Laminin-5, a major adhesive ligand for epithelial cells, undergoes processing of its γ2 and α3 chains. This study investigated the mechanism of laminin-5 processing by keratinocytes. BI-1 (BMP-1 isoform inhibitor-1), a selective inhibitor of a small group of astacin-like metalloproteases, which includes bone morphogenetic protein 1 (BMP-1), mammalian Tolloid (mTLD), mammalian Tolloid-like-1 (mTLL-1), and mammalian Tolloid-like 2 (mTLL-2), inhibited the processing of laminin-5 γ2 and α3 chains in keratinocyte cultures in a dose-dependent manner. In a proteinase survey, all BMP-1 isoenzymes processed human laminin-5 γ2 and α3 chains to 105- and 165-kDa fragments, respectively. In contrast, MT1-MMP and MMP-2 did not cleave the γ2 chain of human laminin-5 but processed the rat laminin γ2 chain to an 80-kDa fragment. An immunoblot and quantitative PCR survey of the BMP-1 isoenzymes revealed expression of mTLD in primary keratinocyte cultures but little or no expression of BMP-1, mTLL-1, or mTLL-2. mTLD was shown to cleave the γ2 chain at the same site as the previously identified BMP-1 cleavage site. In addition, mTLD/BMP-1 null mice were shown to have deficient laminin-5 processing. Together, these data identify laminin-5 as a substrate for mTLD, suggesting a role for laminin-5 processing by mTLD in the skin.

Proteolysis of the extracellular matrix is emerging as a key mechanism in processes such as wound healing and tumor metastasis (1, 2). Although most studies have investigated the role of serine proteases and matrix metalloproteases, members of the astacin and ADAM (a disintegrin and metalloprotease) families have also been implicated in this process (1, 2). Laminin-5, the major component of epithelial basement membranes, is a heterotrimeric protein consisting of α5, β3, and γ2 subunits (3, 4). Laminin-5 undergoes extracellular proteolysis of the α3 chain from a 200- to a 165-kDa form and of the γ2 chain from a 155- to a 105-kDa form (5). Through its interaction with α6β4 (6, 7), α6β4 (8), and α2β1 integrins (9), laminin-5 supports epithelial cell adhesion (3, 10), and migration (11, 12).

Several proteases have been implicated in laminin-5 processing. Exogenous addition of matrix metalloprotease 2 (MMP-2) cleaved the γ2 subunit of rat laminin-5 (12). A subsequent study suggested that membrane type 1 matrix metalloprotease (MT1-MMP) may play a role in cleaving laminin-5 (13). Cleavage of laminin-5 by plasmin converted the α3 chain into the 165-kDa form observed in human breast and rat epithelial cells and capable of nucleating hemidesmosomes (14). Bone morphogenic protein 1 (BMP-1) has also been implicated in laminin-5 proteolysis. N-terminal sequencing of the 105-kDa γ2 chain obtained from human keratinocytes revealed a cleavage site that matched the minimal consensus sequence of this metalloprotease (15). In vitro studies demonstrated that BMP-1 cleaved the recombinant γ2 short arm at the predicted site and that the enzyme cleaved both the α3 and γ2 chains of whole laminin-5 to generate characteristic 165- and 105-kDa fragments, respectively (15). Thus, the proteases reported to cleave laminin-5, only BMP-1 was shown to process both chains, whereas plasmin was reported to process only the α3 chain, and MMP-2 and/or MT1-MMP were reported to process only the γ2 chain of rat laminin-5.

