Inactivating Mutation of the Mouse Tissue Inhibitor of Metalloproteinases-2 (Timp-2) Gene Alters ProMMP-2 Activation*

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To understand the biologic function of TIMP-2, a member of the tissue inhibitors of metalloproteinases family, an inactivating mutation was introduced in the mouse Timp-2 gene by homologous recombination. Outbred homozygous mutants developed and procreated indistinguishably from wild type littermates, suggesting that fertility, development, and growth are not critically dependent on Timp-2. Lack of functional Timp-2, however, dramatically altered the activation of proMMP-2 both in vivo and in vitro. Fully functional Timp-2 is essential for efficient activation of proMMP-2 in vivo. No evidence of successful functional compensation was observed. The results illustrate the duality of Timp-2 function, i.e. at low concentrations, Timp-2 exerts a “catalytic” or enhancing effect on cell-mediated proMMP-2 activation, whereas at higher concentrations, Timp-2 inhibits the activation and/or activity of MMP-2.

Tissue inhibitors of metalloproteinases (TIMPs) constitute a family of at least four distinct but homologous proteins that are believed to play a role in the regulation of matrix degradation and remodeling in both normal and disease states (1, 2). TIMPs inhibit the catalytic activity of matrix metalloproteinases (MMPs) and in some cases also inhibit the proteolytic activation of MMP zymogens required for attainment of catalytic activity. Among members of the TIMP family, the primary structure is highly conserved. However, TIMPs 1, 2, 3, and 4 differ somewhat in inhibitory selectivity against the various MMPs. The TIMPs also display greatly different patterns of expression (1, 3). Timp-2 is widely expressed in the body and MMPs. The TIMPs also display greatly different patterns of activity. Among members of the TIMP family, the primary structure is highly conserved. However, TIMPs 1, 2, 3, and 4 differ somewhat in inhibitory selectivity against the various MMPs. The TIMPs also display greatly different patterns of expression (1, 3). Timp-2 is widely expressed in the body and occurs either in free form or bound to (inactive) proMMP-2 (4, 5). It inhibits in the nanomolar range virtually all MMPs against which it has been tested by formation of classical binary non-covalent complexes (6). A number of studies have suggested that Timp-2 regulates the proteolytic activation of proMMP-2 on the cell surface by a membrane-type MMP-dependent process (7–9). The suggested model implicates Timp-2 as a bridging molecule, tethering proMMP-2 through binding between the COOH-terminal ends of the two molecules and binding between the MT-MMP and TIMP-2 NH2-terminal ends (7). In this way Timp-2 may play a biological role in the activation of MMPs as well as through the inhibition of MMP-activity (10). To analyze the significance of the various proposed roles of Timp-2, we targeted the mouse Timp-2 gene for mutation by homologous recombination.

MATERIALS AND METHODS

Isolation and Characterization of the Mouse Genomic Timp-2 Locus

Screening of a axfII mouse genomic DNA library with human Timp-2 cDNA (11) yielded 2 overlapping clones, each of which contained exons 2, 3, and 4 of the mouse Timp-2 gene. To obtain the two terminal Timp-2 exons, primer pairs were designed to the 5′ (sense, CTGCCG-CCTTGGACAAAGA; antisense, TGCAATTGGCAAAACGCCTGTT) and 3′ (sense, CTTGACATCGAGGACCCGTAAGAAG; antisense, CAT-GGGGTCGACTGGAACCTC) untranslated regions of the mouse Timp-2 cDNA (12). PCR of those probes was used to screen pools of 129/OLA mouse genomic DNA PI phase clones (Genome Systems, St. Louis, MO) to obtain the entire Timp-2 locus. Three clones were obtained. Due to the size of intron 1 (>50 kb), they have not been fully sequenced. All DNA sequencing was performed by the National Institute of Dental and Craniofacial Research (NDICR) sequencing core facility using an Applied Biosystems Inc. automated DNA sequencer. Primers for the various sequencing reactions were provided either by the sequencing core or by the University of Alabama at Birmingham Cancer Center oligonucleotide synthesis core facility. Sequencing results were confirmed by multiple overlapping reactions on each strand. Genetics Computer Group software was used to analyze all sequence data (13).

