Matrix Metalloproteinase-3 Releases Active Heparin-binding EGF-like Growth Factor by Cleavage at a Specific Juxtamembrane Site*

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Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is synthesized as a membrane-anchored precursor that is cleaved to release the soluble mature growth factor. The two forms are active as juxtacrine and paracrine/autocrine growth factors, respectively. The enzymes that process the HB-EGF transmembrane form are unknown. Accordingly, an in vitro assay was established using a fusion protein in which alkaline phosphatase (AP) replaced the transmembrane and cytoplasmic domains of HB-EGF (HB-EGF JM-AP). The fusion protein was anchored to agarose beads coated with anti-AP antibodies. Several matrix metalloproteinases (MMPs) were tested for the ability to release soluble HB-EGF in the in vitro system. MMP-3 released soluble 12-kDa immunoreactive and mitogenic HB-EGF within 30 min. On the other hand neither MMP-2 nor MMP-9 had any cleavage activities. A non-cleavable mutant was prepared by replacing the juxtamembrane (JM) region of HB-EGF with the JM region of CD4. The mutant HB-EGF, which in its full-length form was as active a juxtacrine growth factor as was the wild type HB-EGF in vivo, was not cleaved by MMP-3 in the in vitro assay. The C-terminal portion of the cleaved HB-EGF JM-AP that remained attached to the anti-AP beads was N-terminally sequenced and the MMP-3 cleavage site was determined to be Glu151-Asn152, a site within the JM domain. MMP-3 treatment also released soluble HB-EGF in vivo from MC2 cells expressing transmembrane HB-EGF precursor, at a level of about 2-fold above control. It was concluded that MMP-3 cleaves HB-EGF at a specific site in the JM domain and that this enzyme might regulate the conversion of HB-EGF from being a juxtacrine to a paracrine/autocrine growth factor.

A number of growth factors and cytokines are synthesized as membrane-anchored precursor proteins that can be proteolytically cleaved to release soluble factors. Examples are TGF-α, a tumor necrosis factor-α, the c-kit ligand, and colony stimulating factor 1 (1). Conversion of insoluble precursor to soluble released factor by enzymatic cleavage may constitute an important post-translational modification that regulates growth factor activity and bioavailability. For example, the transmembrane precursor form of TGF-α is a juxtacrine growth factor that mediates signaling, proliferation, and adhesion of neighboring cells (2, 3). On the other hand, soluble TGF-α can diffuse freely and is a potent paracrine and autocrine growth factor (4).

There has been great interest in identifying the enzymes responsible for proteolytic cleavage of cytokine and growth factor precursors, one reason being that release of these factors often contributes to pathological processes. For example, interleukin-1β-converting enzyme has been identified as an intracellular cysteine protease that releases mature interleukin-1β, thereby contributing to inflammatory disease (5). Release of soluble TGF-α, an oncogenic growth factor is mediated by a yet unidentified protease that cleaves at an Ala-Val site in the juxtamembrane (JM) region of the precursor (4). Apopain, the product of the pro-apoptotic gene, ced-3, is a cysteine protease related to interleukin-1β-converting enzyme and is required for programmed cell death in Caenorhabditis elegans (6). Hydroxy-amic acid-based derivatives which are specific inhibitors of metalloproteinases have been used to identify precursor-cleaving proteases. For example, TACE, a metalloproteinase member of the mammalian adamalysin family, specifically cleaves the tumor necrosis factor α precursor to release a potent proinflammatory and immunomodulatory cytokine implicated in numerous inflammatory conditions and cachexia (7–11). Metalloproteinases have also been implicated in the proteolytic release of TGF-α (12), the β-amyloid precursor protein (12), the FGF1 receptor ectodomain (13), the interleukin-6 receptor ectodomain (12, 14), the human thyrotropin receptor ectodomain (15), and the lymphocyte i-selectin adhesion molecule (16–18).

Our laboratory has been analyzing the structural and biological properties of heparin-binding epidermal growth factor-like growth factor (HB-EGF) since its discovery (19–21). HB-EGF is a member of the EGF family that is structurally homologous to EGF, TGF-α, amphiregulin, the neuregulins, betacellulin, and epiregulin (1, 22–24). HB-EGF was first identified in the conditioned medium of human macrophages as a soluble heparin-binding potent mitogen for fibroblasts, smooth muscle cells, keratinocytes but not for endothelial cells (19, 20, 25). It activates HER1/erbB1 as does EGF, TGF-α, amphiregulin, and betacellulin. However, HB-EGF also activates HER4/}