BMP-1, first identified in osteogenic extracts of bone (16), is a metalloprotease of the astacin family (17). It has substantial homology with proteins involved in morphogenetic patterning such as Tolloid in Drosophila and Xolloid in Xenopus, and the mammalian enzyme may function both in patterning and in modifying components of the extracellular matrix (18). The first identified activity for BMP-1 was the cleavage of the C-terminal propeptide from procollagen types I–III in the process of collagen deposition in fibrillogenesis (19). Three additional mammalian enzymes homologically homologous to BMP-1 have been reported: mammalian Tolloid, an alternatively spliced variant of the Bmp1 gene (mTLD) (20); mammalian Tolloid-like 1 (mTLL-1) (21) and mammalian Tolloid-like 2 (mTLL-2) (18). All of these BMP-1 isoenzymes share a similar domain structure comprising an N-terminal prodomain, an astacin-like metalloprotease domain, and several EGF (epidermal growth factor) and CUB domains (a widespread module found in developmentally regulated proteins), thought to be involved in...
mediating protein-protein interactions (17) (Fig. 1). mTLD, mTLL-1, and mTLL-2 also have additional C-terminal EGF and CUB domains not found in BMP-1 (18, 20, 21) (Fig. 1). The overall sequences of these three proteins have between 72 and 75% identity to one another, and identity is even higher in conserved regions such as the metalloprotease domain (18). In addition to procollagen C-protease activity, the BMP-1 isoenzymes have been reported to process prolyl oxidase, probrinogen (22), and procollagen VII to their mature forms (23, 24), and to cleave Chordin (18).

In the present study, we have utilized a novel inhibitor of the BMP-1 family to investigate processing of laminin-5 by the BMP-1 isoenzymes expressed in keratinocytes. We demonstrate that treatment of keratinocytes with this inhibitor significantly reduced the cleavage of both the α3 and β2 chains. A comparison of the enzymatic activities of the four BMP-1 isoenzymes on laminin-5 revealed that all forms are able to cleave both the α3 and β2 chains. However, only mTLD was present in significant quantities, at both the RNA and protein levels, in cultured keratinocytes under the conditions used in the study of laminin-5 cleavage. In addition, a defect of laminin-5 processing was demonstrated in skin lacking mTLD/BMP-1. Interestingly, neither MMP-2 nor MT1-MMP was able to cleave the β2 chain of human laminin-5, although they cleaved the rat chain, most likely reflecting sequence differences between the two proteins. Together, these data suggest a novel role for mTLD in processing laminin-5 in keratinocytes and the skin.

EXPERIMENTAL PROCEDURES

Enzymes and Inhibitors—Plasmin (United States Biological, Swampsport, MA or Calbiochem) and purified MMP-2 and MT1-MMP catalytic domains (Chemicon, Temecula, CA) were purchased from commercially available sources. BI-1 (a kind gift of FibroGen, Inc.), a selective hydroxamic acid inhibitor of BMP-1 and its isoenzymes, was dissolved in Me2SO.

A fluorogenic peptide containing a consensus BMP-1 cleavage site (49) was used to assess the inhibitory activity of BI-1 on BMP-1. In a similar assay, a fluorogenic peptide with the sequence Cmc-L-G-L-Dpa/Dnp-A-R-NH2 (where Mca is 7-methoxycoumarin-4-acetic acid and Dpa is dinitrophenylalanine) (excitation 360 nm, emission 465 nm; Bachem, catalog No. M-1895) was used to measure the inhibitory activity of BI-1 and the appropriate fluorogenic peptide substrate (50 μM); the increase in fluorescence was used to determine initial rates. MMP-2 activity was also verified by examining its ability to digest chicken type I [3H]procollagen at 25 °C, using pro-MMP-2 as a substrate and examining its conversion to the mature form. The digests were analyzed by Western blot as below using antibody Ab-3 (Calbiochem) against MMP-2, which recognizes both the 66- and 72-kDa forms. All control reactions were run in buffer consisting of 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM CaCl2, 0.02% Brij 35.

Protein Analysis—Proteins were separated by SDS-PAGE using NOVEX gels (Invitrogen) and analyzed by Western blot as described previously (26). Goat anti-rabbit or goat anti-mouse secondary antibodies were from Amersham Biosciences and were used according to the manufacturer’s instructions. Blots were developed using a standard ECL kit (Amersham Biosciences) unless indicated otherwise.