Construction of the Targeting Vector and Creation of Transgenic Mice

Using the vector pNTK (14) as the framework for the construction of a targeting vector, a 2.2-kb BamHI fragment upstream of mouse Timp-2 exon 2 and a 6-kb HindIII fragment from a 9-kb EcoRI subclone downstream of mouse Timp-2 exon 3 were cloned in as the 2 homology regions. The construct was linearized with SalI, purified, and electroporated into the embryonic stem cell line HM-1 (15). Electrooporated cells were subjected to 300 μg/ml Geneticin (Life Technologies, Inc.) and 2 μg/ml ganciclovir (Roche Molecular Biochemicals) selection. Purified DNA of surviving colonies was genotyped by both PCR and Southern blot analyses. For PCR analysis, two primer pairs were designed to amplify sequences spanning the 5′ homology region. The first was made to the endogenous locus: sense, ATTTCCGACATGTTTATGGAA; antisense (sequence deleted in mutant locus), GCCCTGGTGTGTCTCATAG. The second was made to the mutant locus sense, AAGG-GAAAGCTTTCGAGA; antisense (phosphoglycerate kinase promoter), CTTTACTTGGAGGAGAGTTTGGTGTG. Detection of the mutant locus by Southern blot analysis was observed as a 1.8-kb decrease in size (7.5 to 5.7 kb) of the EcoRI band, hybridizing to a 1-kb XbaI/BamHI probe just upstream of the 5′ homology region. The overall targeting efficiency was 9/72 (~13%). Five of the 9 homologous recombinant...
binants were injected into 72-h-old blastocysts from C57BL/6 mice (NCI-Frederick, Frederick, MD) and implanted into pseudopregnant B6D2 or C57BL/6 X DBA females (NCI-Frederick, Frederick, MD). Offspring were mated to generate heterozygous animals for the targeted gene in either an out-bred background to C57BL/6 females (NCI-Frederick) or in a mixed background to 129/R1/SvJ females (The Jackson Laboratory, Bar Harbor, ME). Heterozygous animals were subsequently interbred to generate homozygous mutant progeny in both genetic backgrounds.

RNA Analysis

A mouse multiple tissue Northern blot (CLONTECH Palo Alto, CA) was hybridized with a 250-base pair probe corresponding to the mouse Timp-2 gene located on mouse chromosome 11 (described above as the proximal probe for the Timp-2 gene locus). The manufacturer’s protocol was followed. For analysis of gene targeting, total RNA was isolated from newborn mouse tissues by snap-freezing in liquid N2 followed by extraction in TRIzol (Life Technologies). Purified RNA was subjected to Northern analysis by conventional methods (16).

In this case, the 5' Timp-2 probe could not be used due to cross-hybridization with 28 S rRNA. Therefore, this blot was probed with 170 base pairs of KpnI fragment from the mouse Timp-2 cDNA containing portions of exons 4 and 5. The novel splice junction was cloned by reverse transcription-PCR using the primer pair sense, CCTCCTGCTGTTAGCCTCCTGCCT- GTG (exon 1), and antisense, TGACCGAGTCCAATCAGGGCCTCTC (exon 4). The amplification product was cloned into pCRIIscript (Stratagene, La Jolla, CA) and sequenced.

Cell Culture

Skins of 14.5-day-post-coitus embryonic and newborn mice were floated dermis side down on 0.25% trypsin in Hanks’ balanced salt solution (Life Technologies) at 4 °C for 8 h. The epidermis was discarded, and the dermis was dissociated by forcing it through a syringe. The cell suspension was seeded in Dulbecco’s modified Eagle’s medium (Life Technologies) with 50% heat-inactivated fetal calf serum (HyClone Laboratories, Inc., Logan, UT). The suspension concentration was gradually reduced to 10% for maintenance. HT1080 cells (ATCC, Manassas, VA) were also maintained in Dulbecco’s modified Eagle’s medium + 10% heat-inactivated fetal calf serum.

