BMP-1/Tolloid-like Metalloproteases Process Endorepellin, the Angiostatic C-terminal Fragment of Perlecan*

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Endorepellin, the C-terminal domain of the heparan sulfate proteoglycan perlecan, possesses angiostatic activity. The terminal laminin-like globular (LG3) domain of endorepellin appears to possess most of the biological activity on endothelial cells. LG3 protein has been detected in the urine of patients with end-stage renal disease and in the amniotic fluid of pregnant women with premature rupture of fetal membranes. These findings suggest that proteolytic processing of endorepellin and the generation of LG3 might have biological significance.

In this study, we have identified specific enzymes of the bone morphogenetic protein-1 (BMP-1)/Tolloid family of metalloproteases that cleave LG3 from recombinant endorepellin at the physiologically relevant site and that cleave LG3 from endogenous perlecan in cultured mouse and human cells. The BMP-1/Tolloid family of metalloproteases is thereby implicated in the processing of a major basement membrane proteoglycan and in the liberation of an anti-angiogenic factor. Using molecular modeling, site-directed mutagenesis and angiogenic assays, we further demonstrate that LG3 activity requires specific amino acids involved in Ca²⁺ coordination.

Perlecan is a proteoglycan widely expressed during early stages of development, in most vascularized tissues, and in the avascular cartilage (1–3). It plays significant roles in vasculogenesis and tumor angiogenesis (4, 5). The unique modular structure of perlecan allows it to interact with other basement membrane components and various growth factors during vasculogenesis (1, 6). Genetic evidence of roles for perlecan in vasculogenesis derives from the complex phenotype of perlecan null mice, most of which perish at day 10.5 of embryogenesis due to increased blood vessel pressure (7). Shortly after birth, surviving mice die with extreme vascular and cephalic abnormalities (7, 8), as well as a high incidence of cardiac outflow malformations (9).

The perlecan protein core is a large, modular protein of ~470 kDa composed of five structural domains (10). The 85-kDa C-terminal domain V, renamed endorepellin in light of its recently discovered angiostatic activity (11), is the major cell-binding domain. The structure of endorepellin is organized in a pattern akin to the LG³ domain repeats of agrin and laminin, i.e. a sequential series of LG domains, which, in the case of endorepellin, are interconnected by short epidermal growth factor-like repeats (12). Indeed, endorepellin displays binding affinities similar to those reported for agrin and various laminins (13, 14).

We have recently discovered that the C-terminal laminin-like globular (LG3) domain of endorepellin harbors most of its anti-angiogenic activity (15). Notably, LG3 fragments cleaved at the amino acid residues identical to those generated in vitro by 293-EBNA cells in either human (11) or mouse (13, 14) have been detected in the urine of patients with end-stage renal disease (16) and in the amniotic fluid of pregnant women with premature rupture of fetal membranes (17). These findings suggest that proteolytic processing of endorepellin and the generation of LG3 might have biological significance. In this study, we demonstrate that enzymes of the bone morphogenetic protein 1 (BMP-1)/Tolloid-like family of metalloproteases specifically liberate LG3 by cleaving endorepellin between Asn⁴¹⁸⁶ and Asp¹⁹⁷, the same site at which cleavage produces LG3 in vivo. Moreover, such proteinases also directly liberate LG3 from endogenous perlecan in cultured mouse and human cells. Mutation of Asp¹⁹⁷ prevents processing of endorepellin, further confirming the specificity of this cleavage site. To better understand the molecular determinants of LG3 biological activity, we have generated a three-dimensional model and performed site-directed mutagenesis of key amino acid residues involved in Ca²⁺ coordination. Manipulation of this site reduced the ability to bind Ca²⁺ and abrogated LG3 activity on endothelial cells.

