characterization of METH-1/ADAMTS1 processing reveals two distinct active forms*

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METH-1/ADAMTS1 is a member of a newly described family of genes that contain metalloprotease, disintegrin, and thrombospondin-like motifs. We have recently shown that METH-1 protein is a potent inhibitor of angiogenesis. Here, we demonstrate that secreted human pro-METH-1 is processed in two consecutive steps to release both p87 and p65 active forms. The p87 form lacks the N-terminal prodomain and p65 results from an additional processing event in the C-terminal end. Generation of p87 was blocked with specific inhibitors of furin, and incubation of pro-METH-1 with purified furin released the p87 fragment but not p65. Generation of p65 required preformation of p87 and was suppressed by inhibitors of matrix metalloproteases. We demonstrate that matrix metalloproteases 2, 8, and 15 were able to release p65 when p87 was used as substrate. This second processing step removes two thrombospondin repeats from the carboxyl-terminal end of p87-METH-1 and alters the affinity of the protein to heparin and endothelial cultures. Furthermore, this deletion was associated with a reduced activity upon suppression of endothelial cell proliferation. We hypothesize that METH-1 processing is relevant for the modulation of the anti-angiogenic properties displayed by the protein.

METH-1/ADAMTS1 belongs to the recently described metalloproteasin/ADAMTS (A disintegrin and metalloproteinase with thrombospondin motifs) family of proteins (1). The mouse ADAMTS1 gene was first identified as a transcript expressed highly in a cachexigenic colon tumor cell line (2), and it has been suggested to be an active metalloprotease by means of the protease trapping mechanism of α2-macroglobulin (3). More recently, it has been shown that targeted disruption of the mouse ADAMTS1 gene resulted in growth retardation and defects in fertility and morphogenesis (4). We cloned the human ortholog of ADAMTS1 (named METH-1) and a second homologous gene (METH-2/ADAMTS8) (1). Recombinant METH-1 and -2 proteins display anti-angiogenic properties and inhibit endothelial but not smooth muscle or fibroblast proliferation (1). Other members of the ADAMTS family have been implicated in processing of extracellular matrix components (5–7), and gonadal morphogenesis of Caenorhabitis elegans (8). Additional cDNAs showing conservation of the METH-1 domain structure have been cloned (ADAMTS5, -6, and -7), but their function remains unknown (9).

The structure of all ADAMTS members include a signal sequence for targeting to the secretory pathway, a prodomain, a catalytic motif related with the reprolysin subfamily of metalloproteases, a putative disintegrin domain, and a carboxy-terminal region containing a variable number of type I repeats (properdin- or thrombospondin-like). Several members of this family appear to be processed upon secretion (1, 3, 6, 7). By analogy to proteins of the metalloprotease family, it is likely that this processing is required for functional activation. For example, all matrix metalloproteases (MMPs) are synthesized as inactivezymogens in which the amino-terminal prodomain is responsible for maintaining latency of the proteolytic activity. Activation involves removal of the prodomain by endoproteolytic cleavage that may be performed by distinct MMPs, furin, plasmin, and other proteases (10). Sequence analysis of the metalloprotein/ADAMTS family of proteins reveals a well conserved furin consensus cleavage site between the prodomain and the catalytic motif. Furin is a ubiquitously expressed calcium-dependent serine protease that is involved in cleaving various precursor proteins (11). This processing has been shown to occur with a deletion mutant form of ADAMTS1 (3). Furthermore, the mature form of aggrecanase-1/ADAMTS4 lacks the prodomain, indicating processing at this site (6). In addition to removal of the prodomain, secondary processing can also take place and may also be important for activity. Recent studies on aggrecanase-1/ADAMTS4 suggest the possibility that a C-terminal fragment of this protein may function as a competitor for the activity of the mature enzyme (12). Also, active forms of aggrecanase-2/ADAMTS5 were identified as 64- and 50-kDa fragments. These forms are smaller than the predicted protein (~75-kDa) that should result upon sole removal of the prodomain (7). Finally, processed forms of METH-1 and METH-2 resulting from expression of the full-length cDNAs

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1 The abbreviations used are: MMP, matrix metalloprotease; BAEC, bovine aortic endothelial cells; ECM, extracellular matrix; TSP, thrombospondin type I repeat; TIMP, tissue inhibitors of metalloproteinases; APMA, p-aminophenylmercuric acetate; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
have been shown to display angi-inhibitory activity (1). Regulation of these proteolytic events is directly relevant to the mechanism of action of these proteins and their capacity to interact with cells and extracellular matrix molecules.