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*The abbreviations used are: TGF-α, transforming growth factor-α; EGF, epidermal growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; MMP, matrix metalloproteinase; pro-MMP, precursor of MMP; PMA, phorbol 12-myristate 13-acetate; APMA, p-aminophenylmercuric acetate; FCS, fetal calf serum; GPS, glutamine/penicillin/streptomycin; PAGE, polyacrylamide gel electrophoresis; TM, transmembrane; JM, juxtamembrane.
erB4, in common with neuregulin and betacellulin (26). Activation of HER4/erbB4 by HB-EGF results in chemotaxis but not proliferation, unlike activation of HER1/erbB1 which leads to both activities. The significance of HB-EGF heparin binding was demonstrated in that the chemotactic activity of HB-EGF for smooth muscle cells is mediated by interactions with cell surface heparan sulfate proteoglycans acting as low affinity receptors (21). HB-EGF has been implicated as a participant in a variety of processes such as wound healing, blastocyst implantation, smooth muscle cell hyperplasia, atherosclerosis, and tumor growth (27–31).

The transmembrane precursor form of HB-EGF precursor has several biological properties. As a purified protein, recombinant transmembrane HB-EGF is a chemotactic and mitogenic factor (32). In co-culture, it is a juxtacrine proliferative factor for neighboring cells (33, 34), and a juxtacrine adhesion factor for mouse blastocysta (29). It is also the unique receptor for diphtheria toxin (35, 36). Transmembrane HB-EGF synthesis is induced during skeletal muscle cell differentiation, and its gene expression is activated in these cells by MyoD (37). Release of soluble HB-EGF has been found to occur both constitutively and in a regulated manner. Macrophages and T lymphocytes are among the cell types that release HB-EGF constitutively (19, 20, 38). On the other hand, in the case of adherent cells such as MDA-MB-231 (39) and Vero (34), the membrane-anchored precursor is the predominant form and processing is inducible, for example, by addition of phorbol esters. Phorbol ester treatment of these cells renders them diphtheria toxin-resistant, suggesting that the transmembrane precursor has been fully converted into the soluble form (39).

The cleavage sites for HB-EGF processing and the enzymes responsible are not known. The endopeptidase, furin, has been implicated in the rapid constitutive release of the HB-EGF N-terminal propeptide (40). The enzyme(s) that release soluble HB-EGF from the cell surface may be matrix metalloproteinases (MMP) since phorbol ester-induced HB-EGF secretion is inhibited by hydroxyamine-based acid derivatives that are MMP inhibitors (41). Considering that the HB-EGF cleavage enzymes might be MMP-related, we devised an in vitro assay and tested several MMPs for their ability to cleave and release HB-EGF. In this report we demonstrate that MMP-3 (stromelysin-1) but not MMP-2 nor MMP-9, cleaves HB-EGF to release soluble immunoreactive and mitogenic HB-EGF. Furthermore, cleavage occurs specifically in the juxtamembrane region at a Glu231-Asn152 site. In addition, a non-cleavable HB-EGF mutant was generated to exclude artifacts of nonspecific cleavage in measuring juxtacrine activity and determining juxtamembrane cleavage sites.

EXPERIMENTAL PROCEDURES

Cell Culture—Cell culture reagents were purchased from Life Technologies, Inc., Invitrogen, or agarose (Sigma). Mouse hematopoietic 32D cells and EP170.7 cells, which are 32D cells transfected with the EGF receptor, were kindly provided by Dr. Jackie Pierce (National Institutes of Technology, Gaithersburg, MD). Mouse hematopoietic 32D cells and WEHI-3 cells conditioned medium (42). Zymography—Native MMP-3 (greater than 98% homogeneity by SDS-PAGE electrophoresis and Western blot) was purchased from Bio-Genesis Ltd. (Sandown, NH). Human recombinant MMP-2 and MMP-9 were purchased from Biogenesis Ltd. and provided by Dr. R. Fridman (Wayne State University School of Medicine, Detroit, MI). The MMPs were activated prior to the cleavage experiments. To activate pro-MMPs, 200 ng/ml of each enzyme were incubated with APPA at a final concentration of 1 mM for 1 h at 37 °C. Pro-MMP-3 was activated in the same manner with the exception being an increased incubation time of 3 h. All zymographic reagents were obtained from Bio-Rad with the exception of APMA which was obtained from Sigma. Substrate gel electrophoresis was carried out as described previously (43). Briefly, Type I gelatin or casein was added to the standard Laemmli acrylamide polymerization mixture at a final concentration of 1 mM. MMP samples were mixed with substrate sample buffer (10% SDS, 40% sucrose, 0.25 mM Tris–Cl, pH 6.8, and 0.1% bromphenol blue) and loaded without boiling into wells of a 4% acrylamide Laemmli stacking gel in a mini-gel apparatus. Polyacrylamide gels were run at 15 mA/gel while stacking and at 20 mA/gel during the resolving phase at 4 °C. After electrophoresis, gels were soaked and incubated at 37 °C in substrate buffer (50 mM Tris–Cl buffer, pH 8, 5 mM CaCl2, and 0.02% NaN3). After incubation, gels were stained for 15–30 min in 0.5% Coomassie Blue R-250 in acetic acid/isopropl alcohol:H2O (1:3:6), destained in H2O, and photographed.

