Individual Timp Deficiencies Differentially Impact Pro-MMP-2 Activation*

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Membrane-type matrix metalloproteinases (MT-MMPs) have emerged as key enzymes in tumor cell biology. The importance of MT1-MMP, in particular, is highlighted by its ability to activate pro-MMP-2 at the cell surface through the formation of a trimeric complex comprised of MT1-MMP/tissue inhibitor of metalloproteinase-2 (TIMP-2)/pro-MMP-2. TIMPs 1–4 are physiologically MMP inhibitors with distinct roles in the regulation of pro-MMP-2 processing. Here, we have shown that individual Timp deficiencies differentially affect MMP-2 processing using primary mouse embryonic fibroblasts (MEFs). Timp-3 deficiency accelerated pro-MMP-2 activation in response to both cytochalasin D and concanavalin A. Exogenous TIMP-2 and N-TIMP-3 inhibited this activation, whereas Timp-3 containing matrix from wild-type MEFs did not rescue the enhanced MMP-2 activation in Timp-3−/− cells. Increased processing of MMP-2 did not arise from increased expression of MT1-MMP, MT2-MMP, or MT3-MMP or altered expression of TIMP-2 and MPP-2. To test whether increased MMP-2 processing in Timp-3−/− MEFs is dependent on Timp-2, double deficient Timp-2−/−/−/− MEFs were used. In these double deficient cells, the cleavage of pro-MMP-2 to its intermediate form was substantially increased, but the subsequent cleavage of intermediate-MMP-2 to fully active form, although absent in Timp-2−/−/− MEFs, was detectable with combined Timp-2−/−/−/−/− deficency. Timp-4 associates with MMP-2 and MT1-MMP in a manner similar to Timp-3, but its deletion had no effect on pro-MMP-2 processing. Thus, Timp-3 provides an inherent regulation over the kinetics of pro-MMP-2 processing, serving at a level distinct from that of Timp-2 and Timp-4.

Matrix metalloproteinases (MMPs) are fundamental to biological processes because of their ability to cleave and remodel the extracellular matrix (ECM). MT1-MMP is one of six cell surface membrane-type MMPs (MT-MMP). Its activity and regulation have been widely studied in the context of cell surface MMP activation, cell migration, and invasion (1–3), independently and in conjunction with other cell adhesion molecules (4–10). A key function of MT1-MMP is to process pro-MMP-2, whose activity also correlates with an invasive propensity in several cancers and is predictive of poor survival (reviewed in Refs. 11–14). Understanding the regulation of pro-MMP-2 processing by MT1-MMP and other members of the MT-MMP family is central to defining their role in cancer biology.

The classical model for the cell surface activation of MMP-2 is through the formation of a trimeric complex comprising MT1-MMP, TIMP-2, and pro-MMP-2 (15). The transmembrane MT1-MMP interacts via its N-terminal domain to the N terminus of TIMP-2, forming a “receptor” onto which pro-MMP-2 (72 kDa) binds. Pro-MMP-2 is initially cleaved to its intermediate form (64 kDa) by an adjacent active MT1-MMP. The second stage of MMP-2 processing, resulting in its fully active form (62 kDa), involves an autocatalytic event that requires an MMP-2 molecule in trans (16). It is known that of the six MT-MMPs, MT2−, 3−, 5−, and 6-MMP (17–21) also have the capacity to activate pro-MMP-2. Alternative mechanisms of MMP-2 processing at the plasma membrane, such as the urokinase plasminogen system (22, 23) or TIMP-independent processing involving MT2-MMP, have also been suggested (24). Although the dynamics of the trimeric complex have been well studied, new insights into its regulation and control are continually being discovered. More recently, cell surface processing of pro-MMP-2 is reported to occur through formation of a trimeric complex comprised of MT3-MMP, TIMP-3, and pro-MMP-2 (25), although TIMP-3 is known not to form similar complexes with MT1-MMP (26).