METH-1 and METH-2 are the only metalloprotein/ADAMTS members for which anti-angiogenic properties have been attributed (1). Angiogenesis represents a complex process that requires the presence and activation of a wide variety of molecules, including growth factors, extracellular matrix components, receptors, and proteolytic enzymes (13). Metallopro- 

EXPERIMENTAL PROCEDURES

Expression Vectors and Purification of Recombinant Proteins—Full-length METH-1 cDNA was cloned into pcDNA3.1 expression vector (Invitrogen, San Diego, CA). METH-1(MET-1; 

METH-1 was cloned by removing the 3′-chelate affinity chromatography. For purification, conditioned medium was passed through heparin Sepharose CL-6B (Amersham Pharmacia Biotech) columns, washed and eluted with increasing concentrations of NaCl (500 mM to 2 M). Zn2+/chelate affinity chromatography was performed using His(6)NTA agarose (Qiagen, Valencia, CA) and reconstituted in the following buffer: 100 mM Hepes, pH 7.5, 1 mM CaCl2. The digestion was performed at 37 °C with 1.4 μg/ml of purified recombinant furin as described previously (15). Reaction was stopped by the addition of 2× sample buffer (0.1% Triton X-100, 6.8% SDS, 20% glycerol, 1 mg/ml bromophenol blue, and 0.7 M 8-mercaptoethanol) and analyzed by immunoblot.

Treatment with Proteinase Inhibitors—Stable 293T cells expressing METH-1 were plated on 12- or 24-well plates. At 70% confluence, cells were rinsed and incubated with fresh serum-free medium in the presence or absence of different inhibitors. Conditioned medium was collected after 18 h for MMP treatment when the medium was collected at 18 h, and analyzed for the presence of METH-1 by immunoblot. Proteinase inhibitors included (a) the furin inhibitor AT-EK1 (14); (b) the MMP inhibitors doxycycline, minocycline and MMP inhibitor 1 (Calbiochem), TIMP-1 and TIMP-2 (Chemicon International; Te- mecula, CA), and 1,10-phenanthroline and EDTA (Sigma); and (c) apro- 

Protein Sequencing—Conditioned medium from stable 293T cells expressing the METH-1(3′-4′) construct was collected, and the C-termin- nal fragment containing the Myc-His tag was purified using Ni2+- 

Proteins were visualized by staining with Ponceau S (0.2% solution in 1% acetic acid); excised fragments were sent for sequencing. pLSHL-Hygro (Invitrogen) for further selection with hygromycin.

Treatment with MPPs—Purified p87-METH-1 protein or conditioned medium containing p87-METH-1 was treated with p-aminophen- 

METH-1/ADAMTS1 Processing

Localization Assays—Quiescent BAEC and human dermal fibroblast samples were analyzed by immunoblot analysis. All samples were analyzed by immunoblot.

Immunoblot Analysis—Samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were incubated with polyclonal antibody raised against recombinant METH-1 (1). After incubation with the appropriate peroxidase-conjugated secondary antibody, signal detection was performed with chemiluminescence (SuperSignal kit; Pierce).

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Purification of recombinant METH-1 protein included heparin and Zn2+/chelate affinity chromatography. For purification, conditioned medium was passed through heparin Sepharose CL-6B (Amersham Pharmacia Biotech) columns, washed and eluted with increasing concentrations of NaCl (500 mM to 2 M). Zn2+/chelate affinity chromatography was performed using His(6)NTA agarose (Qiagen, Valencia, CA). Binding of Zn2+ and equilibration of the column was performed following the manufacturer’s recommendations. Bound proteins were eluted with increasing concentrations of NaCl and NH4Cl or directly with EDTA. Specific binding to Zn2+ has been verified by using alternative cations chelated to the same resin. Further purification was achieved by 10–20% sucrose gradient centrifugation. Final separation and purity was assessed by immunoblot analysis and gel staining with GelCode® (Pierce). Concentration of protein was determined using the DC protein assay (Bio-Rad) and by reading at 280 nm.

