within the intracellular compartment. In contrast, the laminin 5 present in the culture medium following 10, 30, and 60 min of chase showed conversion of the 200-kDa α3 chain to a 165-kDa form. The conversion was obvious even after 10 min of chase, indicating that the first processing event occurs very rapidly after secretion.

To determine the extent of processing after more extended periods of chase, cells pulse-labeled for 10 min were then chased at 1.5-h intervals. At zero time, radioactive medium was replaced with unlabeled medium, and incubation was continued for 1.5 h. At that time, the spent medium was replaced with fresh nonradioactive medium, and the chase was continued to
Fig. 1. Laminin 5 is normally processed by keratinocytes grown in high [Ca\textsuperscript{2+}] medium containing 10% fetal bovine serum. Fourth passage neonatal human foreskin keratinocytes were pulse-labeled for 10 min with \[^{35}S\]methionine/cysteine and chased in nonradioactive medium for 0, 10, 30, 60, and 180 min. Cell layers and media were separately harvested, and laminin 5 was isolated by immunoprecipitation using polyclonal anti-laminin 5 sera or monoclonal anti-laminin α\textsubscript{3} (BM-165) and analyzed by SDS-5% PAGE, following disulfide bond reduction, and autoradiography. The cell sheets (lanes 1–4) contain only unprocessed laminin 5 at time 0 (lanes 1 and 3) and at 3 h (lanes 2 and 4) when laminin 5 is immunoprecipitated using polyclonal anti-laminin 5 (lanes 1 and 2) or monoclonal anti-laminin α\textsubscript{3}, BM-165 (lanes 3 and 4). Laminin 5 was immunoprecipitated from medium using monoclonal anti-laminin α\textsubscript{3} (BM-165) following 10 (lane 5), 30 (lane 6), and 60 (lane 7) min of chase. Increasing amounts of laminin 5 accumulated in the medium with longer chase times, but even at 10 min, processing of laminin 5 α\textsubscript{3} is evident. Processing of γ\textsubscript{2} was detectable at 60 min, but the processing product remains only a very minor fraction of the total.

Fig. 2. Time course of laminin 5 processing from 0 to 6 h. Cultured keratinocytes were pulse labeled as described in the legend to Fig. 1, and the chase medium was harvested and replaced each 1.5 h. Laminin 5 that accumulated within the indicated times (lanes 1–4) was immunoprecipitated using monoclonal anti-laminin α\textsubscript{3} (BM-165) and analyzed by SINS-5% PAGE, following disulfide bond reduction and autoradiography. Lane 5, pooled fractions 1 and 2. Lane 6, pooled fractions 1–4. Laminin α\textsubscript{3} processing to 165 kDa is largely complete by 6 h, whereas processing of γ\textsubscript{2} remains less than 50% complete.

Fig. 3. Inhibition of laminin 5 processing by protease inhibitors. Keratinocytes grown on porous culture substrates were labeled with \[^{35}S\]methionine/cysteine for 1 h and chased in nonradioactive medium for an additional 3 h in the presence of the indicated protease inhibitors. Processing products that accumulated in the medium were immunoprecipitated using monoclonal anti-laminin α\textsubscript{3} (BM-165) and analyzed by SDS-5% PAGE, following disulfide bond reduction, and autoradiography. C, no added inhibitor; Hep, 100 μg/ml heparin; Leup, 100 μg/ml leupeptin; Aprot, 100 μg/ml aprotinin; Chym, 100 μg/ml chymostatin; 20 μM o-PT, 20 μM ortho-pheno-phenanthroline; 1 mM o-PT, 1 mM ortho-phenanthroline. Only the higher concentration of ortho-phenanthroline inhibited the processing of both α\textsubscript{3} and γ\textsubscript{2}. Heparin appears to partially inhibit the processing of α\textsubscript{3} but has no effect upon the processing of γ\textsubscript{2}.