Production of Full-length AP-HB-EGF Fusion Proteins with Wild Type or Mutated JM Domains—For juxtacrine co-culture and in vivo MMP-3 cleavage experiments, expression vectors for the synthesis of full-length HB-EGF with both wild type and mutated JM domains were used. A full-length HB-EGF expression vector in which the AP has been inserted between Leu169 and Thr180 in the N-terminal region of the mature HB-EGF domain (Fig. 1A) has been previously described and is designated here as AP-HB-EGF (29). To obtain a putative non-cleavable HB-EGF mutant, the JM domain of HB-EGF spanning from Pro149 to Thr160 was replaced with the amino acid sequence of the corresponding JM domain of CD4 (CD4-JM) (44). The overall strategy was to substitute the HB-EGF JM domain with a cloning cassette so as to be able to introduce any desired amino acid sequence such as the CD4-JM domain. To do this, an HB-EGF mutant was constructed which contained a JM domain (Ser147-Ile162) with a spacer sequence containing two BamBI restriction sites using polymerase chain reaction (PCR). The following oligonucleotides were used: oligo A, 5′-CCCGAAGCTTCCGCAATGAGCT- GTCGTCGTCGTCG-3′ containing nucleotides 1–18 of the human HB-EGF cDNA preceded by a HindIII restriction site; oligo B, 5′-GCTCGAGACG- GTCTCTCGCCATGACACCTCTCTC-3′ containing nucleotides 422–441 of the human HB-EGF cDNA followed by an EcoRI site; and oligo C, 5′-GCTGTAGACCCTCTAGCTGGCGGTTGCTTG- TG-3′ containing nucleotides 487–504 of the human HB-EGF cDNA preceded by an XbaI site and a BamBI site; oligo D, 5′-CCCGAATTCTAGTGGGAAATTTGGCATC-3′ containing nucleotides 610–625 of the human HB-EGF cDNA followed by an EcoRI site. Both PCR products were subcloned into a pcR3 vector (Invitrogen) and sequenced. The HindIII/XbaI fragment and the XbaI/EcoRI fragment were then released and ligated into the HindIII-EcoRI site of the expression vector pCDNA3 (Invitrogen) and the DNA sequence of the product was confirmed. The resulting construct, pHB-EGF Ser147-Ile162, facilitated substitution of Ser147-Ile162 with any desired amino acid sequence. To prepare specifically the HB-EGF CD4-JM mutant (Fig. 1A), two 52-mer oligo nucleotides: 5′-GCTGAGCTCTAAAGTCTGCCACATGCGAC- ACCCGCGTGAGGCAACATCTC-5′ and 5′-CCAGGATGTTGCTGCGTAC- ACGGCTGTTGACGACTTGGCAGAATTCTG-3′, were synthesized and annealed to each other. The resulting oligonucleotide encoded Ser147-Leu166 of human HB-EGF followed by LyS166-Pro167 of the human HB-EGF (CD4-JM) (44), followed by Thr169-Ile166 of human HB-EGF. This oligonucleotide was ligated to the BamBI sites of pHB-EGF Ser147-Ile162, Ile162 and the DNA sequence of the product, pHB-EGF CD4-JM was confirmed. To prepare the AP-HB-EGF CD4-JM fusion protein, the 4.5-kilobase pair BamBI fragment of pHB-EGF CD4 Lys166-Pro167 was used to obtain a full-length expression vector in which the AP has been inserted between Leu29 and Thr85 in the N-terminal region of the mature HB-EGF domain (29) (Fig. 1A).
T-3 pVL1392 (Invitrogen) was digested with Hin and Xba was prepared by substituting the CD4-JM domain described above for ental AP coding (tag-1) plasmid (45) were ligated into the linearized Sepharose beads and the bound material was released with 2.5M NaCl. MMP addition by changing the buffer using ultrafiltration (Centricon 3, Eagle's medium without phenol red and exposed to various concentrations of MMP activated with APMA. The APMA was removed before MMP addition by changing the buffer using ultrafiltration (Centricon 3, Amicon). The supernatant fractions were incubated with heparin-Sepharose beads and the bound material was released with 2.5M NaCl and assayed for AP activity.