The construction, expression, and purification of the serpin variant AT-EK1 was performed as described previously (14). Briefly, the cDNA encoding histidine-tagged AT-EK1 was subcloned in the prokaryotic expression vector pHEx-31 (Qiagen, Valencia, CA). Transformed BL21 cells were grown at 30 °C in TB medium, and expression was induced by 1 mM isopropyl-1-thio- 

RESULTS

Processing of METH-1 Protein—We have previously described the anti-angiogenic effects of a processed form of METH-1 (p65), suggesting that cleavage of the unprocessed protein is required for its anti-angiogenic activity. We therefore examined the proteolytic activity of recombinant furin (15).

Proteins were visualized by staining with Ponceau S (0.2% solution in 1% acetic acid); excised fragments were sent for sequencing. Samples were N-terminally sequenced on an Applied Biosystems Proc- 

Proliferation Assays—Quiescent BAEC were trypsinized and plated onto 24-well dishes in Dulbecco’s modified Eagle’s medium supplemented with 0.1% fetal calf serum, 2 ng/ml fibroblast growth factor-2, and 50 ng/ml vascular endothelial growth factor, in the presence or absence of recombinant METH-1 protein. A pulse of [3H]thymidine (1 μCi/well) was added during the last 8 h prior to harvesting. Cells were washed and treated in 10% trichloroacetic acid. Incorporation of [3H]thymidine was determined by scintillation counting, as described previously (16).

Localization Assays—Quiescent BAEC and human dermal fibroblast samples were trypsinized and plated onto six-well plates in Dulbecco’s modified Eagle’s medium supplemented with 0.1% fetal calf serum, 2 ng/ml fibroblast growth factor-2, and 50 ng/ml vascular endothelial growth factor, in the presence or absence of recombinant METH-1 protein. After 48 h of treatment, conditioned medium was concentrated using heparin Sepharose CL-6B, and dishes were rinsed twice with phosphate-buffered saline. Cell layer was extracted directly with loading buffer. The presence of METH-1 in conditioned medium and cell layer samples was assessed by immunoblot analysis.

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METH-1 (p110) in the cell layer (Fig. 1B). This form corresponds to the unprocessed pro-METH-1 (Fig. 1A). When we examined the conditioned medium, three METH-1 species (110, 87, and 65 kDa) were detected. By analysis of the deduced amino acid sequence of METH-1, the 87-kDa fragment (p87) would correspond to a mature form following the removal of the prodomain alone. The smaller 65-kDa fragment (p65) was consistently the most predominant form detected in the medium. p65 appeared to result from a second proteolytic event. We also detected both p110 and p87 forms associated with the ECM, but the levels were significantly reduced compared with those in the cell layer (data not shown).

To gain insight into the presence of specific domains in the processed forms of METH-1, we used heparin and Zn\(^{2+}\)-chelate affinity chromatography. For ADAMTS1 it has been determined that all of the TSP motifs found at the C terminus of the protein are important for heparin binding (17) and are likely to be the sites that confer heparin affinity to METH-1. Purification of conditioned medium containing METH-1 by heparin affinity chromatography revealed different affinities for each one of the processed forms (Fig. 2A). Using a continuous salt gradient (NaCl), the first form to be eluted was p65 (at 450 mM NaCl). Subsequently, p87 and p110 eluted at considerably higher salt concentration (800 mM NaCl) (Fig. 2B). The lower affinity of p65 for heparin was an indication that a portion of the carboxyl end of METH-1 containing all or some of the TSP repeats had been removed.

METH-1/ADAMTS1 contains a consensus zinc binding motif (HELGHVFNMPHID), which defines the metalloprotease domain. To evaluate the presence of this Zn\(^{2+}\) binding site in the three METH-1 forms, we subjected heparin-purified fractions to affinity chromatography on HiTrap-Zn\(^{2+}\) columns. Metal chelate affinity chromatography has been used previously to purify metalloproteases (18, 19) based on their ability to coordinate to metal ions. All forms of METH-1 were capable of binding the HiTrap resin, suggesting that the Zn\(^{2+}\) binding motif is present (Fig. 2C). These results indicate that the processing events described occur at both upstream and downstream positions of the core protein that maintains the metalloprotease domain.