EXPERIMENTAL PROCEDURES

Proteolytic Processing by BMP-1/Tolloid-like Proteinases—For in vitro cleavage assays, purified recombinant endorepellin/domain V (11) was incubated with recombinant human BMP-1, mammalian Tolloid (mTLD), and mammalian Tolloid-like-1 and -2 (mTLL-1 and mTLL-2) with C-terminal FLAG-tags that had been produced in 293-EBNA cells, purified on anti-FLAG affinity columns, and quantified as described (18). Cleavage assays involved overnight incubation of ~0.6 pmol endorepellin with ~0.3 pmol of protease at 37 °C (18). For analysis of LG3 production by cultured cells, mouse embryo fibroblasts (MEFs) were prepared from 13.5 days after conception from either wild type or

1 The abbreviations used are: LG, laminin-like globular; BMP-1, bone morphogenetic protein-1; mTLD, mammalian Tolloid; mTLL-1 and mTLL-2, mammalian Tolloid-like-1 and -2; HUVEC, human umbilical vein endothelial cell; MEF, mouse embryonic fibroblast; Ad-ER, adenovirus-endorepellin; PBS, phosphate-buffered saline; EBNA, Epstein-Barr nuclear antigen.
collagen-coated chamber slides (Nalge Nunc) in complete medium (11). HUVECs (passages 4–8) were cultured on

Following drying, the membranes were exposed to x-ray films for 18–24 h. Images were processed with Adobe PhotoShop 7.0 and analyzed

Biomedicals) for 1 h at 5° C, washed with the same buffer for 5 min, in the same buffer containing 1 mCi of45Ca2+

were used at dilutions of 1:2000 and 1:20,000, respectively. N-terminal sequences were determined by automated Edman degradation at the Harvard University Microchemistry Facility. Preparation of adenosvirus-endorepellin (Ad-ER) and transaction experiments were performed as described previously (15).

Comparative Modeling of Endorepellin LG3 and Mutant Design—The LG3 domain was aligned to the sequence of the murine laminin α2LG5 module (24), and then the 3D structure was modeled using the Bioworks software package. The model structure was assessed for quality with ANOLEA (25) and the Swiss-PDB Viewer (27). Placement and refinement of the calcium ion and subsequent mutations were performed using the Swiss-PDB Viewer. All structures were analyzed using the Swiss-PDB Viewer and Chimera (29).

Expression and Purification of Recombinant Proteins and Mutant Species—We cloned either the full-length endorepellin or its terminal LG3 module (G1192–S4391) into the pcDNA3-Pu vector containing the BmA signal sequence and a C-terminal His tag. This vector was used as a template to introduce Ile or Ala point mutations with the QuikChange® XL site-directed mutagenesis kit (Stratagene). All the mutant constructs were sequenced and stably transfected into human embryonic kidney 293-EBNA cells. Transfectants were selected in medium containing G418 (250 μg ml−1) and puromycin (500 ng ml−1) for at least 4 weeks. Isolation and purification of recombinant protein was as described previously (11). Protein purity was assessed by Coomassie Blue staining, and identity and size were verified by SDS-PAGE and immunoblotting using either anti-endorepellin or anti-His tag antibodies.

Circular Dichroism Spectroscopy and 4Ca2+ Overlay Assays—CD spectra were recorded at 22 °C using a Jasco J-500c spectropolarimeter. The path length of the CD cells was 0.1 mm, and CD was expressed in terms of ellipticity [θ] in degree-cm2-dmol−1. Samples were dialyzed into various concentrations (0.5–1 mg ml−1) and analyzed. At least four scans were performed for each spectrum.

The ability of the recombinant LG3 and its mutants to coordinate Ca2+ was tested by 4Ca2+ overlay assay (30). Different protein concentrations were slot-blotted onto nitrocellulose membranes. The membranes were washed in PBS at different pHs (7.4, 6.5, and 5.5). To remove any possible Ca2+ contaminants, the buffers were pre-eluted through a Chelex® (Bio-Rad) column. The membranes were incubated in the same buffer containing 1 mM 4Ca2+ (13 mM mg−1 Ca, MP Biomedicals) for 1 h at 25 °C, washed with the same buffer for 5 min, and then washed again with 50% ethanol (v/v) for another 5 min. Following drying, the membranes were exposed to x-ray films for 18–24 h. Images were processed with Adobe Photoshop 7.0 and analyzed using NIH Image 1.59 software.