Cell Culture—For the cell-based laminin-5 cleavage assay, human primary keratinocytes (NIHEK, BioWhittaker, or Cascade Biosciences, Portland, OR) were grown in Epilife medium (Cascade Biosciences) containing 0.06 mM CaCl2. When testing BI-1 in cell-based assays, we supplemented the medium with extra CaCl2 to a final concentration of 0.3 mM. The α2-deficient keratinocytes, LSV5 cells (27), transfected with plasmids encoding wild-type or α2-pNC mutant α2 chains (a kind gift from Dr. G. Meneguzzi, Nice, France (28)) were cultured in a 1:1 mix of defined keratinocyte serum-free medium (Invitrogen) and Medium 154 (Cascade Biosciences) (29). Rat bladder epithelial 804G cells were cultured as previously described (30). Primary dermal fibroblasts were obtained from BioWhittaker and grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum.

Recombinant BMP-1 Isoenzymes—Purified BMP-1 and conditioned media from cell lines stably expressing FLAG-tagged mTLD, mTLL-1, and mTLL-2 were the kind gifts of FibroGen, Inc. The mTLD, mTLL-1, and mTLL-2 proteins were affinity purified over a FLAG-agarose column essentially as described (16). Concentrations of purified enzymes were assessed using BCA assays and by direct comparison of Coomassie-stained band intensity with protein standards of known concentration. The activity of the purified enzymes was assayed by measuring the cleavage of a fluorogenic peptide containing a consensus BMP-1 cleavage site (49). The ability of BMP-1, mTLD, and mTLL-1 to cleave type I [3H]procollagen was verified as described previously (18).

Antibodies—A laminin-5 polyclonal rabbit antibody (pKal) was raised against laminin-5, purified from human keratinocyte conditioned media by antibody affinity chromatography as described previously (5). Monoclonal antibody 10562 against the β2 chain of human laminin-5 was from Chemicon. The rabbit polyclonal antibody 1084 directed at domains IV and V of the mouse laminin-2 chain (31), was the kind gift of Dr. Rupert Timpl, Martinsried, Germany. The polyclonal antiserum S.E.-85 and S.E.-144 directed against the α3 and β2 chains of laminin-5, respectively (32), were a kind gift from Dr. G. Meneguzzi, Nice, France.

The following affinity purified rabbit polyclonal antibodies raised against peptides from BMP-1 isoenzymes were kind gifts of FibroGen, Inc.: pAb 585 against the BMP-1 C terminus (33), pAb 586 against the mTLD C terminus (33), pAb 531 against the mTLL-1 catalytic domain, pAb 517 against the BMP-1 and mTLD catalytic domain, and pAb TLL2 against the mTLD catalytic domain. The reactivity of each antibody against peptide 1084 was determined by antibody affinity chromatography as described previously (5). Recombinant BMP-1 (49) was used as positive controls and analyzed by Western blot with the panel of anti-BMP-1 isoenzyme antibodies.

Immunoprecipitation of Unprocessed Laminin-5 and Enzyme Assays—For immunoprecipitation, human primary keratinocytes were grown to 70% confluency in six 225-cm² flasks, washed with Hank’s balanced salt solution (BioWhittaker) and Cys/Met-free keratinocyte growth medium (KGM; BioWhittaker), and then starved in the same medium for 30 min at 37 °C. Cells were labeled for 24 h in Cys/Met-free FRM supplemented with 0.1 mM EasyTag EXPRESS (PerkinElmer Life Sciences) protein labeling mix. After labeling, cells were lysed with ice-cold radioimmunoprecipitation assay buffer (5). Cell debris was removed by centrifugation at 14000 × g for 30 min at 4 °C. The supernatant was snap-frozen and stored at -80 °C until its use in immunoprecipitation.