**Fig. 1. Processing of METH-1. A, schematic structure of METH-1 showing the major structural domains and potential N-glycosylation sites (inverted triangles). The arrow indicates the cleavage site after the signal peptide. B, 293T cells were transiently transfected with a full-length METH-1 construct. Cell layer and conditioned medium were harvested and analyzed by immunoblot. In the cell layer, a major band at 110 kDa was detected. This form corresponds to unprocessed p110 pro-METH-1. In the conditioned medium, three major bands at 110, 87, and 65 kDa were detected. These correspond to unprocessed p110 and two processed forms of METH-1, p87 and p65, respectively.

**Fig. 2. METH-1 purification by heparin and Zn\(^{2+}\)-chelate affinity chromatography. A, conditioned medium from stable cells expressing METH-1 was subjected to heparin affinity chromatography. After washing, samples were eluted with increasing concentrations of NaCl (gradient 150 mM to 2 M). The graph represents the elution profile of total conditioned medium through a heparin-Sepharose column. B, an immunoblot of fractions showed elution of p65 at lower concentrations of salt. Higher salt concentrations released both p87 and p110 forms. C, fractions that resulted from heparin purification, with different proportions of METH-1 forms, were dialyzed and subjected to Zn\(^{2+}\)-chelate affinity chromatography. Bound proteins were washed and eluted. Fractions containing bound protein were analyzed by immunoblot. All forms, p110, p87, and p65, were able to bind to the Zn\(^{2+}\)-chelated column.

**Furin-dependent Processing of METH-1—**It has been shown that furin plays an important role in maturational processing of a diverse group of precursor enzymes. Most often, pro-protein cleavage by furin occurs in the trans-Golgi network, but recent studies have demonstrated that the enzyme can contribute to extracellular processing as well (11). Like other members of the metalloprospondin family, METH-1 contains a consensus furin cleavage sequence (RKKR235). Furthermore, cleavage of the mouse ortholog ADAMTS1 has been shown to be impaired in LoVo cells, which are furin-deficient (3). This processing was restored when LoVo cells were transfected with furin expression constructs. To investigate the location and timing of a furin-like cleavage event in METH-1, we analyzed whether pro-METHOD-1 processing was inhibited when the furin inhibitor AT-EK1 was co-expressed. The AT-EK1 molecule is a mutant serpin derived from the previously characterized furin inhibitor AT-PDX (14) but where the reactive site has a RIRR se-
sequence resembling the RKKR cleavage of METH-1. These inhibitors function by irreversibly binding to furin and abolishing the enzyme's proteolytic activity. When 293T cells were co-transfected with METH-1 and increasing concentrations of AT-EK1 expression construct, all processing of p110-METH-1 was blocked (Fig. 3A). We deduced that furin inhibition mediated by AT-EK1 directly affected p110 cleavage and that the first processing step was essential to enable the second catalytic event that generates p65-METH-1.

To extend these results, we examined processing of METH-1 in RPE.40 cells. These cells are a mutant Chinese hamster ovary K1 strain lacking furin activity (20). When RPE.40 cells were transfected with the full-length METH-1 cDNA, only unprocessed p110-METH-1 was detected (Fig. 3B). We deduced that furin inhibition mediated by AT-EK1 directly affected p110 cleavage and that the first processing step was essential to enable the second catalytic event that generates p65-METH-1.

To verify that the second cleavage event occurs at the C-terminal end, we analyzed the processing of a METH-1 construct containing a functional Myc tag at the carboxyl-terminal end (METH-1934Myc). This construct lacks the last 16 amino acids of full-length METH-1 yet includes all three TSP repeats (Fig. 4A). Cell layer and conditioned medium of 293T cells stably transfected with this construct were analyzed by immunoblot with specific antibodies for the Myc tag and METH-1 (Fig. 4B). The cell layer presented a unique band that corresponded to the unprocessed METH-1 (p110). As expected, this form was recognized by both Myc and METH-1 antibodies. The analysis of the conditioned medium showed distinct patterns when each antibody was used. Using the METH-1 antibody, we identified p87 but not p65, confirming that this second fragment results from a second processing event at the C-terminal end. The Myc antibody also detected a C-terminal fragment (~22 kDa) that resulted from the cleavage of p87.

