TIMP-2 Is Required for Efficient Activation of proMMP-2 in Vivo*

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Matrix metalloproteinases (MMPs) are synthesized as latent proenzymes. A proteolytic cleavage event involving processing of the cysteine-rich N-terminal propeptide is required for their full activation. Previous in vitro studies indicated that activation of proMMP-2 can occur through formation of a trimolecular complex between MMP-14, TIMP-2, and proMMP-2 at the cell surface. Using TIMP-2-deficient mice and cells derived from them, TIMP-2 was shown to be required for efficient proMMP-2 activation both in vivo and in vitro. The requirement for TIMP-2 was not cell-autonomous as exogenously added TIMP-2 could restore activation of proMMP-2 to TIMP-2-deficient cells. Mutant mice were overtly normal, viable, and fertile on the C57BL/6 background, indicating that both TIMP-2 and activated proMMP-2 are dispensable for normal development.

More than 20 matrix metalloproteinases (MMPs)1 have been described that collectively degrade all extracellular matrix and a number of non-matrix proteins involved in inflammation and cell growth control (reviewed in Ref. 1). The MMPs and their specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs)2 have been associated directly and indirectly with many developmental processes such as branching morphogenesis (2–6), regulation of cell migration (7), apoptosis (8, 9), angiogenesis (10), and regulation of innate immunity (11).2 In addition to their link with developmental events, MMPs have been implicated in several disease processes such as tumor metastasis (12), arthritis (13), and emphysema (14, 15). They are regulated in many ways that include transcriptional (16) and post-translational mechanisms. Two post-translational means of MMP regulation have been described. First, MMPs are synthesized as latent pro-enzymes. A cysteine-rich N-terminal peptide of the latent proenzyme interacts with the Zn(II) of the active site, blocking proteolytic activity of the proteinase inhibitor α2-macroglobulin (18) and by four known MMP-specific tissue inhibitors of metalloproteinases or TIMPs (19–22). The mechanisms by which MMPs are proteolytically processed from their latent to their activated forms and how they are regulated by inhibitors are important to understanding their contributions to normal developmental and pathological processes.

Evidence exists for several mechanisms of proteolytic activation of the latent proMMPs. For example, plasmin can participate in the activation of proMMP-1(23), -3 (24), -7 (25), -9 (26, 27), -13 (28), and -14 (29), whereas furin can activate the membrane-associated proMMP-14 (30), proMMP-11 (31), and others. Already activated MMPs can also contribute to activation of other proMMPs. For example, MMP-3 can activate proMMP-1 (32), and -proMMP-7 (25) and MMP-14 together with MMP-2 can contribute to the activation of proMMP-13 by a cascade mechanism (28).

In biochemical studies designed to elucidate the mechanisms of proMMP-2 activation, it was shown that membrane fractions from proMMP-2-activating cells were able to catalyze the proteolytic processing of latent proMMP-2 and that both the membrane-associated MMP-14 and TIMP-2 participate in the activation (33–35). This process of activation first involves an initial cleavage of proMMP-2 by MMP-14 at the Asn37-Leu38 bond of proMMP-2 followed by an autocatalytic cleavage of the intermediate product at the Asn57-Tyr61 bond, generating the fully active MMP-2 (36). Although TIMP-2 is an inhibitor of MMPs, paradoxically in this mechanism it can function as a co-activator of proMMP-2 as well. Evidence indicates that TIMP-2 participates in the activation of proMMP-2 by MMP-14 by facilitating the assembly of a complex on the cell surface that brings the two MMPs together. In this trimeric complex, the C termini of proMMP-2 and TIMP-2 interact while the N termini of MMP-14 and TIMP-2 interact (34–41). The ability of TIMP-2 to participate in this mechanism is sensitive to the local extracellular matrix environment (42, 43). Furthermore, TIMP-2 may be completely dispensable for proMMP-2 activation since the extracellular domain of MMP-14 can lead to proMMP-2 activation in the absence of any TIMP-2 (44). Such TIMP-2-independent pathways may exist that allow intracellular activation of latent proMMP-2 (45).