TIMPs are the naturally occurring inhibitors of metalloproteinate activity. There are four members of the TIMP family, and each has a specific niche with respect to function. Studies have focused on the dual role of Timp-2 in regulating the processing of pro-MMP-2. A threshold level of Timp-2 is required in relation to MT1-MMP to construct the trimeric complex, which still leaves sufficient MT1-MMP un inhibited to cleave pro-MMP-2. At higher concentrations, Timp-2 prevents MMP-2 processing by inhibiting all free MT1-MMP (27–29). The presence of Timp-2 was initially considered necessary to achieve any form of MMP-2 processing (30, 31), but recently Timp-2−/− cells were shown capable of some processing to the intermediate form (32, 33). Although Timp-1 is known not to inhibit MT1-MMP and does not associate as strongly with MMP-2 (26, 34–36), Timp-4, the most recently discovered member of the family (37, 38), is able to associate with and inhibit both MT1-MMP and MMP-2. It is, however, not able to replace Timp-2 in trimeric complex formation with MMP-2 and MT1-MMP (32, 39, 40). The role of Timp-3, which can inhibit both MT1-
MMP and MMP-2, is currently less well understood with respect to trimeric complex function (25, 26, 34, 39). TIMP-3 is unique among the TIMP family in that it is bound to the ECM rather than remaining a freely soluble protein (41, 42). Additionally, it has a broader inhibition profile that extends to members of the ADAM (a disintegrin and metalloproteinase domain) and ADAM-TS families, proteases that have the potential to control the bioactivity of many growth factors and cytokines (reviewed in Ref. 43). TIMP-3 is also implicated in the regulation of apoptosis (44–46). In the TIMP-3-deficient mouse we have previously observed greater matrix degradation, indicating increased MMP activity, as well as unscheduled MMP-2 activation (44, 47, 48). In the present study we used primary mouse embryonic fibroblasts from TIMP-deficient mice to determine the physiological roles of individual TIMPs in pro-MMP-2 processing. TIMP-3−/− mouse embryonic fibroblasts (MEFs) displayed an accelerated rate of pro-MMP-2 processing, whereas Timp-4−/− MEFs showed no alteration. We further investigated the necessity of TIMP-2 during the activation process in TIMP-3−/− cells using double deficient Timp-2−/− / Timp-3−/− MEFs. This study provides a comprehensive parallel assessment of each TIMP in cell surface processing of pro-MMP-2 and highlights a unique regulatory function of TIMP-3.

MATERIALS AND METHODS

Experimental Animals—Timp-3−/− mice were generated as previously described (47) and backcrossed at least six times into either FVB or C57Bl/6 background. Timp-2−/− and Timp-1−/− mice were generated as previously reported (30, 49) and are in the C57Bl/6 background. Timp-4−/− mice generated in Dr. E. Vuorio’s laboratory (University of Turku, Finland) were maintained in C57Bl/6 background. Controls included mice with identical backgrounds to the experimental animals. All animals were cared for in accordance with guidelines of the Canadian Council for Animal Care.

MEF Isolation and Maintenance of Cells—Primary MEFs were isolated from embryos at day 13.5–15.5 of gestation. Cells were maintained in DMEM + 10% fetal bovine serum. All experiments were performed using non-immortalized MEF cultures maintained for 4–6 passages. Cell stimulation experiments were carried out in 24-well culture dishes, and cells were seeded the night prior to the experiment at a density of 1 × 10^5 cells/well in 1 ml of DMEM + 10% fetal bovine serum. The following day the subconfluent monolayers were washed twice with serum-free DMEM prior to treatment with concanavalin A (con A; 10 or 50 μg/ml) or cytochalasin D (cyto D; 1 μg/ml) (Sigma) in serum-free DMEM supplemented with insulin transferrin, sodium-selenite (Sigma) in a 250-μl volume. At a given time point conditioned medium and lysates (prepared in radioimmune precipitation assay buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% nonidet P40, 1% Triton X-100 + inhibitors) were collected.