To further elucidate the second processing step, we gener-
ated a truncated METH-1 form (METH-1744Myc) by introducing a Myc tag stop cassette at amino acid 744 and thereby removing the last two TSP repeats and part of the spacer region of native METH-1 (Fig. 4A). Transfection of this construct in 293T cells resulted in the detection of an unprocessed form (~90 kDa) in the cell layer (data not shown) and two proteolytic products of 70 kDa (TR.1) and 65 kDa (TR.2) in the conditioned medium (Fig. 4C) that appear to recapitulate the same processing events displayed by full-length METH-1. TR.1 and TR.2 would correspond to p87-like and p65-like processing events, respectively. When the same blot was probed with Myc antibody, only TR.1 was reactive. This result confirmed that the tag was removed by proteolytic cleavage at a position very close to residue 744.

Finally, we were able to purify the C-terminal Myc-tagged fragment resulting from the processing of the METH-1744Myc construct (Fig. 4B) and subsequently submitted it for N-terminal sequencing. The sequencing data showed several potential cleavage sites (at least four) between amino acids 717 and 800. However, the most predominant cleavage occurred at alanine 717.

MMPs Are Involved in the Second Processing Event of METH-1—To investigate the nature of the enzymes that are responsible for the second processing event, we incubated METH-1-producing cells with various protease inhibitors (Fig. 5A). These included several metalloprotease inhibitors (mimocynine, doxycycline, MMP inhibitor I, TIMP-1, TIMP-2, 1,10-phenanthroline, and EDTA) and the serine protease inhibitor aprotinin. Using nontoxic levels, we observed different degrees of inhibition. As expected, EDTA was able to inhibit both processing events because of its known inhibitory activity on MMPs and furin. Also, 1,10-phenanthroline was able to inhibit both cleavages, but the inhibition of the furin processing was not as efficient. Among the more specific MMP inhibitors tested, doxycycline was able to inhibit the second processing event of METH-1 completely. This compound has been described as a broad inhibitor of metalloproteases, but its specificity is not well known. MMPs inhibited by doxycycline include MMP8, MMP9, and MMP13 (21, 22). Finally, partial inhibition of this second cleavage was also observed when a mixture of TIMP-1 and TIMP-2 was used.

To test the ability of several MMPs to process p87-METH-1, we followed two different approaches. First, we used conditioned medium from 293T cells producing METH-1 that was treated previously with heparin. We observed that this treatment resulted in an almost complete inhibition of p87-METH-1 processing to p65 (Fig. 5B). This could relate to the ability of heparin to block cleavage sites, alter conformation of the protein, displace METH-1 from the cell surface and/or to its capacity to regulate the activity of other proteins, including MMPs and serine proteases (23, 24). Equal amounts of this conditioned medium were incubated with specific MMPs and screened for production of p65. A band with similar size to p65 was detected in the presence of MMP2, MMP8, and the membrane-anchored MMP15 (MT2-MMP), whereas MMP1, MMP3, MMP9, MMP14 (MT1-MMP), MMP16 (MT3-MMP), and MMP17 (MT4-MMP) had little or no effect (Fig. 5B). A second approach involved the use of purified p87-METH-1 eliminating the participation of heparin and other potential molecules present in the conditioned medium used above. MMPs used were MMP2 and MMP15. Generation of the p65 form occurred with both enzymes; however, MMP15 was more efficient (Fig. 5C). The potential autacatalytic activity of p87-METH-1 was also addressed using APMA, a known activator of several MMPs. Treatment with APMA did not generate p65-METH-1 (Fig. 5C).

Activity of METH-1 on Endothelial Cells—To determine whether p87 and p65 processed forms were equally active as inhibitors of endothelial cell proliferation, we purified both forms to homogeneity using heparin and metal-chelate affinity chromatography followed by centrifugation on sucrose gradients. The purity of each form was confirmed by immunoblot analysis and staining (Fig. 6A). We observed that both forms promoted a dose-dependent inhibitory effect on endothelial cell proliferation. Inhibition was detected at concentrations as low as 1 nM (Fig. 6B). However, we found that the p87 form was significantly more potent (2-fold) than p65 when used at 10 nM
The three forms of METH-1 were completely inhibited by doxycycline and partially by the combination TIMP-1/TIMP-2. Immunoblot. Both processing events were inhibited by EDTA and partially by 1,10-phenanthroline; the second cleavage (p87 to p65) was not treated with heparin was also analyzed to compare the molecular size of the second processed form. Several MMPs released a clear 65-kDa fragment that co-migrated with the control sample. Processing to p65 was previously blocked by treatment with heparin. After 16–18 h of treatment at 37 °C, samples were evaluated by immunoblot for the presence of METH-1 forms. Conditioned medium from 293T cells expressing METH-1 not treated with heparin was also analyzed to compare the molecular size of the second processed form. The arrows indicate the three forms of METH-1. Min., minocycline; Aprot., aprotinin; Hep., heparin; Cont., control.