If the TIMP-2-dependent mechanism of proMMP-2 activation is the primary means for activating the latent proenzyme in normal tissues or cultured primary cells, then conversion of the latent MMP-2 proenzyme to its active form would fail in tissues or cells deficient for TIMP-2. To test this hypothesis and to study other in vivo functions of TIMP-2 in normal development and disease processes, mice carrying a null mutation on chromosome 11 were generated. Reported here is the development of TIMP-2-deficient mice and studies of proMMP-2 activation in tissues and cells derived from mutant animals.

* This work was supported by the Roswell Park Alliance and National Institutes of Health Grants EY 11279 (to P. D. S.) and CA 16056 (to the Roswell Park Cancer Institute). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase; ConA, concanavalin A; APMA, 4-aminophenylmercuric acetate.

MATERIALS AND METHODS

Generation of Mutant Mice—A genomic DNA library prepared from 129SvEv mice was screened for clones hybridizing to a bovine TIMP2 cDNA probe (46). A Timp2 targeting vector was prepared as described in the results section using these clones and gene targeting performed by standard methods as described (47).

Zymography—Protein gels containing 10% polyacrylamide were co-polymerized with 1 mg/ml gelatin. MMP-containing lung extracts or cell culture-conditioned media were loaded and run at 4 °C at 15–20 mA constant current for 2–4 h in 1× electrophoresis buffer (0.025 M Tris, 0.192 mM glycine, 0.1% SDS, pH 8.5). Gels were washed twice for 20 min each with fresh wash buffer (50 mM Tris, 5 mM CaCl₂, 0.02% NaN₃, 2.5% Triton X-100, pH 7.5). The in-gel gelatinolytic reactions were performed by incubating the gel in incubation buffer (50 mM Tris, 5 mM CaCl₂, 0.02% NaN₃, 2.5% Triton X-100, pH 7.5) at 37 °C for 16–24 h. Gels were stained for 1–2 h in 0.25% Coomassie Blue R, 30% methanol, and 10% acetic acid, then destained in 30% methanol, 10% acetic acid to achieve optimum contrast.

Lung Extracts—Lysetes of mouse lung were prepared by homogenizing 60- to 100-mg tissue pieces, fresh or previously flash-frozen in liquid nitrogen, at 4 °C in 1 ml of radiolabeled precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-Cl, pH 8.0) per 3 mg of tissue. Lysetes were centrifuged at 14,000 × g for 15 min in a Sorvall SS-34 rotor, and the insoluble extracellular matrix-enriched fractions were washed in radiolabeled precipitation buffer then analyzed by zymography directly or stored as aliquots at −80 °C for later analysis. Immediately before loading samples on gels, the extracted matrix-rich pellet was resuspended in 1 μl of 2× sample loading buffer (0.125 M Tris-Cl, pH 6.8, 20% glycerol, 3% SDS, 0.1% w/v bromphenol blue) per 3 mg of sample. Samples were then incubated at 37 °C for 10 min followed by centrifugation at 16,000 × g for 10 min in an Eppendorf microcentrifuge. Twelve to 20 μl of supernatant were loaded per lane.

In Vitro Studies—Primary fibroblasts were prepared from embryos isolated at day 13.5 post-coitus. Cells were plated in 24-well or 48-well plates in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and cultured for 1–2 days until 75–85% confluent. Cells were washed with Hanks’ balanced salt solution three times and cultured for an additional 24 h in serum-free medium (OptiMem, Life Technologies, Inc.). Cultures were supplemented with concanavalin A (ConA, 50 μg/ml) or human recombinant TIMP-1 or -2 protein generously provided by H. Nagase and W. Stetler-Stevenson. Conditioned medium was removed 24 h after culturing in OptiMem, centrifuged three times to remove cells, and analyzed immediately by zymography using 12–20 μl/ lane. For proMMP-2 activation with 4-aminophenyl mercuric acetate (APMA), serum-free conditioned medium from primary embryonic fibroblasts was treated with 20 μg APMA for 3–6 h at 37 °C before analysis by zymography.