TIMP Purification and Inhibition Experiments—Human recombinant TIMP-1 was expressed in Chinese hamster ovary K-1 cells and purified from the conditioned medium. Human recombinant TIMP-2 was expressed in 293-EBNA cells (Invitrogen) using the mammalian expression vector pCEP4 (Invitrogen). TIMP-2 was purified from the conditioned medium as described previously (51). Recombinant N-terminal domain of TIMP-3 (N-TIMP-3) was prepared as described by Kashivagi et al. (52). Each TIMP was added at 10, 25, 50, 75, or 100 nM 1 h prior to the addition of con A. N-TIMP-3 was dissolved in a glycerol-containing buffer (0.125%), and this was included as an exper-

3 I. Koski, W. Cruz-Muñoz, D. Rahkonen, M. Toriseva, R. Kiviranta, V. Kytö, A. Saraste, S. Okoskki, E. Jokinen, V.-M. Kähäri, R. Khokha, and E. Vuorio, manuscript in preparation.
Loss of TIMP-3 Accelerates Processing of Pro-MMP-2—Primary MEFs were treated with cyto D or con A, which are known to induce pro-MMP-2 processing (57–60). MMP-2 activation was studied over the course of 24 h using SDS-PAGE gelatin zymography of cell lysates and conditioned medium. Comparable pro-MMP-2 levels were present at the stage of MMP-2 processing previously shown to be sensitive to inhibition by TIMP-1 (16). This effect was, however, not seen in the absence of TIMP-3. MMP-2 activation was much slower but still accelerated in Timp-3-deficient cells (lanes 14 and 15, 16 and 17). Increased MMP-2 activation was not observed in the unstimulated primary Timp-3−/− MEF cultures. This effect of accelerated MMP-2 processing due to the loss of TIMP-3 was mirrored in analyses performed with cell lysates (Fig. 1B). In fact, as early as 1 h post-stimulation, processing of pro-MMP-2 was observed in the Timp-3−/− MEF lysates, whereas none was apparent in the wild-type cells (data not shown). These data indicate an accelerated rate of MMP-2 processing in the absence of TIMP-3.

Enhanced MMP-2 Processing in the Absence of TIMP-3 Is Inhibited by TIMP-2 and TIMP-3, but Not TIMP-1—We found that active-to-pro ratio of MMP-2 was consistently higher (∼1.8-fold) in Timp-3−/− MEFs. To investigate whether increased activation of MMP-2 in Timp-3−/− MEFs involved the same cell surface mechanism, we added recombinant TIMP proteins to MEF cultures (Fig. 2A). TIMP-1 was not able to inhibit the processing of pro-MMP-2 induced after the addition of cyto D in either the WT or Timp-3−/− cells. It was noted, however, that increasing concentrations of TIMP-1 did slightly decrease the ability of WT cells to process the intermediate form of MMP-2 to the fully active, the stage of MMP-2 processing previously shown to be sensitive to inhibition by TIMP-1 (16). This effect was, however, not seen in the Timp-3−/− cells, and high doses of TIMP-1 up to 100 nM were unable to reduce processing (Fig. 2A, top panel). The addition of either TIMP-2 (middle panel) or N-TIMP-3 (bottom panel) to both WT and
**Timp-3 Deficiency Accelerates Pro-MMP-2 Activation**

**FIGURE 2. TIMP inhibition of MMP-2 processing in Timp-3-deficient MEFs.** A, gelatin zymograms of conditioned medium from wild-type (WT) and Timp-3-deficient (Timp-3−/−) MEFs. Cells were pretreated with recombinant TIMP-1 (top panel), recombinant TIMP-2 (middle panel), and recombinant N-TIMP-3 (bottom panel) at the indicated concentrations 1 h prior to the addition of cyto D (1 μg/ml) or cyto D (1 μg/ml). B, gelatin zymograms of condition medium from wild-type (WT) and Timp-3−/− MEFs. Cells were pretreated with GM6001 (25 μg/ml) for 6 h. 8, gelatin zymograms of condition medium from Timp-3−/− MEFs. Cells were pretreated with GM6001 (25 μg/ml) or PD166793 (25 μg/ml) 1 h prior to the addition of con A (50 μg/ml) or cyto D (1 μg/ml).