Immunoprecipitation. The supernatant was preclerased with gelatin-Sepharose 4B (Amersham Biosciences) overnight at 4 °C using 100 μl of Sepharose/each ml of supernatant. Precleared supernatant was incubated overnight at 4 °C with protein G-Sepharose beads to which K140 had been preadsorbed. Beads were pelleted, washed, and resus-
In this study we have utilized BI-1, a hydroxamic acid-based selective inhibitor of BMP-1 isoenzymes, to investigate the role of these metalloproteases in laminin-5 processing. BI-1 has IC_{50} values of 6, 2, and 4 nM for BMP-1, mTLD, and mTLL-2, respectively compared with IC_{50} values of greater than 50 µM for MMP-2 and MMP-9.

Under the culture medium calcium concentrations used in this study, keratinocytes produced a mixture of unprocessed and processed forms of both the α3 and γ2 chains of laminin-5 in the extracellular matrix (Fig. 2A, i). When cells were seeded and grown in the presence of increasing concentrations of BI-1 for 72 h, the appearance of the unprocessed 200-kDa α3 and 155-kDa γ2 bands indicated that the inhibition was dose-dependent (Fig. 2A, i). The kinetics of cleavage differed for the two chains. Cleavage of the γ2 chain was 85% inhibited at 0.5 µM BI-1 (Fig. 2A, iii). In contrast, processing of the α3 chain appeared more complex. In the absence of the inhibitor, three bands were apparent: the 200-kDa unprocessed form, the 165-kDa form processed at the C-terminal end of the chain, and a 145-kDa form, which had undergone additional processing in domain IIIA of the N-terminal region (Fig. 2A, ii) (5, 15). These forms comprise 14, 52, and 34%, respectively, of the total α3 (Fig. 2B). The addition of BI-1 inhibits both of these cleavage events, with the 145-kDa band reduced to 9% of the total at 5 µM BI-1, whereas the 165-kDa band still comprised 28% of the total α3 at 50 µM BI-1 (Fig. 2B). This may indicate that the cleavage site in the G-domain, which generates the 165-kDa fragment, is particularly labile and sensitive to proteolysis.

Laminin-5 α3 and γ2 Chains Are Substrates for all BMP-1 Isoenzymes—BMP-1 has been reported to cleave both the α3 and γ2 chains of radiolabeled human laminin-5 immunoprecipitated from keratinocyte cell lysates (15). To determine whether other BMP-1 isoenzymes were also capable of catalyzing this reaction, we tested the ability of the panel of BMP-1 isoenzymes to cleave unprocessed laminin-5. Purified recombinant BMP-1, mTLD, mTLL-1, and mTLL-2 used in the digestions were present as single bands on a Coomassiestained SDS-polyacrylamide gel with molecular weights corresponding to those of the mature proteins from which pro-regions had been proteolytically removed (18) (Fig. 3A). Their proteolytic activity was assayed using a fluorogenic peptide containing a consensus BMP-1 cleavage sequence, and all enzymes were found to have similar activity (Fig. 3A). In addition, the ability of BMP-1, mTLD, and mTLL-1 to cleave type I procollagen as reported previously (18) (data not shown). mTLL-2 also cleaved type I procollagen at a low efficacy, similar to the efficiencies of mTLD and mTLL-1 (data not shown).

Radiolabeled, unprocessed intracellular human laminin-5 was immunoprecipitated from keratinocytes and used as a substrate for digestion. Under the conditions of the assay, unprocessed laminin-5 was completely stable in the absence of added enzyme (Fig. 3B). Digestion of unprocessed laminin-5 with each BMP-1 isoenzyme at 100 nM resulted in the appearance of the 165-kDa processed α3 chain and the 105-kDa processed γ2 band. No smaller molecular weight γ2 chain fragments were observed. The laminin-5 cleavage activities using 100 nM BMP-1, mTLD, mTLL-1, and mTLL-2 were abolished in the presence of 10 µM BI-1, and only the unprocessed α3 and γ2 forms were observed.