Protein Analysis

Western Blotting—Serum-free conditioned medium from mouse dermal fibroblasts was precipitated with an equal volume of ethanol and resuspended in gel loading buffer containing 2% SDS, 10 mM dithiothreitol, and 1% β-mercaptoethanol. The samples were boiled and resolved by SDS-polyacrylamide gel electrophoresis (4–12% NuPAGE Bis-Tris gel run with MES buffer, Novex, San Diego, CA), electroblotted onto nitrocellulose, and probed with anti-TIMP-2 monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) followed by exposure to Renaissance solutions (NEN Life Science Products) and autoradiography. The films were stained with Coomassie Blue.

Isolation of proMMP-2 Timp-2 Complexes—Gelatin-Sepharose (0.2 ml) equilibrated with 50 mM HEPES buffer, pH 7.5, 200 mM NaCl, 5 mM CaCl2, 15 mM Na2SO4, was mixed with 100 mL of conditioned fibroblast medium derived from Timp-2 heterozygous mice. The suspension was rotated end over end overnight, and the gel was repeatedly washed with the same buffer. Bound fibronectin was removed by a single wash with 1 x arginine, 50 mM HEPES buffer, pH 7.5, for 30 min. After an additional washing step, proMMP-2/Timp-2 complex was eluted by boiling in gel loading buffer containing 2% SDS under reducing conditions.

Collagen Fibril Dissolution

To analyze the ability of live cells to degrade fibrillar collagen, 24-well tissue culture plates were coated with reconstituted collagen fibrils by polymerizing 0.4 ml/well of a 300 μg/ml neutralized solution of rat tail tendon type I collagen for 2 h at 37 °C (18). The collagen fibril film was air-dried and washed with several changes of distilled water and culture medium. Aliquots (25 μl) of a suspension of 106 cells/ml were dispensed onto the center of each well. The cells were allowed to attach for 5 h, and the wells were washed with Hanks’ balanced salt solution (Life Technologies). Mouse skin fibroblasts were grown in serum-free medium (OptiMEM, Life Technologies) for up to 4 days. HT1080 cells were pretreated for 12 h with 1.6 × 10−7 M phorbol 12-myristate 13-acetate (Sigma), then incubated with either Dulbecco’s modified Eagle’s medium plus 1% fetal calf serum with and without 3 μg/ml purified human TIMP-1 or TIMP-2. The cells were removed with trypsin-EDTA (Life Technologies) supplemented with 1% Triton X-100, and collagen remaining in the wells was visualized by staining with Coomassie Blue.

Preparation of Lung Homogenates—Lungs of 3-week-old mice were homogenized individually in a Dounce homogenizer in 20 ml of radiomimune precipitation buffer (16). The homogenates were subjected to centrifugation at 14,000 × g for 15 min. The pellets were resuspended in 120 μl of gel loading buffer, incubated for 10 min at 37 °C, and microcentrifuged for 5 min. The supernatants were analyzed by zymography.

RESULTS

The structure of the mouse Timp-2 gene locus is shown in Fig. 1, A and B. To generate a mutation of the mouse Timp-2 gene, a targeting vector was designed that removes exons 2 and 3. High percentage chimeric animals produced from five different homologous recombinants were mated to C57BL/6 mice, and chimeras from four of the five clones resulted in germine transmission of the targeted allele. Chimeras with the highest percentage germine transmission for two of the clones were also mated to 129/ReJ mice, and each produced germine transmission of the targeted allele to the inbred background as well. Interbred heterozygotes gave rise to the expected Mendelian distribution of the two alleles in both the out-bred and inbred backgrounds. Genotyping of F1 offspring is shown in Fig. 1C.