**Timp-3**−/−** cells inhibited MMP-2 processing induced by cyto D. However, although the WT cells could be inhibited by the lowest dose (10 nM) of either TIMP-2 or N-TIMP-3, total inhibition of MMP-2 processing in Timp-3−/− cells was only achieved with a much higher concentration (50 nM). These results further strengthen our observations of a more robust and less regulated processing of MMP-2 in the absence of Timp-3.

To investigate whether the processing observed in the absence of Timp-3 is through increased MMP or ADAM activity, we used a broad spectrum metalloproteinase inhibitor, GM6001, as well as an MMP-specific inhibitor, PD166793, at 25 μM 1 h prior to con A or cyto D stimulation. The addition of either GM6001 or PD166793 similarly inhibited the processing of pro-MMP-2 in Timp-3−/− cells at 3 and 6 h (Fig. 2B), suggesting the enhanced pro-MMP-2 processing is through an MMP-specific mechanism.

**Timp-3 Present in the Matrix of WT Cells Does Not Reduce MMP-2 Processing in Timp-3−/− MEFs—**Timp-3 is unique among TIMP proteins in that it is secreted and bound to the ECM rather than remaining soluble in the cell milieu (41, 42). We investigated the role of matrix-associated Timp-3 in MMP-2 processing. The presence of functional Timp-3 in the matrix secreted by WT cells was confirmed by reverse zymography. A band of inhibition at 24 kDa indicated the presence of Timp-3 (Fig. 3A) along with a weaker higher molecular mass band indicating the glycosylated form of Timp-3. As expected, these bands were absent in the matrix derived from Timp-3−/− MEFs. A parallel silver stain gel showed equivalent protein levels in all lanes (data not shown). We asked whether Timp-3 present in the matrix of WT cells would be sufficient to regulate MMP-2 processing and, conversely, whether the absence of Timp-3 in the matrix would allow accelerated pro-MMP-2 activation by the WT cells. We performed the matrix-cell swap experiments as described under “Materials and Methods.” Briefly, MEFs of defined genotypes were allowed to deposit extracellular matrix over 10 days before the removal of the original cell monolayer. A fresh preparation of MEFs was then plated either on their own genotype-matched extracellular matrix or swapped. Stimulation with cyto D was performed 1.5 h later for a period of 4 h. Timp-3-containing matrix deposited by the WT cells was not able to decrease the pro-MMP-2 processing in Timp-3−/− cells following cyto D stimulation (Fig. 3B, lane 8) because the processing was comparable with that of Timp-3−/− cells plated on their own matrix (lane 11). Further, the absence of Timp-3 in the matrix deposited by the Timp-3−/− cells failed to enhance pro-MMP-2 activation in WT cells (lane 12). Control experiments performed in parallel included MEFs plated on tissue culture dishes devoid of matrix, which again revealed greater processing of pro-MMP-2 in Timp-3−/− cells compared with the WT counterparts (lanes 1–4).

We also tested whether equivalent numbers of cells attached to the underlying extracellular matrices and whether the cells remained adherent during the time frame of this assay. We observed comparable levels of attachment across genotypes, matrices, and treatments (Fig. 3C). Together, these data indicate that WT matrix is unable to rescue the accelerated pro-MMP-2 processing in Timp-3−/− cells.