DISCUSSION

In this study, we demonstrate that maturation of METH-1, a member of the metalloproteinin/ADAMTS family, requires two independent and sequential processing events that release two forms of 87 and 65 kDa, respectively. Generation of p87 by a furin-like enzyme removes the N-terminal prodomain, a process that has been common to several ADAM and MMP proteins. The proteolytic deletion of the last two TSP repeats resulting in the formation of p65-METH-1 alters its affinity to the cell layer and its extracellular distribution, an event that is probably significant for the in vivo function of this protein.

A common requisite for activation of matrix metalloproteases is a primary proteolytic event that removes an N-terminal prodomain (10, 25, 26). Studies performed with a truncated form of ADAMTS1, the mouse ortholog of METH-1, showed that the protein was processed by the convertase furin (3). The ability of furin to process a broad range of substrates is facilitated by its expression in many tissues throughout development and its localization in multiple cellular compartments, including the trans-Golgi network, cell surface, and endosomes (11). In addition, some studies have suggested that furin can also exist as a soluble/secreted or “shed” form (27) that could play a role in processing extracellular precursor proteins. Our results reveal that pro-METH-1 is transported via the secretory pathway and is subsequently processed at two different sites (Fig. 7). The absence of processed forms in the cell lysate together with the inhibition of processing upon exogenous administration of specific furin inhibitor AT-EK1 suggests that this processing occurs in the late trans-Golgi pathway or extracellularly. Further studies are required to fully clarify the location where this event takes place, since it is possible that the inhibitor enters the endocytic pathway and inactivates furin within intracellular compartments. In any case, METH-1 adds to the list of proteins that are activated by furin. The high expression of METH-1 in the heart and its known effects on the vasculature (1) make this protein another potential cause for the abnormal vascular phenotype found in animals lacking furin (28).

In addition to removal of the prodomain, we presented evidence that METH-1 protein undergoes a second proteolytic cleavage at the carboxyl end and, thereby, releases a second active form with modified adhesion properties. Importantly, this second processing event is dependent on the first processing step. Since there is no second furin site in METH-1, we speculate that initial processing enables a second catalytic event, most likely through alterations in protein structure or by activation of secondary proteases. For some metalloproteases, it has been shown that a second processing event is also required for modulation of activity. Brooks and colleagues (29) have demonstrated that a naturally occurring carboxyl-termi-
nal fragment of MMP2 could modulate in vivo MMP2 activity by interaction with α,β, integrin. A recent study on the activity of aggrecanase-1/ADAMTS4 discussed the possibility that a C-terminal fragment of this enzyme containing the TSP motif can negatively regulate aggrecan degradation (12). It is interesting to speculate whether the second processing event described for METH-1 may also be involved in the regulation of its activity. Additional experiments using the C-terminal fragment containing the last two TSP repeats are currently being tested.

**Fig. 6.** Effect and localization of different METH-1 forms on endothelial and fibroblast cultures. A, immunoblot and staining showing the complete separation and purification of both p65 and p87-METH-1 forms for functional studies. B, proliferation assay performed with quiescent BAEC during 48 h, using increasing concentrations of both p65 and p87-METH-1 forms. Values were normalized to 100% of growth factors alone. C, after 48 h of treatment of endothelial cells with p65 or p87-METH-1, cell layer and conditioned medium were analyzed by immunoblot. p87-METH-1 was retrieved only from the cell layer, while p65-METH-1 was confined to the conditioned medium. The arrowhead signals degradation products. D, after 48 h of treatment of endothelial and fibroblast cultures with METH-1, cell layer and conditioned medium were analyzed by immunoblot. p87 fragment was distinctly found in the cell layer of BAEC and conditioned medium of human dermal fibroblast (HDF). p65-METH-1 detected in the conditioned medium was the result of minimum processing of p87 and quality of the original preparation.