**Juxtacrine Growth Factor Activity**—Parental MC2 cells and MC2 cells transfected with full-length AP-HB-EGF or the AP-HB-EGF CD4-JM mutant cDNA were plated in 96-multiwell dishes at increasing cell densities and cultured overnight. After washing with Dulbecco's modified Eagle's medium, 10% FCS, and 2M NaCl to remove AP-HB-EGF possibly trapped by cell surface heparan sulfate proteoglycans, cells were fixed with 4% paraformaldehyde/phosphate-buffered saline and washed three times with RPMI 1640, 10% FCS. EP170.7 cells were added at a concentration of 2 x 10^5 cells/200 µl/well and after 42 h, [3H]thymidine (1 µCi/well) was added and the cells were incubated for 6 h. The EP170.7 cells, which grow in suspension, were harvested in a cell harvester (Microbeta Plus, Wallac) and DNA synthesis was measured by assaying the incorporation of [3H]thymidine into DNA (38). To compare the specific activities (DNA synthesis/amount of cell surface HB-EGF AP) of the wild type and CD4-JM mutant transgenic mice, the HB-EGF juxtacrine growth factors, cell surface AP expression levels were determined after fixation of the cells with 4% paraformaldehyde/ phosphate-buffered saline. The AP values were 0.456 OD₄₁₀/min/3.45 x 10⁶ cells and 0.95 OD₄₁₀/min/3.96 x 10⁶ cells for wild type and mutant HB-EGF, respectively, equivalent to a ratio of 0.56 wild type/mutant cell surface HB-EGF.

**In Vitro Assay for Cleavage and Release of Soluble HB-EGF**—To establish an in vitro assay for identifying enzymes that can release soluble HB-EGF, fusion proteins were prepared containing the mature HB-EGF domain, either the HB-EGF JM domain or the CD4-JM domain, and plaecental AP replacing the transmembrane and cytoplasmic domains at the C terminus (Fig. 3). This construct was designated as HB-EGF-AP. To do this, the sequence corresponding to the first 149 amino acids of the human HB-EGF cDNA (19) was amplified by PCR using synthetic oligonucleotide primers, 5'-GCTCTAGAGCATGAAGC-GTCCTGCCGTGC-3' and 5'-CGAAGCTTGGGTGTGGTCATAGGG-TG-3'. The PCR product was digested with XbaI and HindIII and ligated to XbaI and HindIII sites of pUC18. The baculovirus transfer vector pVL1392 (Invitrogen) was digested with BamH I, treated with Klenow enzyme and cut again with XbaI. In a single step the XbaI-HindIII HB-EGF fragment and the HindIII-HpaI fragment of a human placental AP coding (tag-1) plasmid (45) were ligated into the linearized pVL1392 (pVLHB-EGF-AP). A putative non-cleavable mutant was prepared by substituting the CD4-JM domain described above for the HB-EGF JM domain at position Pro²⁵-Thr²⁷. To generate this fusion protein, synthetic oligonucleotide primers were synthesized and ligated to the following oligonucleotides: 5'-TGAGTCTCAAGAGTTCTGACCACATGTCCTCACCACCCG-TCGACCAACAACCA-3' and 5'-CAGAATTCAGCAAGGGCTGATCACG- GTGGGGCAGCTCGTGGTGCGA-3'. The resulting oligonucleotide possesses overhanging ends that could be ligated to CellII and HindIII sites. The plasmid pVLHB-EGF-AP was cut by both CellII and HindIII and ligated with the synthetic oligonucleotide to obtain pVLHB-EGF CD4-JM-AP. To obtain recombinant HB-EGF fusion proteins, the baculovirus expression system was used (32). Briefly, plasmids pVLHB-EGF-AP and pVLHB-EGF CD4-JM-AP were used to generate recombinant baculovirus. Sf9 cells growing in a late logarithmic phase in serum-free medium (SF900) were infected with recombinant baculovirus clones. Conditioned medium (500 ml) was harvested 96–120 h post-infection and applied to a TSK heparin column (25 ml, TosohHaas, Tokyo, Japan) which was washed extensively with 20 mM HEPES, pH 7.2, 0.2 mM NaCl. Proteins were eluted with a 0.2–2.0 M NaCl linear gradient. The elution of the fusion proteins was monitored by measuring AP activity using a Sigma diagnostic kit with naphthol phosphate as a substrate. After incubation at room temperature for 0.5–6 h, absorbance at 410 nm was measured in a 96-well multiwell plate reader. The peak fractions were pooled and diluted to 0.2 M NaCl with 20 mM Hesp, pH 7.2. A second round of TSK-heparin FPLC was performed and fractions were assayed for AP activity. SDS-PAGE with silver staining and Western blot (32) with anti-HB-EGF antibody number 197, kindly provided by Dr. Judy Abraham (Scrios, Sunnyvale, CA) was used to ascertain the purity and size of the fusion proteins, which were expected to be about 72 kDa. Active purified fusion protein fractions were pooled and used for the in vitro HB-EGF cleavage assay.