Sequences of Cleavage Products—In an attempt to identify the enzymes responsible for laminin 5 processing, the N-terminal sequences of the 200-, 165-, and 145-kDa forms of the α\textsubscript{3} chain and the 105-kDa form of the γ\textsubscript{2} chain were obtained. These are summarized in Table I. The N terminus of the 200-kDa α\textsubscript{3} chain indicates that the signal peptide cleavage actually occurs between glycine residue 16 and tyrosine residue 17 (23). The N-terminal sequence of the 165-kDa α\textsubscript{3} is identical, indicating that the initial proteolytic event occurs within the C terminus. Deglycosylation of the α\textsubscript{3} chain reduces the mass of α\textsubscript{3} peptides by approximately 10%. With this in mind, comparison of the mass of the 165-kDa α\textsubscript{3} chain to its predicted sequence suggests that the cleavage occurs within do-

\[^{2}\]H. Welgus and R. E. Burgeson, unpublished results.

\[^{3}\]M.-F. Champliaud and R. E. Burgeson, unpublished data.
main G4. We do not know the exact site of cleavage, because we have been unable to isolate the peptide lost following this cleavage event. The second α3 cleavage to 145 kDa was found to lie within domain IIIA, 11 residues prior to the beginning of domain II. The α3 cleavage sites are highly conserved between mouse and human. Cleavage of the γ2 chain was found to occur within domain III at the beginning of the 2nd EGF-like repeat. The cleavage sequence is identical in mouse and human and has a low level of sequence identity with the equivalent sequence of the γ1 chain. A schematic diagram of the processing sites within laminin 5 is shown in Fig. 4.

**Binding Properties of Cleavage Products**—Laminin 5 binds heparin-Sepharose (34), and the major heparin-binding site in the laminin α1 chains is within domain G4 (35). If the heparin-binding domain of laminin 5 is also within domain G4, then the α3 chains processed within the G domain should have reduced heparin affinity. To test this prediction, radiolabeled keratinocyte medium was applied to heparin-Sepharose and eluted with increasing concentrations of NaCl. Because α3 undergoes cleavage within the short arm as well as within the G domain, this procedure also enabled us to test the possibility that the α3 short arm is involved in heparin binding. The cleavage within the short arm occurs rarely in cell culture, but for unknown reasons some preparations of keratinocyte medium contain significant amounts of laminin 5 molecule processed at the N terminus but not at the C terminus. We used such a preparation for this experiment. Following heparin-Sepharose fractionation, laminin 5 was immunoprecipitated using BM-165, which followed by a question mark. A second cleavage occurs in α3 within domain IIIa (arrow). The new N-terminal sequence (bold) was identified by amino acid sequencing. This sequence does not contain the BMP-1 consensus sequence. γ2 is cleaved by BMP within domain III (arrow). The new N-terminal sequence was identified by amino acid sequencing (bold).

The second bound fraction elutes between 0.45 and 0.6 M NaCl. These fractions contain α3 chains of 200 and 180 kDa, β3 chains, and 155-kDa γ2 chains. Our interpretation of these results is that laminin 5 contains at least two heparin-binding sites with different affinities. One site resides within the short arm of γ2. A second binding site is within the G4/G5 domains of α3 and has greater affinity than the γ2 short arm site. The presence of the α3 short arm appears to increase the heparin affinity as well. Consistent with this observation, the laminin form found in the 0.5 M NaCl fraction is exclusively the unprocessed molecule, containing the α3 short arm, the intact G domain, and the γ2 short arm. The α3 short arm may itself contain a third heparin-binding site or may stabilize the conformation of the γ2 chain short arm heparin-binding region. **BMP-1 as a Potential Proteolytic Cleaving Enzyme**—The cleavage sequence within the γ2 chain matches the minimal consensus sequence for the cleavage of procollagen C-propeptides by BMP-1 (2), in that Asp is at the P-1' position and Tyr is at the P-3 position. Further support for a possible role of BMP-1 in laminin 5 processing was provided by comparing the sensitivity of BMP-1 proteolytic cleavage activity and laminin 5 γ2 chain processing with various inhibitors in cell culture (Fig. 4). Both are inhibited by divalent cation chelating reagents, whereas leupeptin has no effect on either activity.