Phenotype of Timp-2-deficient Mice—Mice homozygous for the mutant Timp-2 locus developed without immediately apparent (0–120 weeks) macroscopic or histologic abnormalities in the out-bred background. They were able to repeatedly procreate, produce, and nurture seemingly healthy offspring. Lifespan based on 70 mouse (half wild type, half mutant) appeared unaltered beyond 2 years regardless of gender. Because TIMP-2 is involved in the cellular activation of proMMP-2, we sought to determine whether this process was altered in mutant mice. Zymography of matrix-enriched lung extracts (Fig. 2) showed that although most of the proMMP-2 was already converted to active form in normal littermates, only a small fraction of the proenzyme was processed in the lungs of mutant mice, suggesting that the mutation had resulted in impairment of MMP-2 activation in vivo. We then asked whether the TIMP-2 mutation also impaired the autocatalytic activation of proMMP-2 by organonemeric intermediates with MT1-MMP-free conditions. To determine this skin fibroblast cultures were derived from 14.5-day-post-coitus embryos and neonates for each genotype and background. Fig. 3 shows that proMMP-2 from homzygous mutant cells is converted significantly faster (>30 fold) that than from wild type cells. Although full activation required overnight (16 h) incubation of wild type medium, this process was completed in 30 min in the TIMP-2 mutant media (Fig. 3A). The rate of conversion was again reduced by addition of exogenous TIMP-2 (Fig. 3B).

The observation that TIMP-2 deficiency abrogates the physiologic (presumably MT1-MMP-dependent) activation of proMMP-2 in mutant Timp-2 mice but significantly accelerates the MT1-MMP-independent, organonemeric-induced autocatalytic conversion of the enzyme illustrates the duality of
TIMP-2 function in MMP-2 activation. Previous studies suggest that TIMP-2 in a biphasic manner promotes the MT1-MMP-dependent processing of proMMP-2 at low concentrations but inhibits it at higher concentrations (9). Exposure of live cells to concanavalin A has previously been shown to induce or greatly enhance the cell-mediated (and presumably MT1-MMP-dependent) conversion of proMMP-2 to its active form. Homozygous mutant cells displayed a decidedly biphasic response to addition of exogenous TIMP-2. In the range 5 to 100 ng/ml, TIMP-2 greatly accelerated proMMP-2 conversion, whereas concentrations of 500 ng/ml or higher increasingly blocked conversion (Fig. 4). In wild type cells, which by reverse zymography contained in the range 400–500 ng/ml endogenous TIMP-2, conversion of proMMP-2 was inhibited at all concentration.

Expression of Wild Type and Mutant Timp-2 Gene Loci—Mouse Timp-2 transcript is expressed in 2 size species, 1.1 and 3.5 kb, resulting from the use of alternative polyadenylation signals. Expression levels vary among tissues as do the ratios between the two transcripts, but some level of expression can be detected in all tissue types tested (Fig. 5A) as previously

FIG. 1. The mouse Timp-2 gene locus and targeting strategy. A, shown are the positions of the original two overlapping 5′ flanking mouse genomic DNA clones containing Timp-2 exons 2, 3, and 4. The gene targeting construct is also shown. The complete mouse Timp-2 locus isolated from a P1 phage insert is also shown with the uncharacterized portion of intron 1 denoted by //. Restriction endonuclease sites are BamHI (B), EcoRI (E), and XbaI (X). Restriction sites in parentheses are not complete analyses of the portions of the locus shown but are included for the purpose of identification of sites mentioned in the text: H, HindIII; K, KpnI; P, PstI. The positions of the 5′ and 3′ probes used in the isolation of the P1 clone are noted as are the positions of the five numbered Timp-2 exons. The mutant locus (M) can be observed as a 1.8-kb decrease in size of the EcoRI band hybridizing to a probe just upstream of the 5′ homology region as compared with that of the wild type locus (W). This decrease results from a novel site (E) within polylinker sequence just upstream of pgk-NEO introduced into the locus though the recombination event. B, exon (uppercase)/intron (lowercase) border sequences and sizes. C, genotype analyses of Timp-2 gene-targeted mice. Shown are representative genotype analyses of DNA from mice with homozygous wild type (+/+), heterozygous (+/−), and homozygous mutant (−/−) Timp-2 loci by both PCR (left panel) and Southern blot (right panel). Samples run are noted above each lane (for PCR, whether wild type (W) or mutant (M) primers were used is also noted). nt, nucleotides.

FIG. 2. Impairment of proMMP-2 activation in mutant mice. Gelatin zymogram of SDS extract of lungs of four wild type and four homozygous mutant mice.