**Fig. 7.** Summary of processing events and distribution of METH-1 forms. A, general structure of METH-1, indicating the two different processing events described in this report. A first proteolytic step is mediated by furin (consensus sequence: RKKR) to produce p87-METH-1. p65-METH-1 results from a second processing step at the spacer region and is mediated by MMPs-like activity. B, representation of METH-1 processing and effects in endothelial cells. The secreted p110 pro-METH-1 is processed to p87-METH-1 by furin. This form is found in the conditioned medium or loosely associated with the cell layer of the producing cells. A second cleavage by MMPs occurs to yield p65-METH-1. When METH-1 is added to endothelial cultures, p87 localizes on the cell layer, and p65 remains soluble in the medium. Both forms are able to inhibit endothelial proliferation.
We observed that some MMPs, as major enzymes responsible for processing extracellular proteins, were capable of cleaving METH-1, producing a fragment of identical molecular size (65 kDa) to the moiety observed in cell cultures. To support this concept, the addition of TIMP-1 and TIMP-2 to cultures that overexpress METH-1 significantly reduced the processing of p87 to p65. These data confirm the notion that MMPs can also process nontraditional MMP substrates (30, 31) and support a major role of proteolytic enzymes in modulating angiogenesis. Recent studies have shown how proteolysis of collagen XVIII, plasminogen, and antithrombin can release antiangiogenic peptides such as endostatin, angiostatin, and cleaved antithrombin, respectively (32–34). Whether MMPs are the proteases that mediate METH-1 processing in vivo has yet to be confirmed. Very likely, several proteases are involved in its processing in different tissues. Also, the inhibition of the second processing of METH-1 by heparin reaffirms the relevance of this second processing event. One possibility is that heparin allows METH-1 to acquire a specific conformation that enhances its suppressive properties in vivo. These distinct propositions would both be consistent with the angiogenic switch hypothesis during tumor progression (43). Overexpression of matrix metalloproteases could accelerate the cleavage of METH-1, removing this protein from its “environment” and disrupting its ability to suppress local tumor neoangiinarization.

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REFERENCES

1. Vázquez, F., Hastings, G., Ortega, M. A., Lane, T. P., Oikonomou, S., Lombardo, K. C., and Terashima, Y., and Matsuishi, K. (1999) J. Biol. Chem. 274, 23259–23257

2. Kuno, K., Kanada, N., Nakashima, E., Fujiki, F., Ichimura, F., and Matsuishi, K. (1997) J. Biol. Chem. 272, 556–562

3. Kuno, K., Terashima, Y., and Matsuishi, K. (1999) J. Biol. Chem. 274, 18821–18826

4. Shindo, T., Kurihara, H., Kuno, Y., Yokoyama, H., Wada, T., Kurihara, Y., Imai, T., Wang, Y., Ogata, M., Nishimoto, H., Moriyama, N., Oh-hashi, Y., Muto, H., Ishikawa, T., Hamashima, Y., Yasaki, Y., and Matsuishi, K. (2000) J. Clin. Invest. 105, 1345–1352

5. Colge, A., Li, S., Sieron, A. L., Nuñez, B. V., Prokop, D. J., and Lapiere, C. M. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 2574–2579

6. Tortorella, M. D., Burn, T. C., Pratta, M. A., Abbadazzi, I., Hollis, J. M., Liu, R., Rosenfeld, S. A., Copeland, R. A., Decicco, C. P., Wynn, R., Rockwell, A., Yano, F., Duke, J. L., Solomon, R., George, H., Bruckner, R., Nagase, H., Itoh, Y., Ellis, D. M., Ross, H., Wissiall, B. H., Murphy, K., Hillman, M. C., Jr., Hollis, G. F., Newton, B. C., Magolda, R. L., Trzaskos, J. M., and Arner, E. C. (1999) Science 284, 1664–1666

7. Abbadazzi, I., Liu, R., Yano, F., Rosenfeld, S. A., Ross, H., Link, J. R., Ellis, D. M., Tortorella, M. D., Pratta, M. A., Hollis, J. M., Wynn, R., Duke, J. L., George, H., Hillman, M. C., Jr., Murphy, K., Wissiall, B. H., Copeland, R. A., Decicco, C. P., Bruckner, R., Nagase, H., Itoh, Y., Newton, R. C., Magolda, R. L., Trzaskos, J. M., Hollis, G. F., Arner, E. C., and Burn, T. C. (1999) J. Biol. Chem. 274, 23443–23450