To directly test the ability of laminin 5 to serve as a substrate for BMP-1, radiolabeled laminin 5 from the intracellular com-

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**Table 1**

| α3A, 200 kDa | YSSQR-  
| Human*  
| Mouse*  
| α3A, 165 kDa | -CLG YSSQR-  
| Human*  
| Mouse*  
| α3A, 145 kDa | -CLG YSSEQQRV-  
| Human*  
| Mouse*  
| α2, 105 kDa | -EPK DSSPAE-  
| Human*  
| Mouse*  

*Ref. 23.
†Ref. 40.
‡Ref. 41.
§Ref. 42.

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![Schematic representation of the locations of proteolytic processing sites in laminin 5](image-url)
BMP-1, a Processing Enzyme of the Laminin 5 γ2 Chain

FIG. 5. Fractionation of laminin 5 processing intermediates by heparin-Sepharose affinity chromatography. Radiolabeled keratinocyte medium was applied to a heparin-Sepharose affinity column. The bound materials were eluted with a linear NaCl gradient from 0 to 0.6 M in PBS and followed by a 1.5 M final step elution. Laminin 5 was immunoprecipitated from each fraction using a mixture of monoclonal anti-laminin α3 antibodies (BM-165 and BM-4). The precipitated products from each of the indicated fractions were evaluated by SDS-5% PAGE, following disulfide bond reduction, and autoradiography. The bound materials were eluted with a linear NaCl gradient from 0 to 37 °C for 17 h. Aliquots of the incubation mixture were taken at 0, 1, 3, 7, and 17 h and evaluated by SDS-5% PAGE, following disulfide bond reduction and autoradiography. Incubation of the substrate with recombinant BMP-1, but not with recombinant β-galactosidase, results in the loss of 155-kDa γ2 and the appearance of 105-kDa γ2. BMP-1 also converts α3 from 200 kDa to approximately 165 kDa. A similar conversion of α3 also occurs during the control incubation but at a significantly slower rate.

FIG. 6. Cleavage of laminin 5 in vitro by recombinant BMP-1. Intact laminin 5 substrate was immunoprecipitated from the intracellular compartment of radiolabeled cultured keratinocytes as described under “Materials and Methods.” Aliquots of the substrate were incubated with the medium from baculovirus expressing only β-galactosidase (Control β-gal) or with the medium from baculovirus expressing BMP-1 (BMP-1) and incubated at 37 °C for 17 h. Aliquots of the incubation mixture were taken at 0, 1, 3, 7, and 17 h and evaluated by SDS-5% PAGE, following disulfide bond reduction and autoradiography. Incubation of the substrate with recombinant BMP-1, and not with recombinant β-galactosidase, results in the loss of 155-kDa γ2 and the appearance of 105-kDa γ2. BMP-1 also converts α3 from 200 kDa to approximately 165 kDa. A similar conversion of α3 also occurs during the control incubation but at a significantly slower rate.

FIG. 7. Rotary shadowed images obtained from r γ2. The images shown are representative of those obtained by transmission electron microscopy following rotary shadowing. The images suggest the presence of a globular domain and of short rod-like domains, consistent with the predicted domain structure of the γ2 short arm.

FIG. 8. rBMP-1 cleaves r γ2. Incubation of r γ2 with purified rBMP-1 results in the observation of two fragments with electrophoretic mobilities consistent with the predicted N and C termini of cleaved γ2 short arm. N-terminal microsequence determination of the fragment indicated as γ2 chain C terminus matched the predicted sequence of the cleavage site (see text).

Department of keratinocytes was immunoprecipitated and incubated for 1, 3, 7, and 17 h with baculovirus-expressed recombinant BMP-1 or with conditioned medium from cells infected with parental baculovirus expressing β-galactosidase rather than BMP-1. As shown in Fig. 6, in the absence of BMP-1, there appears to be a slow but progressive loss of the α3 200-kDa chain. There is no change in the amount of 155-kDa γ2, nor is there appearance of a band at 105 kDa, the position of fully processed γ2. In contrast, in the presence of BMP-1 there is an obvious appearance of γ2 in the 105-kDa position within the first 1 h of incubation. This appears to represent an essentially complete proteolytic processing of the γ2 chain, because there is no significant increase in the level of 105-kDa product even after 17 h. There is also a very rapid conversion of the α3 chain from 200 to 165 kDa. This processing appears to be complete by 1 h. The BMP-1 processing of laminin 5 is partially inhibited by ortho-phenanthroline or 10 mM EDTA (data not shown). Further, BMP-1 sequences can be amplified from neonatal keratinocyte RNA by reverse transcription-PCR (data not shown), and keratinocytes have been reported to secrete detectable quantities of BMP-1 under certain culture conditions (30), supporting the probability that BMP-1 is involved in the processing of laminin 5 in the cultures reported here.