FIG. 3. Regulation of organomercurial activation by TIMP-2. Samples of culture medium from wild type (+/+), heterozygous (+/−), and homozygous mutant (−/−) mouse fibroblasts were incubated at 37 °C with or without 1 mM p-aminophenyl mercuric acetate (APMA) and in the presence or absence of exogenous TIMP-2 (final concentration, 10 μg/ml). Aliquots were removed at various time points, and 1,10-phenanthroline was added to stop the reactions. Aliquots from the various time points were analyzed by gelatin zymography. A, time course of MMP-2 activation by p-aminophenyl mercuric acetate. B, effect of exogenous TIMP-2 on MMP-2 activation in mutant conditioned medium.
shown (5). Expression from the mutant locus produced two new species of 0.9 and 3.3 kb corresponding to loss of exons 2 and 3 (Fig. 5B). All four species were expressed in heterozygous mice. To verify that a splicing event was taking place on message produced from the mutant locus resulting in attachment of exon 1 to exon 4, reverse transcription-PCR was performed across the splice junction, and the amplification product was cloned and sequenced (Fig. 5C). This confirmed the predicted splicing event and verified that the original reading frame of the message remained intact, suggesting that any protein made following transcription from this locus would contain the correct COOH-terminal sequence downstream of Ala-89 (Fig. 5D).

**Mutant TIMP-2 Is Produced in Targeted Mice**—To determine if the mutant TIMP-2 transcript was being translated, fibroblast-conditioned medium was probed with two anti-TIMP-2 mAbs (Fig. 6B), mAb 67–4H11e, recognizing sequences in the COOH-terminal tail predicted to be present also in any in-frame mutant protein (residues 178–193), and mAb 68–6H4, recognizing epitopes in the deleted region (residues 30–44). An immunoreactive polypeptide of the predicted size (14 kDa) was produced by cells containing the mutant locus and absent in cells that do not (Fig. 6B). The wild type TIMP-2 product (22 kDa), as expected, was not detected in samples from homozygous mutant mice. The reactivity with the two mAbs confirmed that the mutant polypeptide contained the COOH-terminal tail epitope but lacked the deleted epitope in the NH2-terminal domain. This observation provided additional and independent evidence of an in-frame deletion with nucleotide and amino acid sequences in correct register. Densitometry of Western blots suggested that mutant cells secreted in the range 5–10-fold less TIMP-2-like protein than wild type cells on a molar basis.

**Mutant Cells Express <1% of Wild Type TIMP-2 Inhibitory Activity**—Having established that the predicted mutant protein was being expressed and possessed the correct amino acid sequence downstream of the splice site, we sought to assess any residual TIMP-2-type inhibitory function by two different lines of experiments. First, reverse zymography (Fig. 6C) showed no TIMP-2 inhibitory activity at the position of wild type TIMP-2 from homozygous mutant cells, but a barely visible band was noted at the predicted (14 kDa) size, suggesting that this protein might still possess some, albeit minimal, inhibitory activity against MMP-2. The activity of other TIMP bands in the same reverse zymograms did not appear to have changed noticeably because of loss of functional TIMP-2, which all but dispels the notion of compensatory up-regulation of other

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**Fig. 4.** Effect of exogenous TIMP-2 on activation of proMMP-2. Gelatin zymogram of 40-h culture medium from wild type and homozygous mutant fibroblasts grown in increasing concentrations of exogenous TIMP-2. ProMMP-2 activation is induced by exposure to 20 μg/ml concanavalin A (ConA) for the duration of the incubation period.