The in vitro cleavage assay was performed as described schematically in Fig. 3. Excess amounts of purified HB-EGF JM-AP or HB-EGF CD4-JM-AP fusion proteins were incubated with anti-human placental AP-antibody conjugated agarose beads (Sigma) at 4°C for 12 h. The beads were washed extensively with 10 mM Hepes, pH 7.2, 0.2M NaCl, and 5 mM CaCl₂ and boiled in 2 x SDS-PAGE buffer. The dissolved proteins were analyzed by SDS-PAGE and silver staining or subjected to N-terminal sequencing.

**N-terminal Sequencing**—The HB-EGF JM-AP fusion proteins attached to anti-AP coated beads (30 µg/60 µl beads) were incubated for 300 min at 37°C in 250 mM Tris-HCl, pH 7.2, 2.5 M NaCl, and 5 mM CaCl₂ and boiled in 2 x SDS-PAGE buffer. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (ProBlott, Applied Biosystems). The proteins were stained with 0.1% Coomassie Brilliant Blue in 50% methanol and the major 60-kDa band was cut out and subjected to N-terminal microsequencing using a Applied Biosystems model 477A microsequenator as a service provided by Dr. William Lane, of the Harvard Microchemistry Facility (Cambridge, MA).

**RESULTS**

**HB-EGF Juxtacrine Growth Factor Activity**—It has been shown previously that the transmembrane HB-EGF precursor is a juxtacrine growth factor (33, 34). However, those studies could not rule out definitively the possibility that the juxtacrine growth stimulation was merely an artifact resulting from the processing of the membrane-anchored precursor during the co-culture and the subsequent release of active soluble paracrine HB-EGF. To address this possibility, a putative non-cleavable mutant was produced that would resist artifactual proteolytic degradation (Fig. 1). This was done by altering the AP-HB-full-length fusion protein (29) so that 12 amino acids in the JM region of HB-EGF were replaced by 12 amino acids in the JM region of the CD4 + T cell CD4 transmembrane antibody (Fig. 1A). This construct was designated as AP-HB-EGF CD4-JM. The rationale for this substitution was that CD4+ cells release HB-EGF but not CD4 (38), suggesting that the enzymes that cleave HB-EGF do not target the CD4 protein. The ability of the CD4-JM mutant to resist artifactual degradation was tested in cells treated with PMA. PMA has been shown previously to rescue release soluble HB-EGF from its precursor in several cell types including Vero and MDA-MB 231 cells (34, 39). PMA treatment also released soluble HB-EGF from COS7, Chinese hamster ovary, Vero, and MC2 cells transfected with the wild type construct (Fig. 1B). However, PMA did not release AP-HB-EGF from cells transfected with the CD4-JM mutant.
An in Vitro Assay for Proteolytic Cleavage of Transmembrane HB-EGF—The processing of transmembrane HB-EGF, which is a juxtacrine growth factor, into soluble HB-EGF, a paracrine growth factor, represents a post-translational modification that may regulate HB-EGF function. To analyze the enzymatic processing of transmembrane HB-EGF, an in vitro assay was established (Fig. 3). HB-EGF cDNA consisting of mature, TM, transmembrane, and cytoplasmic domains (Fig. 3A) was modified such that the TM and cytoplasmic domains were replaced with placental AP (Fig. 3B). This fusion protein with AP at the C terminus was designated as, HB-EGF JM-AP. It was expressed in a baculovirus/insect cell system, purified by heparin affinity chromatography and immobilized onto agarose beads coated with anti-AP antibodies (Fig. 3C). To analyze for potential cleavage enzymes, samples to be tested were added to the beads for various time intervals at 37°C. The beads were pelleted and the supernatant fractions were assayed for HB-EGF growth factor activity and HB-EGF immunoreactivity in a Western blot analysis. The beads containing the C-terminal remaining portion of the HB-EGF JM-AP fusion protein were subsequently analyzed by SDS-PAGE and N-terminal sequencing as will be described below.
MMP-3 Cleaves HB-EGF in Vitro—Previous studies have demonstrated that phorbol esters induce proteolytic cleavage of transmembrane HB-EGF (34, 39, 41). Furthermore, in Vero, MC2, and U937 cells, the phorbol ester-induced cleavage is blocked by hydroxamic acid-based inhibitors of MMPs (41). Accordingly, we tested several MMPs for their ability to release biologically active and immunoreactive HB-EGF from the immobilized HB-EGF JM-AP fusion protein. After activation of the enzymes with APMA, 5 μg/ml MMP-2, MMP-3, or MMP-9 were added to the substrate at 37 °C and release of HB-EGF was measured by Western blot analysis (Fig. 4). No HB-EGF was released in the absence of MMPs (data not shown). However, after MMP-3 treatment, a single 12-kDa HB-EGF immunoreactive band was detected in the supernatant within 30 min (Fig. 4, lane 1) that comigrated with a recombinant HB-EGF standard (Fig. 4, lane 10). Greater levels of released immunoreactive HB-EGF were detected by 90 and 300 min (Fig. 4, lanes 2 and 3, respectively). In contrast, no immunoreactive HB-EGF was released by MMP-2 or MMP-9 at 30, 90, or 300 min (Fig. 4, lanes 4–6 and 7–9, respectively), or even at 12 h of incubation (not shown).