**Expression of the MMP-2 Cell Surface Activation Molecules Remains Unaltered in the Absence of Timp-3—**The classical model proposed for cell surface processing of MMP-2 involves the formation of a trimolecular complex composed of pro-MMP-2, a member of the MT-MMP family, usually MT1-MMP, and Timp-3. We first determined whether the expression of these molecules was comparable between WT and Timp-3−/− MEFs, both in the resting state and after stimulation with cyto D. Real-time Taqman RT-PCR for MT1-MMP, MMP-2, and Timp-3 was performed on samples collected at 0, 3, 6, and 24 h post-stimulation. Shown as representative are the 6- and 24-h expression profiles of these genes (Fig. 4, A and B). Timp-2 and MMP-2 expression levels were comparable in WT and Timp-3−/− MEFs before or up to 6 h after cyto D treatment, with a trend of increase in Timp-2 expression after 24 h of treatment. At 6 h, the expression of MT1-MMP significantly increased upon cyto D treatment in both the WT and Timp-3−/− cells over their respective untreated controls. Similarly, we found no difference in MT1-MMP levels as a function of genotype (Fig. 4A). At 24 h a significant increase in MT1-MMP expression was found only after cyto D treatment in Timp-3−/− cells compared with the untreated control (Fig. 4B). MT2-MMP, which is also implicated in MMP-2 proc-
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**FIGURE 3. TIMP-3 present in the matrix of WT cells does not reduce MMP-2 processing in Timp-3−/− MEFs.** A 12% SDS-PAGE reverse gelatin zymogram of extracellular matrix harvested from WT or Timp-3−/− MEFs after 7 days in culture. Arrowheads indicate the forms of TIMP-3 visible on the gel. A parallel silver-stained SDS-PAGE gel was used to confirm equal loading of samples and absence of a protein band at the same molecular mass (not shown). 8% SDS-PAGE gelatin zymogram of condition medium samples. Briefly, cells were removed from the underlying matrix (+/+), wild type; −/−, Timp-3 deficient) before plating cells (+/+, wild type; −/−, Timp-3-deficient) of the same genotype or “swapped” with the alternate genotype. These were left to attach for 1.5 h and then stimulated with cyto D (1 μg/ml) for 4 h. Cells seeded on plastic alone before stimulation are included as an experimental control. Arrowheads indicate pro- and active forms of MMP-2. C, phase contrast images of cells at the end of the 4-h stimulation period under each matrix swap condition. Bar, 50 μm.

pressing (24, 61), was also unaltered by a lack of Timp-3 or treatment with cyto D at 6 h (Fig. 4A). Recently, MT3-MMP was highlighted in the context of MMP-2 processing involving Timp-3 (25). MT3-MMP expression was also comparable between Timp-3−/− and WT cells at 6 h (Fig. 4A). In fact, the addition of cyto D led to a trend of decreased MT3-MMP expression at 6 and 24 h and of MT2-MMP at 24 h (Fig. 4, A and B). This trend was similar between Timp3−/− and WT cells.

Next, we determined any corresponding changes in protein levels by Western blotting of cell lysates (Fig. 4C). An increase in MT1-MMP level was apparent 6 h post cyto D treatment in both WT and Timp-3−/− MEFs, consistent with its increased RNA expression, but there was no difference in MT1-MMP as a function of Timp-3 loss. An HT1080 cell lysate stimulated with con A served as a positive control for the identification of MT1-MMP (Fig. 4C). An enzyme-linked immunosorbent-based assay for MT1-MMP activity has recently become available and was used to determine MT1-MMP activity in cell membranes prepared after 6 h of cyto D treatment (Fig. 4D). As expected, MT1-MMP activity significantly increased in both WT and Timp-3−/− MEFs after cyto D treatment, and the magnitude of increase in MT1-MMP activity was notably higher in Timp-3−/− than in WT cells. For instance, MT1-MMP activity rose by 1.8-fold in Timp-3−/− but 1.5-fold in WT cells. Thus, the accelerated and enhanced pro-MMP-2 activation following the deletion of Timp-3 resulted from increased MT1-MMP activity rather than a significant alteration in expression of MMP-2, MT1-MMP, MT2-MMP, MT3-MMP, or Timp-2.