Effects of LG3 Mutations on Endothelial Cell Actin Cytoskeleton and Capillary Morphogenesis—HUVECs (passages 4–8) were cultured on collagen-coated slides (Nalgene) in complete medium (11). After serum starving, the cells were treated, in a blinded fashion, with 50 nM LG3, mutants D4258A, N4327A, the double mutant D4258A/N4327A, or PBS for 10 min. Cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 3 min, and stained with rhodamine-phalloidin (to visualize actin stress fibers) and 4',6-diamidino-2-phenylindole (Sigma). Images were captured with an Olympus DP12 microscope and a SPOT CCD camera and processed with Adobe Photoshop 7.0. Actin stress fibers were assessed from 100 randomly selected cells in a blinded fashion. For capillary morphogenesis, proteins were used at dilutions of 1:2000 and 1:20,000, respectively. N-terminal sequences were determined by automated Edman degradation at the Harvard University Microchemistry Facility. Preparation of adenosvirus-endorepellin (Ad-ER) and transaction experiments were performed as described previously (15).

RESULTS

BMP-1/Tolloid-like Metalloproteases Process Endorepellin—Purification of recombinant metalloprotease (~85 kDa) from 293-EVNA cells often showed a second band of ~26 kDa when purified protein was separated on reducing acrylamide gels (11). Because this product was formed during purification procedures, we speculated that it could be a natural cleavage product of endorepellin produced by endogenous proteases. Amino acid sequencing of the N terminus of the 26-kDa product showed that it resulted from cleavage immediately before the third LG domain of endorepellin, between Asn4196 and Asp4197. The sequence surrounding this putative natural cleavage site (SGGN↓DAPQKY) was searched against the MEROPS data base (merops.sanger.ac.uk) (31) for specific proteases that could cleave this bond. Only two proteases (out of >1800 known protease sequences in the MEROPS data base) were found with the required specificity: the two related metalloproteases, BMP-1 (Merops identifier M12.005) and mTLL-1 (Merops identifier M12.016). To determine whether BMP-1/Tolloid-like proteases were capable of cleaving the LG3 domain from precursors, BMP-1 was incubated with recombinant endorepellin. As mentioned above, recombinant endorepellin contained small quantities of the 26-kDa LG3 that, due to a C-terminal His tag (11, 15), co-purified with full-length endorepellin (Fig. 1A, lane 1). Although the purified endorepellin was stable when incubated alone at 37 °C for 18 h (Fig. 1A, lane 2), incubation with BMP-1 resulted in cleavage to produce an ~60-kDa fragment, representing the N-terminal portion of the molecule (which includes LG1–LG2 and the four epidermal growth factor repeats) and additional quantities of free 26-kDa LG3 domain (Fig. 1A, lane 3). In agreement with these findings, we found that the 60-kDa fragment reacted with an antibody directed against the C-terminal His tag, which should had been lost following cleavage (not shown). Moreover, N-terminal sequencing of the 26-kDa band cleaved by BMP-1 gave the single sequence 4195DAPQKYGAYFHY4208. This sequence is identical to that of free LG3 domain produced by transfected 293-EBNA cells and identical to the sequence of the free LG3 domain found in the urine of patients with end-stage renal disease (16).

Each of the four mammalian BMP-1/Tolloid-like proteases was incubated with endorepellin to determine which might be capable of cleavage to produce free LG3. BMP-1 showed the highest levels of endorepellin-processing activity, with lesser levels shown by mTLL-1 and mTLD (Fig. 1B). The lowest levels of processing were observed for mTLL-2. In fact, reactions with mTLL-2 required exposures of blots longer than that shown in Fig. 1 for detection of the 60-kDa band (not shown).