The supernatants were also tested for HB-EGF growth factor activity (Fig. 5). MMP-3, but not MMP-2 or MMP-9, released HB-EGF that stimulated DNA synthesis in EP170.7 cells with kinetics comparable to the release of immunoreactive HB-EGF. Since all 6 cysteine residues in mature HB-EGF are required for mitogenic activity, release of biologically active HB-EGF by MMP-3 suggested strongly that the cleavage site for MMP-3 must be somewhere downstream of the sixth Cys residue as shown in Fig. 3A.

The three MMPs used in these HB-EGF cleavage experiments were activated by APMA treatment in solution. To ensure that APMA did actually activate these enzymes, especially MMP-2 and MMP-9 which did not release soluble HB-EGF, zymography was carried out with gels into which gelatin was incorporated as described under “Experimental Procedures” (Fig. 6). Prior to activation, the MMP-2 and MMP-3 preparations were primarily in zymogen forms, while the MMP-9 preparation contained both zymogen and active enzyme. Activated MMP-2 and MMP-9 readily degraded gelatin (Fig. 6A). MMP-2-induced gelatin degradation was associated with a 62-kDa band. Activated MMP-9-induced gelatin degradation was associated with an 82-kDa band. Activated MMP-3, which degraded gelatin poorly, degraded casein and this enzymatic activity was associated with a 45-kDa band (Fig. 6B). It was concluded that the APMA-treated MMP-2 and MMP-9 used in these experiments were the lower molecular mass active proteases, but that these activated proteases did not release biologically or immunoreactive HB-EGF from the substrate.

To determine whether cleavage occurred within the juxtamembrane domain, MMP-3 was assayed for the ability to cleave an HB-EGF JM-AP substrate in which the wild type JM (WT-JM) domain was replaced with the mutant CD4-JM domain (CD4-JM) (Fig. 7). While MMP-3 released soluble mitogenic HB-EGF (Fig. 7A, left) and immunoreactive HB-EGF (Fig. 7B, lane 2) from the wild type protein, it did not release mitogenic (Fig. 7A, right) or immunoreactive HB-EGF (Fig. 7B, lane 4) from the CD4-JM protein. In addition, when the proteins still associated with the anti-AP beads were analyzed by SDS-PAGE and silver staining, it was found that MMP-3 reduced the molecular mass of the 72-kDa HB-EGF JM-AP protein (Fig. 7C, lane 1, solid arrow) to about 60 kDa (Fig. 7C, lane 2, open arrow). However, MMP-3, even at a dose of 10 μg/ml, did not diminish the size of the 72-kDa HB-EGF CD4-JM-AP mutant protein (Fig. 7C, lanes 3 and 4). Taken together, these results along with those in Fig. 1, suggest the CD4-JM mutant is non-cleavable both in vitro and in vivo and that MMP-3 processes HB-EGF JM-AP to release soluble HB-EGF at a

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3 M. Suzuki and M. Klagsbrun, unpublished results.
specific site in the JM domain.

Identification of the MMP-3 Cleavage Site—To identify the site at which MMP-3 cleaves HB-EGF JM-AP, the anti-AP-coated beads containing the remaining C-terminal portion of the fusion protein were collected, centrifuged, and the bound HB-EGF proteins were visualized by SDS-PAGE (Fig. 8A). The time course showed conversion of 72-kDa HB-EGF JM-AP to a smaller 60-kDa species by 30 min with complete processing apparent at 300 min. The 60-kDa band generated after 300 min of MMP-3 treatment was transferred to a polyvinylidene difluoride membrane and subjected to N-terminal sequence analysis (Fig. 8C). A single sequence of the first 19 N-terminal amino acids of the cleaved product was obtained, NRLYTYDHTQAYVRSSGGI. This sequence contains amino acids in the JM domain and extends into the N-terminal region of AP. When this sequence was compared with the sequence of HB-EGF JM-AP (Fig. 8B), it was apparent that the cleavage site for MMP-3 in vitro was at Glu\(^{151}\)-Asn\(^{152}\) within the JM region of the HB-EGF precursor.