mTLD, mTLL-1, and mTLL-2 Cleave the γ2 Chain of Laminin-5 at the BMP-1 Site—BMP-1 has been shown to cleave the human laminin-5 γ2 chain at a site identical to that which generates the 105-kDa fragment found physiologically (15). To determine whether other BMP-1 isoenzymes also cleaved this chain at an identical site, we used a mutant γ2 chain, γ2-pNC, in which the four-amino acid BMP-1 site (YSGD) was deleted (28). This construct was stably transfected into keratinocytes lacking the γ2 chain (LSV5) (27) and was used to produce laminin-5 containing the mutant γ2 chain. Likewise, wild-type γ2 was generated by transfection of LSV5 cells with a plasmid encoding the wild-type γ2 chain. Mutant and wild-type laminin-5 were radiolabeled and immunoprecipitated from these cells and used as substrates for digestion. All BMP-1 iso-
enzymes cleaved the γ2 chain of the wild-type laminin-5, whereas none of the enzymes cleaved the γ2 chain of the mutant laminin-5, indicating that all BMP-1 isoenzymes cleave the γ2 chain at the established BMP-1 site (Fig. 3C).

Cleavage of Laminin-5 by MMP-2, MT1-MMP, and Plasmin—In addition to BMP-1, the metalloproteases MMP-2 and MT1-MMP have been reported to cleave the γ2 chain of laminin-5 (12, 13). To clarify the role of these enzymes in laminin-5 processing and to compare their activity with that of BMP-1, we studied their cleavage activity on both human and rat laminin-5. To verify the activity of MT1-MMP and the specificity of BI-1, we digested pro-MMP-2 to its active form with BI-1 (Fig. 4B).

Recently, it was reported that digestion of soluble human laminin-5 γ2 chain with MT1-MMP generated several bands smaller than 105 kDa (36). In our studies with immunoprecipitated laminin-5, the protein used in enzyme digestion reactions is complexed to Sepharose beads. We therefore examined the ability of MT1-MMP to digest soluble laminin-5. No cleavage of the γ2 chain was seen at molar ratios of MT1-MMP to laminin-5 up to 3:1 (Fig. 4C), confirming our results obtained with the immunoprecipitated laminin-5. We were also unable to demonstrate MMP-2- or MT1-MMP-mediated cleavage of laminin-5 deposited upon tissue culture plastic by human keratinocytes, a substrate that is cleaved by BMP-1 isoenzymes (data not shown).

The lack of a clear 80-kDa (γ2p') laminin-5 band in any of our MMP-2 and MT1-MMP digests is contrary to the findings of previous studies but may be explained by the use of human instead of rat laminin-5, as the two share no identity at the MMP-2 cleavage site (residues 582–589 in rat and 601–608 in human). We therefore examined rat laminin-5 from the extracellular matrix of rat bladder epithelial 804G cells, which contains a laminin γ2 chain typically processed to a mixture of 105

FIG. 2. BI-1 specifically inhibits laminin-5 processing in human keratinocytes. A, keratinocytes were incubated for 72 h with increasing concentrations of BI-1. Extracellular matrix was harvested from cells and probed with the following pAbs: pKal to detect laminin-5 (i), SE85 to detect the α3 chain (ii), and SE144 to detect the γ2 chain (iii). B, SE85 and SE144 blots were analyzed by densitometry. i, unprocessed α3 200-kDa band; ii, processed α3 165-kDa band; iii, processed α3 145-kDa band. ii, processed γ2 165-kDa band.

FIG. 3. BMP-1 isoenzymes digest α3 and γ2 chains of laminin-5. A, BMP-1 isoenzymes were purified as described previously (18). The activity of 0.1 μg of protein was determined using a fluorogenic peptide containing a consensus BMP-1 cleavage site and expressed as % cleavage/h ± S.D. B, radiolabeled, immunoprecipitated human laminin-5 was incubated with 100 nM BMP-1 isoenzymes for 2 h in the presence and absence of 10 μM BI-1. Bands were detected by autoradiography. C, radiolabeled human laminin-5 was immunoprecipitated from LSV5 cells containing either the wild-type γ2 chain (g2) or the BMP-1 site mutant γ2 chain (pNC). Wild-type and mutant proteins were digested for 2 h with 100 nM BMP-1 isoenzymes, and the cleaved γ2 bands (105 kDa) were detected by autoradiography.
or presence of 20 chain (Chemicon). D, rat bladder epithelial 804G cells were grown in the absence or presence of 20 μM BI-1 for 16 h. Cells were removed, and matrix laminin-5 was digested with MMP-2, MT1-MMP, or BMP-1 for 4 h. The matrix was solubilized and laminin-5 detected by Western blot with pKal.