To determine whether BMP-1/Tolloid-like proteases might be responsible for the proteolytic processing of perlecan in live cells, we examined conditioned medium of MEFs derived from either wild type or mutant embryos doubly null for the Bmp1 gene, which encodes alternatively spliced mRNAs for BMP-1 and mTLD, and the TflI gene, which encodes mTLL-1. A 26-kDa protein species was readily detectable with anti-endorepellin antibody, but it was not recognized by an antibody directed against the C-terminal His tag, which should had been lost following cleavage (not shown). Moreover, N-terminal sequencing of the 26-kDa band cleaved by BMP-1 gave the single sequence 4195DAPQKYGAYFHY4208. This sequence is identical to that of free LG3 domain produced by transfected 293-EBNA cells and identical to the sequence of the free LG3 domain found in the urine of patients with end-stage renal disease (16).

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BMP-1 Processing of Endorepellin

Fig. 1. Cleavage by BMP-1-like proteases produces the 26-kDa LG3 in vitro and in vivo. A, Western immunoblotting of recombinant endorepellin starting material (~0.6 pmol, lane 1) or recombinant endorepellin incubated for 18 h either by itself (~0.6 pmol, lane 2) or with BMP-1 (~0.3 pmol, lane 3). The membranes were reacted with an antiendorepellin polyclonal antibody. B, Western immunoblotting of recombinant endorepellin incubated alone (Control) or with BMP-1, mTLL-1, mTLL-2, or mTLD, as indicated. C, conditioned media samples of either wild type or doubly homozygous MEFs null for the Bmp1 and Tll1 genes (Bmp1Tll1 null). To show similar levels of loaded samples in each lane of the upper panel of C, samples identical to those of the corresponding upper panel were subjected to immunoblotting analysis using antisera specific for the unreprocessed endorepellin (indicated by α-PDQPE). α-ER, endorepellin antibody. D, Western immunoblotting of media conditioned by WiDr human colon carcinoma cells treated with control medium, Ad-Empty, or Ad-ER as indicated. Duplicate dishes (~5 × 10⁶ cells) were transduced with ~10⁶ plaque-forming units of either Ad-Empty or Ad-ER for 48 h or left untreated. Two days later, comparable aliquots of conditioned media were harvested and processed for immunoblotting using an anti-endorepellin antibody. Notice the presence of LG3 (~26 kDa) in all samples, whereas endorepellin (~85 kDa) is present only in the Ad-ER-transduced cells.

Mutation of Asp4197 Prevents Cleavage of Endorepellin by BMP-1—The sequence of the cleavage site (SGGN↓DAPGQY) is highly conserved between the human and mouse endorepellin, and it has been previously shown that Asp but not Asn is crucial for proteolytic processing of murine perlecian domain V/endo-repellin (14). To test this, we mutated Asp4197 into lle (D4197I) and tested the recombinant protein for biological activity in HUVECs. Both the wild type and mutant species disrupted the actin cytoskeleton at equimolar amounts (200 nM for 10 min) (Fig. 2A), indicating that mutation of this residue did not significantly disrupt its activity. Next, we transiently transfected 293-EBNA cells with either wild type or D4197I mutant cDNAs. The 48-h conditioned media showed the presence of LG3 in the wild type, likely produced by endogenous BMP-1/Toll-1-like proteinases, but not in the mutant species (Fig. 2B). Consistent with these findings, recombinant BMP-1 was not capable of releasing LG3 from D4197I endorepellin under the same experimental conditions in which it was fully active with the wild type endorepellin (Fig. 2C). Thus, we conclude that Asp4197 is essential for BMP-1 processing of human endorepellin.