RESULTS

Generation of TIMP-2-deficient Mice—To generate TIMP-2-deficient mice, a targeting construct was prepared in which a 4.4-kilobase pair Smal genomic DNA fragment containing the first coding exon of Timp2 and additional 5′ genomic sequences was deleted and replaced with a PGKNeo expression cassette. A 5.0-kilobase pair Smal fragment served as the 5′ homologous arm, and a 0.7-kilobase pair Smal to KpnI fragment served as the 3′ arm. The HSTYR marker was fused to the 3′ flank (Fig. 1A). After electroporation of the linearized vector into J1 embryonic stem cells, colonies resistant to G418 and FIAU (1-[2-A]-dideoxy-2-fluoro-β-D-arabinofuranosyl]-5-iodouracil; Bristol Meyers Squibb, Princeton, NJ) were screened by homologous recombination. Genomic DNA was extracted from Southern blots hybridized with HindIII-digested embryonic stem cell DNA using the probe shown in Fig. 1A. Homologous recombinants were identified at a frequency of 20% (Fig. 1B). Chimeric mice prepared by blastocyst injection of mutant cells transmitted the mutation to their progeny, which were genotyped by a PCR assay (Fig. 1C). An assay for Timp2 mRNA in lung from wild-type, heterozygous, and homozygous mutant mice by Northern blot analysis revealed that neither of the two Timp2 transcripts accumulated in homozygous mutant mice, whereas a reduced level of Timp2 mRNA accumulated in heterozygote relative to wild-type animals (Fig. 1D). The Timp2 mutation had no overt effects on viability or fertility of mice when maintained on a C57BL/6 background. Normal litter sizes and Mendelian distributions of progeny were recovered from all crosses involving heterozygous or homozygous mutant mice. TIMP-2 has been shown to have erythropoietin-potentiating activity; however hematocrit analysis of blood from mutant and wild-type mice revealed no differences (data not shown).

TIMP-2-deficient Mice Are defective for proMMP-2 Activation—In vitro studies have demonstrated that TIMP-2 can participate in the activation of latent proMMP-2 through the formation of a trimolecular complex that includes TIMP-2, proMMP-2, and MMP-14 (34–40). To determine if loss of TIMP-2 led to a defect in proMMP-2 activation in vivo, zymography was performed on lung extracts from wild-type mice and animals heterozygous and homozygous for the mutant allele of Timp2. Results using lung extracts from wild-type and heterozygous mutant animals were qualitatively similar, revealing the latent form of proMMP-2 migrating at 68 kDa and the fully activated 62-kDa form. However, extracts from mutant mice lacked the fully activated form of MMP-2; only the 68-kDa latent form was visible (Fig. 2). To determine if the failure of proMMP-2 activation was due to loss of expression of other critical factors believed to be needed for its activation, Northern blot analysis was done using RNA from mutant or wild-type lungs and Mmp2 and Mmp14 as probes. mRNAs encoding both MMPs were easily detected in lungs from mice of both genotypes (Fig. 3). The results indicated that functional TIMP-2 protein is required for efficient activation of latent proMMP-2 in vivo.

Failure of proMMP-2 Activation in Cultures of TIMP-2-deficient Embryonic Fibroblasts—In vitro cell culture systems facilitate additional opportunities for experimental manipulation. Therefore, embryonic fibroblast cultures were established from individual embryonic day 13.5 sibling mouse embryos arising from an intercross between heterozygote and...
proMMP-2 and the activated forms are indicated. Cells prepared from lungs of wild-type (+/+), heterozygous (+/−), or homozygous mutant (−/−) mice were analyzed by zymography. Conditioned medium from (+/+ cells was used as a marker. The positions of latent proMMP-2 and the activated forms are indicated.

FIG. 2. Zymographic analysis of mouse lung extracts. Extracts prepared from lungs of wild-type (+/+), heterozygous (+/−), or homozygous mutant (−/−) mice were analyzed by zymography. Conditioned medium from (+/+ cells was used as a marker. The positions of latent proMMP-2 and the activated forms are indicated.

FIG. 3. Northern analysis of lung RNA. RNA isolated from lungs of wild-type (+/+ or TIMP-2-deficient (−/−) mice was analyzed by Northern blots hybridized to cDNAs for Mmp2, Mt1mmp, and Rpl32 as a loading control.

homozgyous mutant mice. After expanding cells in serum-containing medium, cells were washed and cultured in serum-free medium with or without ConA for an additional 24 h. Conditioned media were analyzed by gelatin zymography for gelatinase activity. The results showed that homozgyous mutant cells were deficient for activation of the 68-kDa proMMP-2 relative to Timp2-expressing cells (Fig. 4A). Extending the time of culture to 48 h revealed that proMMP-2 remained in its latent form in cultures of homozgyous mutant cells (data not shown). This failure of proMMP-2 activation was seen using cells from multiple independent mouse embryos and in fibroblasts from post-partum day 2 neonates (data not shown). The same phenotype was seen using cultured embryonic cells from mice carrying an independent allele of Timp2 (prepared by Caterina et al. (56)). In those mice, the mutant allele gave rise to a mRNA with an internal in-frame deletion that was translated. The mutant protein had residual MMP-inhibitory activity, however, the specific activity was 300 fold lower than that of wild-type protein (56).