The serine protease, plasmin, was reported to cleave the α3 chain of laminin-5 (14). The addition of 100 nM plasmin cleaved the α3 chain of laminin-5 to the characteristic 165-kDa band (Fig. 4B). The size of this band is indistinguishable from that seen for laminin-5 processed by keratinocytes or for laminin-5 digested by BMP-1, mTLL-1, or mTLL-2. Plasmin also appeared to cleave the γ2 chain to the 80-kDa γ2p form without cleavage to an intermediate form.

The major secreted BMP-1 isoenzyme in cultured human keratinocytes is mTLD—To identify which BMP-1 isoenzyme is secreted by human keratinocytes, we analyzed keratinocyte conditioned media by Western blot. Keratinocytes have been reported to secrete predominantly mTLD in its proenzyme form (Fig. 4B). When concentrated conditioned keratinocyte medium was analyzed using these antibodies, a doublet at ~130 and 140 kDa was detected with pAb 517 and pAb 586 antibodies only (Fig. 5A, lanes 1 and 3). No bands were detected with any of the other antibodies that were not also present in the serum only control (Fig. 5A, lanes 2, 4, and 5). From this pattern of reactivity, we conclude that mTLD is the major BMP-1 isoenzyme found in keratinocyte conditioned media. The molecular masses of the two bands are in the range of the proenzyme, predicted from sequence data to be 143 kDa, and the mature form of mTLD, reported to be 130 kDa (18). A minor 100-kDa band detected with pAb 517 and pAb 586 is likely a degradation product of mTLD.

Expression of RNA levels for the BMP-1 isoenzymes in primary keratinocytes was also assessed using quantitative PCR, with placental and fibroblastic RNA as positive controls for BMP-1 and mTLD. A suitable positive control RNA could not be...
found for mTll-2, but primers were validated using a plasmid containing the mTLD cDNA. In agreement with the detection of only mTLD in conditioned keratinocyte media, this isoenzyme was the most abundant form in keratinocytes, expressed at levels approximately two orders of magnitude greater than BMP-1 and mTLL-1 (Table II). Human dermal fibroblasts also expressed significant levels of mTLD. No mTLL-2 was detected in any of the samples analyzed.

**mTLD/BMP-1-deficient Skin Shows Defective Laminin-5 Processing**—Given that all BMP-1 isoenzymes are capable of cleaving both the α3 and γ2 chains of laminin-5, the presence of mTLD in keratinocyte conditioned media suggests that mTLD may be the enzyme responsible for cleaving laminin-5 in skin. To further evaluate this possibility, we examined mTLD/BMP-1-deficient skin prepared from embryos homozygous null for the Bmp1 gene (37), which encodes both proteases, using pAb 1084 directed at domains IV and V of the laminin γ2 chain. This antibody recognizes only those laminin-5 molecules that contain an unprocessed γ2 chain (38). Increased unprocessed laminin γ2 chain was noted at the dermal-epidermal junction in mTLD/BMP-1-deficient skin compared with wild-type skin in E18 mouse embryos by IDIF microscopy (Fig. 6, A and C). In contrast, expression of total laminin-5, detected by the pKal pAb, was similar in both samples (Fig. 6, B and D). As a next step, we examined the localization of laminin-5 in mTLD/BMP-1-deficient and wild-type skin using immuno-electron microscopy. As with IDIP studies, increased unprocessed extracellular laminin γ2 chain was noted in mTLD/BMP-1-deficient skin (Fig. 6, F and G) relative to wild-type skin (Fig. 6E). Extracellular unprocessed laminin-5 appeared to localize predominantly to the lamina densa. Although blister formation was not clinically apparent in mTLD/BMP-1-deficient mouse skin, at the ultrastructural level the lamina densa was often not well opposed to overlying hemidesmosomes, and hemidesmosomes were often rudimentary in appearance in mTLD/BMP-1-deficient skin (e.g. Fig. 6F). When complete separation of the epidermis and dermis was noted in deficient skin, it appeared to occur in the plane of the lamina lucida with unprocessed laminin γ2 chain localizing to the dermal side of the skin separation (e.g. Fig. 6G).