Three-dimensional Molecular Model of Endorepellin LG3 Module and Mutational Analysis—LG3 domain was aligned with the murine laminin α2LG5 domain (extracted from Protein Data Bank number 1dyk) (24). The alignment showed 24% identity (Fig. 3A), typical of homology among LG domains (25). Although LG modules possess only modest sequence identity, they are predicted to be well conserved structurally and display certain conserved sequence elements, in particular a conserved pair of Cys residues and a core of hydrophobic residues that correspond to β-strands of α2LG5 (25). The recent crystal structure of the tandem laminin α2LG4–LG5 domains (24) demonstrates the predicted conservatism of structure. Indeed, laminin α2LG4 and LG5, despite possessing only 24% sequence identity (much like LG3–α2LG5 alignment), superimpose structurally with a Cα root mean square deviation of only 1.1 Å, using 139 of 181 Cαs for the superposition. This pattern of low overall sequence identity but conserved structure is evidenced in structural comparisons of other LG domain-containing proteins. For instance, Protein Data Bank number 1h30 and Protein Data Bank number 1kdz possess sequence identities similar to the LG3/1dyk alignment yet superimpose with each other with only 1.08 Å root mean square deviation over a combined total of 540 Cαs used for analysis. The predicted structural similarity among LG modules combined with sequence alignments and known crystal structures of prototypical LG domains (24, 26) allowed us to construct a comparative model of the endorepellin LG3 domain based on the α2LG5 structure. The comparative model (Fig. 3B) consists of a β-sandwich or jellyroll composed of 14 antiparallel β strands arranged in two sheets (24, 26). Areas of predicted secondary structure in our LG3 model (Fig. 3A, arrows) are also areas of high identity/homology between the LG3 model and the α2LG5 template structure. Additionally,
binding site mutations in the H9251 nate Ca2+ corresponding position in the template structure, can still coordi-
only in the wild type endorepellin.

transiently transfected with either wild type endorepellin or its mutant following an 18-h incubation with recombinant BMP-1. The exper-
imental conditions are the same as in Fig. 1

Bar,6-diamidino-2-phenylindole.

0.1% Triton X-100, and stained to visualize actin stress fibers with

BMP-1.

This provides a molecular explanation for the lack of LG3 affinity to heparin

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showing any appreciable effects.

Only wild type LG3 caused a significant disassembly of the

actin stress fibers (p

0.001, Fig. 5) with none of the mutants

Comparable values were obtained at pH 7.4, whereas binding at pH 5.5 was barely detectable (not shown). Thus, mutation of either a single or a double amino acid residue in the Ca2+
pocket impairs binding to 45Ca.

LG3 Mutants Become Non-functional—We have recently shown that endorepellin LG3 domain disrupts endothelial cell

actin stress fibers and focal adhesions and that this activity is

highly dependent on proper extracellular Ca2+ concentration (15). Because cytoskeletal dynamics play a key role in angiogenesis, we tested whether mutations of the Ca2+-binding site would alter LG3 biological activity. Toward this end, HUVECs

cells were grown to subconfluency on collagen I and treated with equimolar amounts (50 nM) of wild type LG3, D4258A, N4327A, or D4258A/N4327A for 10 min in a blinded assay. Only wild type LG3 caused a significant disassembly of the

actin stress fibers (p < 0.001, Fig. 5) with none of the mutants showing any appreciable effects.

Next, we investigated whether mutations of the Ca2+-coordinating site would affect capillary morphogenesis on Matrigel. HUVECs (104) were treated with PBS and LG3, D4258A, N4327A, or D4258A/N4327A (50 nM each) for 24 h in 1% serum. In this assay of angiogenesis, only wild type LG3 caused an appreciable disruption of capillary morphogenesis (Fig. 6).
Quantification of the mean tube length showed a significant ($p < 0.001$) inhibition by wild type LG3. Similar results were obtained by quantifying the mean tube area (not shown). Collectively, these results indicate that disruption of the Ca$^{2+}$-coordinating site within LG3 is responsible for loss of function and block of its anti-angiogenic activity.