It was possible that failure of proMMP-2 activation in mutant cultures resulted from an alteration that affected the ability of the proMMP-2 protein to become activated by any mechanism. To test this possibility, conditioned media from cultured cells were treated with APMA to activate by chemical means latent proMMP-2 before analysis by zymography (Fig. 4B). APMA treatment was able to activate partially the latent proenzyme in both mutant and Timp2-expressing cultures. The approximately equal accumulation of the activation intermediate in both cultures is consistent with previous observations that APMA treatment generally does not lead to complete proMMP-2 activation (48) and indicates that loss of TIMP-2 did not render proMMP-2 insensitive to activating stimuli.

Loss of proMMP-2 Activation by TIMP-2-deficient Cells Can Be Restored by Exogenously Added TIMP-2—The effect of TIMP-2 loss on proMMP-2 activation could be cell-autonomous, requiring TIMP-2 production by a cell for the cell to activate proMMP-2. Alternatively, the defect in proMMP-2 activation by mutant cells may be complemented by exogenously added TIMP-2. To distinguish between these possibilities, recombinant human TIMP proteins were added to mutant or TIMP-2-producing cells. The addition of TIMP-2 to cultured cells stimulated proMMP-2 activation by homozgyous mutant cells at TIMP-2 concentrations ranging from 0.15 to 180 ng/50 μl, resulting in accumulation of the proMMP-2 activation intermediate (Fig. 5, first panel). In Timp2-expressing cells, the addition of TIMP-2 stimulated proMMP-2 activation at TIMP-2 concentrations between 0 and 0.15 ng/50 μl. At concentrations higher than 1.5 ng/50 μl, exogenously added TIMP-2 inhibited proMMP-2 activation (Fig. 5, third panel). This is consistent with observations that efficient proMMP-2 activation is highly sensitive to the stoichiometric ratios of TIMP-2 to other components of the activation complex (35, 49). As a control, human TIMP-1 was added to cultures of TIMP-2-producing and -deficient cells. TIMP-1 was unable to stimulate proMMP-2 activation in TIMP-2-deficient cells at concentrations as high as 180 ng/50 μl (Fig. 5, second panel). This is consistent with previous observations that TIMP-1 cannot participate in promoting proMMP-2 activation (36). However, at concentrations above 5 ng/ml, TIMP-1 did inhibit proMMP-2 activation in TIMP-2-producing cells, confirming that TIMP-1-sensitive, MMP-mediated proteolysis is required for proMMP-2 activation (Fig. 5, fourth panel).

DISCUSSION

Mice carrying a targeted mutation in Timp2 were generated. No Timp2 mRNA was detected in tissues from mutant animals, indicating the mutation was null. Loss of TIMP-2 did not adversely affect normal mouse development, viability, or fertility on the C57BL/6 background. However, loss of the inhibitor did result in a dramatic reduction in activation of the latent proenzyme, proMMP-2, in lung tissue in vivo and cultured primary fibroblasts in vitro. This demonstrates that TIMP-2 is not only a potential component of the proMMP-2 activation mechanism, as indicated by several published reports, but in fact it is...
required for normal activation of the latent proenzyme in vivo. It cannot be ruled out that TIMP-2-independent mechanisms exist for activating proMMP-2. However, if such mechanisms exist, they are far less efficient at inducing proMMP-2 activation than the mechanism involving TIMP-2 in the tissue and cells analyzed in this study. Failure of proMMP-2 activation in and of itself was expected to have minimal developmental consequences, consistent with the lack of a developmental phenotype in mice deficient for MMP-2 (50). However, any phenotypes seen in MMP-2-deficient mice may also be expected to be seen in TIMP-2-deficient mice.