To test whether the site at which recombinant BMP-1 processes γ2 matches the site at which γ2 is normally processed in keratinocyte cultures, a recombinant γ2 short arm, comprising domains III–V (r γ2), was prepared in a mammalian expression system. The recombinant product produces the expected image by rotary shadowing (Fig. 7), suggesting that the short arm is correctly folded. Incubation of r γ2 with purified rBMP-1, from a mammalian expression system, produced only two γ2 fragments, of the expected size (Fig. 8), indicating that BMP-1 cleavage is specific for a single site at or near the site processed in keratinocyte cultures. Microsequencing of the smaller, C-terminal fragment resulted in the N-terminal sequence -NP-DIEA, which matches the predicted sequence DENPDIECA and confirms that BMP-1 cleaves at the identical site used in keratinocyte cultures.

Immunolocalization of BMP-1 in Vivo—If processing of laminin 5 involves the BMP-1 enzyme in vivo, then one would expect to see the enzyme localized to the basement membrane zone at times when the epithelial basement membrane is being actively assembled. Using a polyclonal antibody raised against recombinant BMP-1, we examined the localization of antigen in frozen sections of neonatal foreskin (data not shown). Antibody was localized along the dermal-epidermal junction, although it localized more strongly surrounding mesenchymal cells in the dermis. To examine the epithelial basement membranes at earlier stages of skin development, we then examined frozen sections of skin from a bovine embryo corresponding in age to a human fetus at the early-mid second trimester. As shown in...
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**Fig. 9. BMP-1 and laminin 5 localization at the dermal-epidermal junction of fetal calf skin.** Fetal calf skin was excised from a fetus determined to be an age equivalent to the human first to second trimester transition by crown-to-rump measurements. Cryosections of calf skin were immunostained for BMP-1 with polyclonal antiserum (top left panel) or preimmune serum (bottom left panel), and for laminin 5 using polyclonal anti-laminin 5 (top right panel) or preimmune serum (bottom right panel). BMP-1 localizes to the basal keratinocytes and to the outer root sheath cells of the hair follicles in apposition to laminin 5 within the dermal-epidermal and hair follicle basement membranes. Minimal dermal staining for either component is seen at this developmental stage, aside from that seen within or adjacent to hair follicular structures.

Fig. 9, reactivity with this antibody was seen predominantly within the basal epithelial cell layer. The enzyme cannot be visualized within the papillary dermis either because its concentration is below that detected by the reagents or because its diffusion is restricted. The technique is not of sufficient resolution to determine whether the enzyme is present within the dermal-epidermal junction basement membrane. Minimal reactivity was seen within the mesenchymal tissues. The results fit well with the distribution of laminin 5 in the same animal as detected with anti-laminin 5 antibodies.

**DISCUSSION**

The proteolytic processing of laminin 5 in vitro first occurs within domain G4. The eliminated fragment has not yet been identified, but it does not remain disulfide bonded to the major α3 fragment, in contrast to the laminin α2 cleavage product, which does remain covalently associated with processed α2 (20). The loss of at least part of G4 and all of G5 restricts the ability of laminin 5 to bind heparin, consistent with the presence of a putative heparin-binding site in G5. The physiological significance of the loss of heparin binding is unclear, but because this cleavage occurs upon secretion and the fully processed molecule retains cell binding capacity, the lost fragment is not essential to cell binding.