**DISCUSSION**

The selective inhibitor BI-1 has facilitated the study of laminin-5 processing in vitro. BI-1, at the concentrations used, inhibits the BMP-1 isoenzymes but does not inhibit other metalloproteases such as MMP-2 and MT1-MMP, and thus it functions as a tool for distinguishing the effect of these enzymes in cellular processes. *In vitro* digestion assays of unprocessed laminin-5 with BMP-1 isoenzymes, analysis of conditioned keratinocyte media with a panel of antibodies against these isoenzymes, and quantitative PCR studies together suggest that mTLD mediates laminin-5 cleavage in primary keratinocytes.

Interestingly, the α3 and γ2 chains are not equivalent in terms of sensitivity to proteolysis. Processing of the γ2 chain was inhibited at substantially lower concentrations of BI-1 than either of the α3 chain cleavages. Similarly, in cell culture there is a lag in γ2 processing relative to α3 processing (5, 15). The kinetics of this cleavage may reflect a greater accessibility of the protease to the α3 chain cleavage site than to the γ2 cleavage site or the fact that cleavage of the α3 chain cooperatively enhances γ2 chain cleavage. The physiological significance of these kinetics may be linked to the differing roles played by the individual chains in influencing cellular processes such as adhesion, migration, and matrix assembly.

BMP-1 isoenzymes have been shown to be generally similar in their substrate specificities, with all enzymes capable of processing pro-lysyl oxidase (18) and procollagen (16) (and this report) to their mature forms. It is probable that these similarities reflect the need for redundancy in the biological functions of the BMP-1 isoenzymes. This was demonstrated previously in *Bmp1* knockout mice in which deficiency of BMP-1 and mTLD...
did not prevent processing of type I and type VII procollagens, indicating the involvement of several isoforms in these activities (24, 37). The similarity of the BMP-1 isoenzyme activities is likewise indicated by the fact that all enzymes are processed at the y2-chain at the same established BMP-1 cleavage site.

The presence of mTLD, and the apparent absence of the other BMP-1 isoforms in keratinocyte media by Western blot analysis, as well as the predominant expression of mTLD in primary keratinocytes and fibroblasts by quantitative PCR analysis all suggest that mTLD is likely active in cleaving laminin-5 in vivo. TGFβ1 stimulation was not required to detect the mTLD bands in keratinocyte media in this study despite a previous report that the unprocessed form of mTLD is detected only in response to TGFβ1 (33). The differing results could reflect the 3–5-fold greater amount of conditioned media loaded in the current study than in the previous report (33), as no specific bands were detected when less than this amount of media was analyzed.

BMP-1 and laminin-5 were previously shown to co-localize at the dermal-epidermal junction of fetal calf skin (15). The polyclonal antibody used in that study was raised against BMP-1 but is reactive against both BMP-1 and mTLD.3 Furthermore, BMP-1/mTLD localized to basal keratinocytes in normal human skin, whereas mTLL-1 expression was absent in basal keratinocytes (24). These results are consistent with a role for mTLD in the dermal-epidermal basement membrane and, coupled with our findings of defective laminin-5 processing in mTLD/BMP-1-deficient mice, support the hypothesis that mTLD is involved with the processing of the laminin-5 y2 and α3 chains in vivo.