**DISCUSSION**

We report for the first time that enzymes belonging to the BMP-1/Tolloid-like proteinase family specifically cleave the C-terminus of perlecan protein core and present genetic evidence that such cleavage is likely to occur in vivo. Thus, cleavage by BMP-1/Tolloid-like proteinases could serve to liberate the LG3 module from basement membranes, cell surfaces, and extracellular matrices in tissues. Given the size of its monomeric protein core ($\sim$470 kDa), it is not surprising that sites susceptible to in vitro proteolytic processing have been detected in mammalian perlecan (34, 35). Proteolytic processing by BMP-1/Tolloid-like is quite specific since these proteases cleave at a single site in the entire 85-kDa endorepellin molecule. Moreover, this processing is readily demonstrated in cell-free experiments using recombinant substrates and proteases, as well as by using mutant cells that show an absence of such processing in the absence of functional alleles for the genes of BMP-1/Tolloid-like proteinases. We further demonstrate by site-directed mutagenesis that Asp$^{4258}$ is essential for BMP-1 processing of human endorepellin, and we predict that this will also occur in the murine endorepellin because of the fully conserved sequence in and around the cleavage site. Previous studies have shown that BMP-1/Tolloid-like proteinases play a key role in the formation of various extracellular matrices by directly processing to maturity various collagens (18, 20, 36–38), key enzymes such as lysyl oxidase, basement membrane components such as laminin 5, and proteoglycans such as biglycan (39, 40). In this study, we expand the range of activities specifically generate free LG3 modules. Rather, these cleavages occurred in upstream regions, to produce fragments containing portions of domains III and IV (34, 35). Proteolytic processing by BMP-1/Tolloid-like is quite specific since these proteases cleave at a single site in the entire 85-kDa endorepellin molecule. Moreover, this processing is readily demonstrated in cell-free experiments using recombinant substrates and proteases, as well as by using mutant cells that show an absence of such processing in the absence of functional alleles for the genes of BMP-1/Tolloid-like proteinases. We further demonstrate by site-directed mutagenesis that Asp$^{4258}$ is essential for BMP-1 processing of human endorepellin, and we predict that this will also occur in the murine endorepellin because of the fully conserved sequence in and around the cleavage site. Previous studies have shown that BMP-1/Tolloid-like proteinases play a key role in the formation of various extracellular matrices by directly processing to maturity various collagens (18, 20, 36–38), key enzymes such as lysyl oxidase, basement membrane components such as laminin 5, and proteoglycans such as biglycan (39, 40). In this study, we expand the range of activities...
similar structure with a minimum peak at 212–214 nm (\textit{H}1,002 to and nuclei with 4,6-diamidino-2-phenylindole. (\textit{H}1,032 and stained to visualize actin stress fibers with rhodamine-phalloidin for 10 min as indicated, fixed, permeabilized with 0.1% Triton X-100, in overlay assays. Binding of 45Ca to recombinant protein was examined using autoradiography and quantification by NIH image software. The HUVECs were treated with various recombinant proteins (50 nM) LG3. (D4258A, N4327A, and D4258A/N4327A). Lanes were loaded with 2–5 gene products could conceivably play a role in the course of predicted from the molecular modeling. All spectra were corrected using PBS alone. 26 kDa). calibrated with standard proteins. The migration is similar in all lanes (\textit{H}1,015/260 degrees cm2 dmol\(^{-1}\)). The curves present typical \(\beta\)-sheet spectra, as predicted from the molecular modeling. All spectra were corrected using PBS alone. C, specificity of 45Ca binding activity by LG3 and its mutants in overlay assays. Binding of 45Ca to recombinant protein was examined using autoradiography and quantification by NIH image software. The experiment shown was performed at pH 6.5. Similar results were obtained at pH 7.4, whereas no substantial binding was detected at pH 5.5 (not shown). The values represent the mean \(\pm\) S.E. of three determinations using 25–50 pmol of each protein.