Because the proMMP-2 activation defect observed in cultures of Timp2 mutant cells could be suppressed by exogenously added recombinant human TIMP-2 protein, the requirement for TIMP-2 in proMMP-2 activation is not cell autonomous. The effects of added TIMP-2 protein on proMMP-2 activation in the culture systems used were either stimulatory or inhibitory, depending upon the concentration of TIMP-2 protein in the cultures. This is consistent with observations that the stoichiometry of the various co-activators and of the latent proMMP-2 protein is critical in governing proMMP-2 activation. Although exogenously added human TIMP-2 could partly restore proMMP-2 activation to homozygous mutant cells, the activation products that accumulated were intermediates and not fully active MMP-2. The reasons for the partial activation are not clear. There may be important species differences between mouse and human TIMP-2. The specific preparations of human TIMP-2 used may have been only partly active. Finally, the cellular location where TIMP-2 acts to promote proMMP-2 activation, is dependent upon the presence of TIMP-2. These studies are ongoing. Other TIMPs may also play a direct role in activation of proMMPs. TIMP-4 is able to interact with the C-terminal region of proMMP-2 in a manner similar to that of TIMP-2 (53). Whether it, like TIMP-2, is required for efficient proMMP-2 activation is not known.

Other important functional differences between TIMPs 1 and 2 have been revealed during analysis of mutant mice. Although TIMP-2 loss resulted in defects in proMMP-2 activation, this was unaffected by a mutation within Timp1. Also, although TIMP-1-deficient animals are hyper-resistant to corneal infections with Pseudomonas aeruginosa by a complement-dependent mechanism, TIMP-2-deficient mice have normal immune responses (11, 54). It is possible, however, that other MMP-sensitive immune response mechanisms, possibly involving defensin production (55), are altered.

Acknowledgments—We are grateful to Drs. Hideaki Nagase and William Stettler-Stevenson for generously sharing recombinant TIMP proteins and Drs. Birkedal-Hansen and John Caterina for sharing cells and unpublished results.

REFERENCES

**Fig. 5.** Zymographic analysis of conditioned medium from primary cells grown in the presence of exogenously added human TIMPs. Embryonic day 13.5 fibroblasts isolated from Timp2-expressing (+/-) or Timp2 mutant (-/-) siblings were cultured for 24 h in serum-free medium with (+) or without (-) 50 μg/ml Con A, and the indicated quantities of human TIMPs (hTIMP) were added to 50 μl cultures. The positions of latent proMMP-2 (closed triangle) and the activated forms (open triangle) are indicated.

**TABLE 1.** Differences are likely to result from the particular structural features of the two TIMPs. One specific feature that may allow TIMP-2, but not TIMP-1, to participate in proMMP-2 activation is the 10-residue-long negatively charged C-terminal tail of TIMP-2, which is only 3 residues long in TIMP-1 (41). This tail may enable TIMP-2 to form salt bridges with specific positively charged residues at the junction of C-terminal hemopexin domains III and IV of pro-MMP-2, which are required for proMMP-2 to form a complex with TIMP-2 (37, 51). A second structural difference between TIMPs 1 and 2 that may enable TIMP-2 to participate in proMMP-2 activation lies at the N terminus. Residues within the unique long N-terminal A-B beta-hairpin loop of TIMP-2 are required for specific interactions with MMP-14 (52). TIMP-1 is 7 residues shorter in this domain, which significantly shortens the A-B beta-hairpin. This and other loop structure differences between the two TIMPs cause them to associate very differently with MMPs and may prevent TIMP-1 from interacting with MMP-14 in a way that enables it to replace TIMP-2 as a co-activator of proMMP-2 (41).

Activation mechanisms for proMMPs other than proMMP-2 have been described as cell surface events requiring membrane type MMPs. For example, like proMMP-2, proMMP-13 can be activated by a mechanism involving MMP-14 and MMP-2 (28). It is not yet known if efficient proMMP-13 activation, like proMMP-2 activation, is dependent upon the presence of TIMP-2. These studies are ongoing. Other TIMPs may also play a direct role in activation of proMMPs. TIMP-4 is able to interact with the C-terminal region of proMMP-2 in a manner similar to that of TIMP-2 (53). Whether it, like TIMP-2, is required for efficient proMMP-2 activation is not known.
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doi: 10.1074/jbc.M001270200 originally published online May 25, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001270200

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