MMP-3 Treatment of Cells Expressing AP-HB-EGF—To determine whether MMP-3 could process transmembrane HB-EGF in vivo, MC2 cells transfected with full-length AP-HB-EGF were incubated with increasing concentrations of MMP-3 for 2 h (Fig. 9). At 5 \(\mu\)g/ml MMP-3, there was about a 2-fold increase in released AP-HB-EGF from the cells compared with non-MMP-3 treated controls. On the other hand, AP-HB-EGF was not released at all from cells expressing the AP-HB-EGF fusion protein containing the CD4-JM mutant (data not shown).

**DISCUSSION**

The proteolytic processing of the HB-EGF transmembrane precursor to the mature soluble form is a key step in converting this juxtacrine growth factor into a paracrine/autocrine one. We have identified MMP-3 as one of the possible proteases responsible for this processing event based on the demonstration that MMP-3 releases soluble bioactive HB-EGF from an insoluble substrate in vitro consisting of mature HB-EGF, the HB-EGF JM domain, and AP at the C terminus replacing the transmembrane and cytoplasmic domains (HB-EGF JM-AP). Half-maximal release at 5 \(\mu\)g/ml MMP-3 occurs within 30 min. As might be predicted, the proteolytic cleavage occurs within the extracellular JM domain. The evidence for this is that (i) immunoreactive 12-kDa HB-EGF, the expected size, was released and was mitogenic for cells expressing EGF receptor (HER1/erbB1). Proteolytic cleavage in the mature HB-EGF domain would lead to irreversible loss of growth factor activity. Release of the 12-kDa soluble HB-EGF occurred concomitantly with a 12-kDa reduction in the mass of the HB-EGF JM-AP fusion protein. (ii) No release of soluble HB-EGF occurred in vitro when the HB-EGF JM domain was replaced with the corresponding JM region of the CD4 protein. This mutant was shown to be a non-cleavable mutant in vitro since when the replacement was made in the full-length HB-EGF and the construct was transfected into several cell types, the cells were resistant to the proteolytic cleavage induced by phorbol ester. Furthermore, the full-length HB-EGF with the CD4-JM mutant had juxtacrine growth factor activity in co-culture compa-
rable to wild type transmembrane HB-EGF, indicating that the CD4-JM replacement has no adverse effects on overall structure and function beyond the JM domain; and (iii) N-terminal sequencing of the fusion protein that remains anchored to the anti-AP-coated agarose beads indicated that the specific cleavage site is Glu\(^{151}\)-Asn\(^{152}\) which is within the proposed JM domain of HB-EGF, 10 residues upstream of the TM domain. No other N termini were detected.

Previous results, based on amino acid composition analysis of C-terminal tryptic peptides of mature HB-EGF released by phorbol ester treatment of Vero cells, suggested that the cleavage site might be at Pro\(^{149}\)-Val\(^{150}\) (34). However, this analysis might have been incomplete. Alternatively, after cleavage at Glu\(^{151}\)-Asn\(^{152}\) by an MMP-3-like enzyme, there may be additional enzymes available in vitro, for example, neutral endopeptidase (46) that further process mature HB-EGF by removal of C-terminal Val\(^{150}\) and Glu\(^{151}\). Cleavage by MMP-3 at Glu\(^{151}\), Asn\(^{152}\) of the HB-EGF Pro\(^{149}\)-Val\(^{150}\)-Glu\(^{151}\)-Asn\(^{152}\) sequence is consistent with the demonstration that MMP-3 is highly effective in cleaving a Pro-Val-Glu-norvaline (Nva) synthetic fluorogenic peptide substrate at the Glu-Nva site with a \(k_{\text{cat}}/K_m\) of 218,000 s\(^{-1}\)m\(^{-1}\) (47). This substrate is among the most highly hydrolyzed fluorescent MMP-3 substrates yet described. In addition, the HB-EGF cleavage substrate sequence of Leu\(^{148}\), Pro\(^{149}\)-Val\(^{150}\)-Glu\(^{151}\)-Asn\(^{152}\)-Arg\(^{153}\) with cleavage occurring at Glu-Asn, is compatible with stromelysin (MMP-3)-sensitive hexamer sequences that were generated based on bacterio-phage peptide display analysis (48). One of these sequences, Ile-Pro-Phe-Glu-Gln-Arg (with Gln and Gln at the P1 and P2 positions, respectively), resembles the HB-EGF cleavage site sequence, and has a relatively high \(k_{\text{cat}}/K_m\) for MMP-3. Overall, the synthetic hexamers that have relatively high \(k_{\text{cat}}/K_m\) values also have predominantly a Pro residue at position P1\(^{3}\), an Arg residue at position P2\(^{3}\), and often a Glu residue at position P1, positions compatible with those in the HB-EGF cleavage substrate sequence.