**Fig. 5.** Analyses of mouse Timp-2 RNA transcripts. A, mouse multiple tissue Northern blot (2 μg poly(A) mRNA/lane) probed with a portion of the mouse Timp-2 cDNA. The probe recognizes both the 1.1-kb and 3.5-kb forms of the Timp-2 message. A third transcript is seen only in testis. B, Northern blot analysis of total RNA from wild type (1/2), heterozygous (1/1), and homozygous mutant (2/2) neonates. Transcripts being made from the mutant Timp-2 locus splicing together exons 1 and 4 form two new RNA species with a decrease in size directly related to the loss of exons 2 and 3. C, ethidium bromide-stained gel shows the inserts resulting from PstI/KpnI digestion of the cloned splice junctions from RNA of wild type and homozygous mutant mice; reverse transcription-PCR was performed with primers spanning the proposed novel splice junction from the mutant; the respective expected products were cloned, sequenced, and compared. D, sequence of mouse Timp-2 with the portion missing in the mutant in lowercase. The positions of the primers used to PCR from exon 1 to exon 4 are underlined, and the PstI and KpnI sites used to clone the resulting products are marked. The amino acid sequence is also shown with that missing in the mutant italicized. The stop codon is designated by an asterisk. bp, base pair(s).
TIMPs. Densitometry of reverse zymograms performed on serial dilutions of culture medium from (-/-), (+/-), and (+/+), mice suggested that the residual TIMP-2-type inhibitory activity released by the cells was in the range 100–300-fold (190 ± 90) lower than that of wild type cells. Second, we assessed the loss of function of TIMP-2-type inhibitory activity in a cell-based assay. When attached, HT1080 cells efficiently degrade fibrillar type I collagen by a process that is inhibitable by TIMP-2 but not by TIMP-1 (Fig. 6D). Concentrated culture medium from wild type fibroblasts inhibited degradation of the collagen fibril film to a level comparable with that obtained by the addition of purified TIMP-2, whereas similarly concentrated medium from homozygous mutant cells did not (Fig. 6D).

Since the induced mutation largely destroyed inhibitory activity against MMP-2, we asked whether the proMMP-2 binding activity, which resides in the intact COOH-terminal part of the molecule, might still be intact (20). We addressed this question by gelatin-Sepharose affinity isolation of proMMP-2 and any (mutant or wild type) TIMP-2 protein already bound to the proenzyme. Fig. 7 shows that products from both alleles were bound to proMMP2. Moreover, comparison of the ratio of mutant and wild type TIMP-2 secreted by heterozygous cells before and after gelatin-Sepharose purification showed that mutant TIMP-2 bound to proMMP2 more or less to the same extent as wild type TIMP-2. Based on densitometry of Western blots, we surmised that the mutant locus on a molar basis was 5–6-fold less TIMP-2-like protein than the wild type locus in the same cells. These findings suggest that the COOH-terminal domain of the mutant TIMP-2 polypeptide adopts a configuration (perhaps by independent folding) that leaves intact the proMMP-2 binding activity.

**DISCUSSION**

Our findings show that TIMP-2-deficient mice display no gross anatomical or microscopic abnormalities and retain reproductive capability despite severe impairment of proMMP-2 activation in vivo and in vitro. Although the mode and effect of binding of TIMPs to activated MMPs is well understood and based on a straightforward paradigm, the highly specific binding of TIMP-2 to the MMP-2 zymogen (and TIMP-1 to the MMP-9 zymogen) is not. Zymogen binding is mediated predominantly or exclusively by the COOH-terminal domain of the inhibitor, whereas inhibitory binding involves the NH2-terminal domain (20, 21). Our findings show that lack of functional TIMP-2 severely impairs the endogenous activation of MMP-2 in vivo, a process that is presumably mediated by a membrane-bound MMP such as MT1-MMP. This observation provides strong evidence that the role of TIMP-2 in the activation proMMP-2, which has largely been deduced from in vitro studies (9), is indeed an important biological function. It is of note that the subtlety of the TIMP-2 deficient phenotype is not dissimilar to that of the MMP-2-deficient mouse (22).

Our approach to targeted mutation of the Timp-2 locus deleted exons 2 and 3 but preserved the downstream reading frame intact. We reasoned that the mutant protein encoded by this construct, if synthesized and secreted, would be severely impaired in inhibitory activity for the following reasons. (i) The deleted amino acid sequence (residues 18–89) contains two of the major sites of interaction of TIMPs with active MMPs (6, 23) (Fig. 6A), and (ii) manipulation of the corresponding region of TIMP-1 around and including Cys-70 (41) would either impair the endogenous activation of MMPs by TIMP-1 and proMMP-2, as has been deduced from in vitro studies (9), is indeed an important biological function. It is of note that the subtlety of the TIMP-2 deficient phenotype is not dissimilar to that of the MMP-2-deficient mouse (22).