**Timp-3 is an External Regulator of the Trimolecular Complex—**Timp-2 also functions as a critical linker molecule within the trimolecular complex as well as being an inhibitor (27–29, 62, 63). Cells deficient in Timp-2 were initially reported as defective in their ability to process pro-MMP-2 (30, 31). More recent work has highlighted that, although full MMP-2 processing is impaired, processing to the intermediate form does occur in the absence of Timp-2 (32, 33). Because Timp-3-deficient cells exhibit enhanced MMP-2 processing to the intermediate and fully active forms, we asked whether the role of Timp-3 in MMP-2 processing is in an inhibitory capacity or whether it can promote MMP-2 processing independently of Timp-2-dependent formation of the trimolecular complex.

Panels of MEFs deficient in individual (Timp-1−/−, Timp-2−/−, Timp-3−/−), and double deficient (Timp-2−/−/−/−, Timp-3−/−/−) TIMPs, along with wild-type controls, were stimulated for 3, 6, and 24 h in culture with con A and cyto D. Both conditioned medium and cell lysates were collected and their MMP-2 status analyzed by gelatin zymography (Fig. 5). Conditioned medium samples from Timp-2−/− cells did not display any processing until 6 h when barely detectable processing to the intermediate form first became evident with cyto D treatment (Fig. 5A, middle panel, lane 8). This processing to the intermediate form was more substantial by 24 h with both cyto D and con A treatment (Fig. 5A, bottom panel, lanes 8 and 9). Comparison of MMP-2 species between Timp-2−/− and Timp-2−/−/−/−/− MEFs revealed a far greater rate of processing in the latter. Con A induced processing to the intermediate form within 3 h (Fig. 5A, top panel, lane 12), and both inducers resulted in higher levels of intermediate MMP-2 at 6 h in the double deficient cells (middle panel, lanes 11 and 12). There was even the appearance of fully processed MMP-2 in the conditioned medium of Timp-2−/−/−/−/−/− cells by 24 h after con A stimulation (bottom panel, lane 12). The processing of pro-MMP-2 in the cell lysates further emphasized the observations seen using conditioned medium. Further, the appearance of the fully active form of MMP-2 was more apparent in
FIGURE 4. Expression of the MMP-2 cell surface activation molecules remains unaltered in the absence of TIMP-3. Analysis of gene expression of MT1-MMP, MT3-MMP, MMP-2, and TIMP-2. RNA samples were collected at 6- (A) and 24-h (B) time points and RNA expression analyzed using real-time Taqman RT-PCR. Cell type and stimulation conditions were as follows: white bars, WT unstimulated; white striped bars, WT + cyto D (1 μg/ml); gray bars, Timp-3−/− unstimulated; gray striped bars, Timp-3−/− + cyto D (1 μg/ml). *, p < 0.05. C, Western blot of cell lysates for MT1-MMP collected at 6 h from WT (lanes 1 and 2) and Timp-3−/− (lanes 3 and 4) cells, control (lanes 1 and 3), or treated with cyto D (1 μg/ml) (lanes 2 and 4). Both full-length and the 45-kDa form of MT1-MMP are indicated. HT1080 treated with con A is run as a positive control. D, MT1-MMP activity assay. Membrane preparations from WT and Timp-3−/− MEFs were assessed for their MT1-MMP activity using a Biotrak Activity Assay System as described under “Materials and Methods.” Open triangles, WT unstimulated; closed triangles, Timp-3−/− unstimulated; open squares, WT + cyto D (1 μg/ml); closed squares, Timp-3−/− (1 μg/ml).
the cell lysates after con A treatment of Temp-2−/−/TimP-3−/− than in Temp-2−/− cells (Fig. 5B, middle and bottom panels, lanes 12 and 9). Controls for these experiments included parallel analyses with WT and TimP-3-deficient MEs (lanes 1–6), where the pattern of heightened MMP-2 processing in Temp-3−/− compared with WT MEs was as that described for Fig. 1. The inclusion of Tmp-1−/− MEs further confirmed the lack of Tmp-1 involvement in the processing of MMP-2, with a pattern of activation similar to that seen in WT cells (Fig. 5A and B, all panels, lanes 1–3 compared with lanes 13–15).