Although MMP-3 is very effective in releasing soluble HB-EGF from the anchored substrate, MMP-2 (72-kDa gelatinase A) and MMP-9 (92-kDa gelatinase B) have no effect at all, even at 5 \(\mu\)g/ml enzyme in a 5-h digestion period and even though it can be demonstrated that these 2 gelatinases are highly active in degrading gelatin usingzymography.

The HB-EGF JM Glu-Asn cleavage site differs from other known MMP cleavage sites. MMP-3 has been shown to cleave cross-linked fibrin at a Gly-Ala site (49) and insulin B chain at Ala-Leu and Tyr-Leu sites (50), none of which are present in the HB-EGF JM region. In general, there are no significant homologies between the various MMP-3 cleavage sites that are found in proteoglycans, several types of collagen and protease inhibitors (47, 51). Other metalloproteinase cleavage sites have also been identified of which none are present in the HB-EGF JM. For example, MMP-2, but not MMP-9, releases an active soluble ectodomain of FGFR1 by hydrolysis at a Val-Met site which is within the FGFR1 JM domain, eight residues upstream of the TM domain (13). MMP-2 hydrolyzes the \(\beta\)-amyloid precursor at Lys-Leu, Leu-Met, and Met-Val sites (52, 53). Galectin-3, a cell surface lectin involved in cell-cell and cell-matrix interactions in tumor metastases is cleaved at an Ala-Tyr site by both MMP-2 and MMP-9 (54).

MMP-3 also releases soluble HB-EGF in vitro, albeit to a limited extent. MMP-3 treatment of MC2 cells expressing an AP-HB-EGF fusion protein results in about a 2-fold increase in released soluble HB-EGF compared with non-treated controls. The efficiency of release in vitro is limited, with only about 5% of cell surface AP-HB-EGF being cleaved compared with the situation in vitro in which complete release can be achieved. Possible explanations are that the cleavage site is close to the cell membrane and may not be as readily accessible as it is in the in vitro assay and/or that TIMP-like inhibitors of MMP-3 associated with the cells may be inhibiting enzymatic activity. Alternatively, MMP-3 might not be the only cleavage enzyme for HB-EGF in vitro. HB-EGF is processed constitutively by macrophages (19, 20) and T cells (38) and in response to PMA treatment by many cell types including breast carcinoma cells (39). Whether these cleavage processes are mediated by the same enzymes and whether MMP-3 is an enzyme that is involved in these processes remains to be determined. For example, besides secreted proteases such as MMP-3, other enzymes that are membrane-bound might be involved in the processing of HB-EGF as has been demonstrated for tumor necrosis factor-\(\alpha\) (10, 11).

The significance of MMP-3-induced cleavage of the HB-EGF precursor might be that this post-translational modification would increase the bioavailability of a potent growth factor that has been shown to be involved in physiological and pathological processes (reviewed in Ref. 55). Increased levels of MMPs including MMP-3, enhance the extracellular matrix degradation and remodeling that accompanies cell migration, tumor invasion, and wound healing (51, 56). Previously, MMP-3 has been shown to increase the bioavailability of bFGF by releasing it from basement membrane perlecan by cleaving the core protein (57). HB-EGF is a potent mitogenic factor for fibroblasts and keratinocytes, cells involved in wound healing (27, 28) and for tumor cells (31). It is also a potent chemotactic and mitogenic factor for smooth muscle cells (19, 21), activities which have been linked to smooth muscle cell hyperplasia in atherosclerosis, restenosis, and pulmonary hypertension (30, 31, 58). Enhanced levels of MMP-3 in the proliferating epidermis of wounds (59), in tumors (60–62), in atherosclerotic plaques (63, 64), and in aortic aneurysm and occlusive disease (65) could lead to an increase in HB-EGF paracrine growth factor activity in these tissues. Furthermore, EGF and TGF-\(\alpha\) have been demonstrated to increase MMP-3 production (66, 67), suggesting the possible existence of an autocrine amplification loop wherein EGF-like growth factors, perhaps HB-EGF itself, increase MMP-3 levels in turn increasing secreted HB-EGF levels.

In summary, we have demonstrated that MMP-3 cleaves HB-EGF at a specific Glu-Asn site in the HB-EGF JM domain. This finding suggests a new role for MMP-3 in enhancing growth factor bioavailability in physiological and pathological processes.

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