When exposed to concanavalin A, fibroblasts acquire the ability to rapidly and efficiently activate both endogenous and exogenous proMMP-2 (19). This process is likely mediated by MT1-MMP in a ternary plasma membrane-bound complex with TIMP-2 and proMMP-2. Our findings that the proMMP-2-activating capacity of cells derived from mutant animals was impaired (although the proMMP-2 binding function of mutant
TIMP-2 was not) are consistent with that model. Moreover, the dual activating/inhibiting role of TIMP-2 on proMMP-2 activation was clearly demonstrated by add-back experiments using increasing levels of exogenous, wild type TIMP-2. Activation of proMMP-2 was first accelerated at low concentrations of TIMP-2, then increasingly inhibited by a molar excess of TIMP-2 in a manner similar to that observed by Kinoshita et al. (9).

Recent studies by Overall et al. (21) identify the TIMP-2 binding site on the proMMP-2 hemopexin domain and added further evidence that the binding of TIMP-2 to the MMP-2 zymogen, unlike inhibitory binding, is mediated between the two COOH-terminal domains. This observation and our finding that the mutant TIMP-2 retains binding activity with proMMP-2 raises the question of whether the mutant TIMP-2 may still participate in an activation-promoting binding reaction or perhaps act as a competitive blocking agent. Side-by-side comparisons with cells derived from the "Soloway" null mice described in the accompanying paper (30), however, yielded essentially identical outcomes and failed to identify any dominant (negative or positive) effects of the mutant TIMP-2 species. While, however, because the low level of expression of the mutant TIMP-2 (5–10-fold less than wild type on a molar basis), it is not possible at present to resolve the question of whether the binding of mutant TIMP-2 promotes MMP-2 activation or competitively blocks it, but for the same reasons it does not appear to play a role in expression of the phenotype.

We asked the question of whether loss of TIMP-2 inhibitory function also regulates other MMP-2 activation processes, and indeed it does, but in an entirely different manner. Conformation-induced autolytic activation following exposure to organo-mercurials, which results in opening of the cystine switch (27), was accelerated by as much as 30-fold. We interpret these findings to indicate that the regular (wild type) activation process is slowed significantly by the inhibitory function of TIMP-2, then increasingly inhibited by a molar excess of TIMP-2 in a manner similar to that observed by Kinoshita et al. (9).

TIMP-2 inhibition (see accompanying article (30)), which independently created a mouse line with a targeted disruption of the Timp-2 locus that lacks TIMP-2 transcripts altogether. Results from our laboratories have shown similar if not identical phenotypes. This further suggests that the mutant TIMP-2 produced in our line is unable to compensate for the loss of wild type TIMP-2 activity. As well, the possibility that there might be feedback regulatory mechanisms at work in the Soloway line due to the absence of TIMP-2 transcript can be dismissed due to the production of stable transcripts in our line. These two TIMP-2-deficient mouse lines will provide valuable resources for further investigations into the role played by TIMP-2 in various pathologic processes.

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Fig. 7. Mutant (Mut.) TIMP-2 binds to proMMP-2. Western blot stained with mAb 67–4H11. Lane 1, concentrated culture medium from heterozygous (+/−) fibroblasts before gelatin-Sepharose affinity isolation. Lanes 2–5, bound protein eluted by boiling in gel loading buffer containing 2% SDS.

Finally of note is that many of the results presented here have been obtained in cooperation with the Soloway laboratory (see accompanying article (30)), which independently created a mouse line with a targeted disruption of the Timp-2 locus that lacks TIMP-2 transcripts altogether. Results from our laboratories have shown similar if not identical phenotypes. This further suggests that the mutant TIMP-2 produced in our line is unable to compensate for the loss of wild type TIMP-2 activity. As well, the possibility that there might be feedback regulatory mechanisms at work in the Soloway line due to the absence of TIMP-2 transcript can be dismissed due to the production of stable transcripts in our line. These two TIMP-2-deficient mouse lines will provide valuable resources for further investigations into the role played by TIMP-2 in various pathologic processes.
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