Previous studies have shown that MMP-2 (12) and MT1-MMP (13) each cleaved the laminin-5 y2 chain, in apparent contradiction to our study. However, these previous studies used rat laminin-5, whereas our study examined human laminin-5. The sequence of the rat y2 chain at the MMP-2 cleavage site (residues 582–589) (12) has very low identity with the corresponding region of the human protein (residues 601–608), which likely explains the observed lack of cleavage of human laminin y2 chain by MMP-2 in our experiments. It is quite possible that MMP-2 and MT1-MMP cleave the same uncon-

3 D. S. Greenspan, unpublished data.  

served site on the rat laminin y2 chain, because each enzyme yields a similar 80-kDa product; however, the sequence of the MT1-MMP cleavage site has not been reported. Although MMP-2 and MT1-MMP cleaved rat laminin y2 chain to the 80-kDa fragment, neither enzyme, even at concentrations as high as 300 nM, could convert laminin-5 y2 chain to the 105-kDa fragment (12, 13), which is the only processed product of the laminin y2 chain found in human tissues (5).

MMP-2 and MT1-MMP are known to be expressed in human keratinocytes (39–42). However, laminin-5 processing in keratinocyte cultures was completely inhibited by BI-1, which we have shown to have no effect on MMP-2 or MT1-MMP activity. These findings, coupled with the observation that the sequence of the human y2 chain in vivo cleavage site exactly matches that of the BMP-1-cleaved y2 chain in vitro (15), suggest that neither MMP-2 nor MT1-MMP is likely to play a role in processing the human laminin y2 chain. Interestingly, the addition of BI-1 to rat 804G cells inhibited the generation of the y2p but not y2p' fragments, which suggests that one of the BMP-1 isoenzymes may be cleaving rat laminin-5 in addition to MMP-2 or MT1-MMP in these cells. The relative contribution of each of these enzymes to laminin-5 cleavage in the rat model is an area deserving of further investigation.

Although neither MMP-2 nor MT1-MMP cleaved the human laminin y2 chain, both enzymes, as well as all other proteases examined in this and other reports, were able to cleave the α3 chain to a fragment indistinguishable in size from the 165-kDa product observed in the extracellular matrix of cultured keratinocytes. This panel included a serine protease, plasmin, and members of the astacin and matrix metalloprotease families. This suggests that the α3 cleavage site lies on a portion of the chain that is highly sensitive to proteolytic attack, consistent with electron micrograph data that places the cleavage site in an accessible spacer region between globular domains G3 and G4 (43). Recently, the C-terminal peptide released after cleavage of the 200-kDa α3 chain was isolated from the conditioned medium of human keratinocytes (44). The N-terminal sequence of this fragment suggested that a member of the MMP, astacin, or ADAM families could act as the processing enzyme of this chain (44). In a separate report, partial sequencing of two fragments of 35 and 37 kDa released by cleavage of the α3 chain indicated that cleavage occurred in the region between the G3 and G4 domains (45). Although MMP-2 and MT1-MMP do cleave the α3 chain in our in vitro assays, this may not be physiologically relevant, as these enzymes are not inhibited by BI-1, which prevents most laminin-5 processing when added to keratinocyte cultures. This observation does not preclude a role for these enzymes in the upstream activation of another protease involved in cleavage of the α3 chain. Our results hint at a heretofore unsuspected complexity of laminin-5 processing in which different enzymes may cleave the α3 and y2 chains and in which α3 chain processing itself may involve more than one protease.

The involvement of proteases of the MMP family and plasmin in modifying the extracellular matrix in the process of carcinoma invasion is well established (1, 2). It has been suggested that members of the astacin family may also play a role in these processes (1). Proteolytic modification of laminin-5 structure has been shown to be an important process underlying keratinocyte migration, wound healing, and tumor invasion. In particular, increased expression of laminin-5 is often observed at the margins of squamous cell carcinoma tumors (46–48), and thus processing of such laminin-5 by BMP-1 isoenzymes may be a critical step in invasion. Therefore, specific inhibitors of BMP-1 isoenzymes, such as BI-1, may prove to be invaluable in the study of laminin-5 processing in squamous cell carcinoma invasion.

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