The second cleavage of α3, from 165 to 145 kDa (1), produces a new N-terminal sequence within EGF-like repeat 2 of domain IIIa, near the domain IIIa-II border. The cleavage occurs between disulfide bonded cysteine pairs, so the two cleaved fragments are predicted to not be bridged by a disulfide bond. The 145-kDa α3 chain is not efficiently cleaved by BMP-1 because of a conformational restriction imposed by the presence of domains I and II.

Data summarized herein strongly support the likelihood that the laminin 5 γ2 chain is processed by BMP-1 in vivo. The cleavage sequence fits well with the consensus for procollagen C-propeptide processing by BMP-1. Moreover, incubation of the biosynthetic precursor of laminin 5 with recombinant BMP-1 causes a rapid conversion of the 155-kDa γ2 chain to 105 kDa, and the processing of the 200-kDa α3 to approximately 165 kDa. Cleavage of a recombinant γ2 short arm occurs at the predicted physiological site and appears to occur only at that site. Thus, we are confident that BMP-1 is directly responsible for the processing of γ2, and it may be responsible for direct cleavage of α3. However, in cell cultures of keratinocytes, the processing of α3 precedes that of γ2. Further, the processing of α3 in cell culture occurs efficiently in low calcium containing medium, but the processing of γ2 requires at least 1.0 mM Ca²⁺.

One explanation for the observed processing of α3 by BMP-1 may be that α3 contains a BMP-1 cleavage site, and this site is relatively close to, but C-terminal of, the cleavage site recognized by the physiological enzyme. In that case, the BMP-1 activity would occur within the cleaved region of the G domain, subsequent to the initial processing of α3 and would be undetected. We have examined the α3 G domains for the consensus sequence YXXD, and the close match are the sequences FAVDMQTF, beginning at residue 1391 within the third third of G4, and FGHIDGEKG, beginning at residue 1453, midway through G4. Cleavage between V and D would produce a peptide of approximately 35 kDa, whereas cleavage between H and D would produce a product of 28.6 kDa. Either could reasonably fit the observed data. Another possible explanation of the data might be that BMP-1 activates an enzyme that copurifies with intracellular laminin 5. A third possibility is that BMP-1 is responsible for both processing events but that the α3 chain is cleaved more efficiently than the γ2 chain. The latter possibility is consistent with the observation that in vitro cleavage of recombinant γ2 requires relatively high concentrations of rBMP-1. However, we cannot exclude the possibility that the γ2 may be inefficiently cleaved by rBMP-1 because of a conformational restriction imposed by the absence of domains I and II.

We are gratified to find that the antibodies made against recombinant BMP-1 localize to the basal epithelial cells of fetal bovine skin. This observation supports the likelihood that BMP-1 is involved in laminin 5 processing in vivo, as well as in vitro. Antibody localization to cutaneous epithelial cells was quite weak in neonatal human foreskin but showed much stronger staining in the dermis. These observations are consistent with the developmental timing of basement membrane assembly. Although laminin 5 (37) and type VII collagen (38) are expressed early in the development of the dermal-epidermal junction, prior to extensive epidermal stratification, it is not until the equivalent of the transition from the first to the second human trimester that ultrastructural mature hemidesmosomes, anchoring fibrils, and anchoring filaments are abundant. It is at this time that we see strong localization of BMP-1 to calf basal epithelial cells. By birth, the dermal-epidermal junction is essentially equivalent to that of the adult. Thus, it may not be surprising that the amount of BMP-1 at the dermal-epidermal junction is considerably less at this stage. The local-
ization results reported here are novel and, at first glance, appear to contradict the in situ hybridization results reported for murine BMP-1 RNA by Fukagawa et al. (39) where at 16 days post-coital, expression of BMP-1 is strongest in the embryonic mouse dermis. However, it is quite possible that BMP-1 is synthesized in the dermis but then localizes to the epidermis in a fashion reminiscent of the way in which the mesenchymal basement membrane components localize to the dermal-epidermal junction. If so, it will be of great interest to determine the mechanism for localization of BMP-1. Regardless of the results of such future studies, the results presented in the present report show BMP-1 to have both the proper enzymatic activity and spatiotemporal distribution to process laminin 5 during development and thus play a role in assembly of the basement membrane at the dermal-epidermal junction.

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