We provide insights into the nature of the angiostatic LG3 fragment via characterization of a series of LG3 mutants. Evidence is provided that our recombinant mutant proteins maintain proper folding, based on superimposable CD spectra. Nevertheless, we show that all three mutants exhibit a markedly reduced affinity for 45Ca, as determined by overlay assays. This provides a mechanistic explanation for our previous findings that demonstrated a loss of most endorepellin/LG3 activity by reducing extracellular Ca\(^{2+}\) concentrations (15). In support of our conclusions, a crystal structure of laminin \(\alpha2LG4–LG5\) harboring a double mutation in the Ca\(^{2+}\) coordinating residues, much like our double LG3 mutant, did not contain Ca\(^{2+}\) and was essentially isomorphous to the wild type structure. However, it lost Ca\(^{2+}\) binding and its ability to interact with its major cell surface receptor, \(\alpha\)-dystroglycan (32). Thus, it appears that for several LG modules of various extracellular matrix proteins, coordination of Ca\(^{2+}\) ions is essential for full biological activity. Consistent with our findings is the observed Ca\(^{2+}\)-dependent binding of \(\alpha\)-dystroglycan to the C termini of perlecan and agrin (43, 44). The greatest affinity for \(\alpha\)-dystroglycan was observed for domain \(\alpha\) endorepellin \(K_d\) = 3 nM). Although fragments encompassing LG1–LG2 of endorepellin still bound \(\alpha\)-dystroglycan with relatively high affinity \(K_d\) = 40 nM), LG3 showed a near complete absence of binding \(K_d\) = 2 \(\mu\)M). Moreover, we discovered that endorepellin’s LG2, but not LG3, binds specifically to endostatin (11). Thus, LG3 differs in its functionality from other LG modules, and it may be relatively free from binding to two major basement membrane and cell surface constituents, namely collagen XVIII/endostatin (11) and \(\alpha\)-dystroglycan (45). Thus, it is conceivable that this domain is more accessible to BMP-1/Tolloid-like proteinases for processing and release into tissues and circulation.

The successful production of several recombinant LG modules derived from various extracellular matrix proteins and the resolution of their crystal structures as single or paired LGs
(24, 26, 32), have demonstrated that LG modules represent autonomously folding units. The evidence for the endogenous generation of LG3 from endorepellin/perlecan and its existence in vivo is overwhelming. First, in our original study, we noticed that LG3 is easily generated in the 293-EBNA mammalian cell system, particularly when protease inhibitors are reduced or absent (11). N-terminal sequencing of the proteolytically processed LG3 fragment showed that it had been cleaved at a single site precisely between Asn4196 and Asp4197. Second, an identical product was generated from 293-EBNA cells expressing murine endorepellin (14) with a cleavage site between Asp3515 and Asp3516. The sequence around the cleavage site is fully conserved across species, and mutational analysis showed that Asp3515 but not Asn3514 is crucial for processing (14).

Third, an LG3 fragment identical to that generated in the 293-EBNA cell system is found in the urine of patients with end-stage renal disease (16). Because these patients undergo hemodialysis and have essentially no functional kidneys, the LG3 found in their urine could derive from circulating LG3, and it could circulate at relatively high concentrations, insofar as urinary levels of LG3 reached 10 mg liter−1 of urine (16).

Finally, LG3 fragments have been reported to occur in the amniotic fluid of pregnant women with premature rupture of fetal membranes (17), suggesting that LG3 production might be involved in the pathogenesis of this syndrome. Importantly, the N terminus of LG3 fragments found in all of these biological systems is identical to the LG3 N terminus, shown here to be produced via cleavage by the BMP-1/Tolloid-like proteases. In the near future, we plan on pursuing studies with antibodies specific for the cleaved LG3 domain, so as to be able to detect in vivo nascent free cleaved LG3. Additional studies with fluorescent fusion protein constructs will allow for tracking on endorepellin/LG3 on the cell surface in vitro and in tissues in vivo. Purification of both LG3 and of the non-cleavable LG3 D3515I mutant will allow for further structural studies and will likely confirm the validity of the comparative LG3 model, in fold and in calcium coordination, if not in more detail.

In conclusion, we have demonstrated that perlecan protein core, a key constituent of basement membranes and cell surfaces, can be subjected to limited proteolysis by BMP-1/Tolloid-like proteases. The specific cleavage liberates an angiostatic fragment, LG3, from the parent endorepellin molecule; this can occur both in cell-free experiments as well as with live cells and requires Ca2+ coordination. The generation of such processed forms of perlecan would affect angiogenesis in a variety of processes in which vascular and tissue remodeling is predominant.

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