Of the two original reports on Temp-2-deficient mice, one included a Northern blot depicting higher MMP-2 and MT1-MMP RNA levels (30). Using Taqman RT-PCR, we tested whether these molecules were altered in our single and double deficient cells (Fig. 5C). We observed a trend of increased MMP-2 and MT1-MMP expression in Temp-2−/− cells, although this was not statistically significant. Interestingly, the double deficient Temp-2−/−/TimP-3−/− cells showed a trend of decreased expression of these molecules. Altogether, the combined absence of Tmp-2 and TimP-3 enabled processing of MMP-2 to the intermediate form, but efficient conversion to the fully active form required Tmp-2. This suggests that TimP-3 functions externally of the trimolecular complex by regulating MT1-MMP activity.

**TIMP-4 Deficiency Does Not Alter Pro-MMP-2 Processing**—TIMP-4 has properties similar to TIMP-3 with respect to its interactions with MT1-MMP and MMP-2. We therefore hypothesized that a TIMP-4 deficiency would mimic that of TIMP-3 deficiency during pro-MMP-2 processing although it is not matrix bound. After confirmation that WT MEs express TIMP-4 (Fig. 6A), we stimulated Temp-4−/− ME cultures with cyto D for 3 or 6 h. Acceleration of pro-MMP-2 processing did not occur in Temp-4−/− MEs compared with their WT controls (Fig. 6B). Temp-3−/− MEs with their respective WT controls again showed enhanced MMP-2 processing (Fig. 6B). It is possible that the lack of accelerated MMP-2 activation in Temp-4−/− MEs arises from compensation by other TIMPs such as TIMP-2 and/or TIMP-3. Taqman RT-PCR revealed no significant difference in the expression of TIMP-2 or TIMP-3 following 6 h of stimulation with cyto D (Fig. 6C).

**Modeling the Contribution of TIMPs to Pro-MMP-2 Processing**—At the cell surface, MT1-MMP binds Tmp-2, forming a “receptor” complex for pro-MMP-2 docking and subsequent cleavage to intermediate MMP-2 by an adjacent active MT1-MMP (Fig. 7A). The efficiency of this initial stage is in part governed by the concentration of free Temp-2, TIMP-3, and TIMP-4 in the cell milieu. The second stage of MMP-2 processing to the fully active form is through an autocatalytic event. It is
as yet unclear whether this occurs at the cell surface or by soluble MMP-2. Fig. 7B depicts the activation events in the absence of TIMP-2 as proposed by Lafleur et al. (33) whereby pro-MMP-2 is tethered to cell surface independently of MT1-MMP/TIMP-2 and MMP-2 processing is stalled at the intermediate stage. However, the necessity of this cell surface anchor remains open at present. In the absence of TIMP-3 (Fig. 7C), TIMP-2 is available for trimolecular complex formation and we observe accelerated MMP-2 processing through both stages of activation, resulting in fully active form. It likely arises from the removal of TIMP-3, an external inhibitor resulting in more TIMP-free MT1-MMP available for trimolecular complex formation. With a combined TIMP-2/TIMP-3 deficiency (Fig. 7D), MMP-2 processing culminates in a large increase in intermediate MMP-2 and yields a low level of fully active MMP-2. This processing is likely initiated by more TIMP-free MT1-MMP available for generating the intermediate MMP-2, leading to a chance meeting of MMP-2 molecules and their autocatalytic processing to the fully active form. In contrast to TIMP-2 and TIMP-3, deletion of either Timp-1 or Timp-4 does not affect MMP-2 processing.