8. Blelloch, R., and Kimmie, J. (1999) Nature 399, 586–590

9. Hurschelen, T. L., Hirohashi, S., Seldin, M. F., and Apte, S. S. (1999) J. Biol. Chem. 274, 25555–25563

10. Wolsberg, T. G., and White, J. M. (1996) Dev. Biol. 180, 399–401

11. Molloy, S. S., Anderson, E. D., Jean, F., and Thomas, G. (1999) Trends Cell Biol. 9, 28–35

12. Tortorella, M., Pratta, M., Liu, R.-Q., Abbadazzi, I., Ross, H., Burn, T., and Arner, E. (1998) FEBS Lett. 426, 41–46

13. Denault, J. B., Claing, A., D’Orleans-Juste, P., Sawamura, T., Kido, T., Masaki, T., and Leduc, R. (1995) J. Biol. Chem. 270, 13912–13917

14. Van der Stappen, J. W., Hendriks, T., and de Man, B. M. (1992) Int. J. Biochem. 24, 725–735

15. Kuno, K., and Matsushima, K. (1998) J. Biol. Chem. 273, 13912–13917

16. Morita, H., Ishikawa, T., Nagai, R., Yazaki, Y., and Matsushima, K. (2000) J. Biol. Chem. 275, 18821–18826

17. Kuno, K., and Matsushima, K. (1998) J. Biol. Chem. 273, 13912–13917

18. Van der Stappen, J. W., Hendriks, T., and de Man, B. M. (1992) Int. J. Biochem. 24, 725–735

19. Matsuo, C., Fukao, H., and Mateu, O. (1988) J. Cell. Physiol. 134, 253–260

20. Spence, M. J., Sucic, J. F., Foley, B. T., and Moehring, T. J. (1995) J. Biol. Chem. 270, 13912–13917

21. Smith, G. N., Jr., Mickler, E. A., Hasty, K. A., and Brandt, K. D. (1999) Arthritis Rheum. 42, 1140–1146

22. Greenwald, R. A., Moak, S. A., Ramamurthy, N. S., and Golub, L. M. (1992) Mol. Genet. 21, 414–430

23. Hurschelen, T. L., Hirohashi, S., Seldin, M. F., and Apte, S. S. (1999) J. Biol. Chem. 274, 25555–25563

24. Yu, W. H., and Woessner, J. F. J. (2000) J. Biol. Chem. 275, 5556–5562

25. Hurschelen, T. L., Hirohashi, S., Seldin, M. F., and Apte, S. S. (1999) J. Biol. Chem. 274, 25555–25563

26. O’Reilly, M. S., Wiederschain, D., Stetler-Stevenson, W. G., Folkman, J., and Massague, J. (1996) J. Biol. Chem. 271, 11376–11382

27. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. (1997) Cell 88, 277–285

28. O'Reilly, M. S., Wiederschain, D., Stetler-Stevenson, W. G., Folkman, J., and Moses, M. A. (1999) J. Biol. Chem. 274, 29568–29571
34. O'Reilly, M. S., Pirie-Shepherd, S., Lane, W. S., and Folkman, J. (1999) Science 285, 1926–1928.
35. Jin, L., Abrahams, J. P., Skinner, R., Petitou, M., Pike, R. N., and Carrell, R. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14683–14688.
36. Carrell, R. W. (1999) Science 285, 1861.
37. Woods, A., and Couchman, J. R. (1998) Trends Cell Biol. 8, 189–192.
38. Zhang, Y., Cao, L., Yang, B. L., and Yang, B. B. (1998) J. Biol. Chem. 273, 21342–21351.
39. Steinfeld, R., Van Den Berghe, H., and David, G. (1996) J. Cell Biol. 133, 405–416.
40. Kinsella, M. G., Tesi, C. K., Jarvelainen, H. T., and Wight, T. N. (1997) J. Biol. Chem. 272, 318–325.
41. Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. (1997) J. Clin. Invest. 99, 2062–2070.
42. Perrimon, N., and Bernfield, M. (2000) Nature 404, 725–728.
43. Hanahan, D., and Folkman, J. (1996) Cell 86, 353–364.