DISCUSSION

MT1-MMP has emerged as a key metalloproteinase in cancer progression. This membrane-bound protease has the capacity to degrade ECM proteins and is known for its central role in the cell surface activation of the constitutively expressed soluble MMP, MMP-2. This study has undertaken a comprehensive comparison of pro-MMP-2 activation as a function of individual Timp gene deficiencies. We demonstrated that TIMP-3 provides an important inherent control over the kinetics of cell surface activation of MMP-2 by MT1-MMP, serving an inhibitory function distinct from TIMP-2 and TIMP-4. This effect is consistently seen in independent Timp-3−/− mouse chimeras generated from two independent embryonic stem cell clones, multiple independent primary MEF clones, and in different strains of Timp-3−/− mice, including C57BL6 and FVB. Timp-3−/− mice have greater MMP-2 activation during physiological and pathological events, such as during mammary gland involution, and in a model of heart disease (44, 48). Increased MMP-2 activation at the cell surface may be a key mechanism by which TIMP-3 loss contributes to aberrant ECM remodeling.

The addition of con A or cyto D to cell cultures stimulates MMP-2 processing by increasing MT1-MMP activity that is effectively inhibited by the addition of soluble TIMP-2 or N-TIMP-3 (34, 64). TIMP-3-deficient primary MEFs show accelerated kinetics of pro-MMP-2 processing, and inhibition requires ~5-fold higher level of exogenous TIMP-2 or N-TIMP-3 than the WT controls. This demonstrates a more efficient activation mechanism in the absence of TIMP-3. Soluble TIMP-3 (N-TIMP-3), but not TIMP-3 bound to the ECM, rescues the heightened MMP-2 processing in Timp-3−/− MEFs. The increased level of MMP-2 processing in Timp-3−/− cells is not due to altered cell proliferation or apoptosis compared with WT controls (data not shown). Further, the increased MMP-2 processing in Timp-3−/− MEFs does not arise from a significant alteration in expression of TIMP-2, MMP-2, MT1-MMP, MT2-MMP, or MT3-MMP, which have each been shown to play a role in MMP-2 processing (15, 24, 25, 61). However, 24 h of treatment with cyto D stimulates a trend of increased TIMP-2 expression in both WT and Timp-3−/− MEFs. As a regulator of MMP-2 activation, this increase in TIMP-2 production may underlie the comparable levels of active MMP-2 observed at this time point in the two groups.

At the protein level, MT1-MMP increases as a function of the cyto D treatment but not as a function of TIMP-3 deletion. The lack of mouse antibodies to the other above components precluded their assessment at the protein level. Biochemical studies show that TIMP-3 rapidly interacts with MMP-2, although this interaction is weaker than that of TIMP-2 (26) and it regulates pro-MMP-2 processing by inhibiting MT1-MMP (34). There is evidence to show direct TIMP-3 binding with cell surface proteoglycans (26), and it has recently been noted by proteomic screening of MT1-MMP-associating proteins that TIMP-3 associates with MT1-MMP at the surface.4 Altogether, these results demonstrate that TIMP-3 provides an important inherent control over the kinetics of cell surface activation of MMP-2 by MT1-MMP, and we postulate that TIMP-3 closely associated with the cell surface is able to regulate the trimolecular complex.

4 M. Seiki, personal communication.
The loss of TIMP-3 may affect MMP-2 processing through a number of mechanisms. It may simply result in the removal of an inhibitor of both MT1-MMP and MMP-2, thereby increasing their activity and subsequent efficiency of pro-MMP-2 processing. Alternatively, the lack of TIMP-3 may facilitate generation of trimolecular complexes of MT1-MMP with TIMP-2 and pro-MMP-2 by increasing the amount of TIMP-free MT1-MMP at the cell surface. It also remains possible that the lack of TIMP-3 increases the availability of free MT3-MMP, which can activate pro-MMP-2 through TIMP-2 (25). Interestingly, although MT1-MMP expression significantly increases upon cyto D treatment, that of MT3-MMP decreases. Other possibilities such as alterations in MT-MMP trafficking or MMP-2 clearance, although not investigated in this study, should not be ruled out.

Conversion of pro-MMP-2 to its fully activated state is a two-step process via an intermediate form of MMP-2. We used a genetic approach to map the relative roles of TIMP-2 and TIMP-3 in the trimolecular complex. There was no active MMP-2 in cyto D-treated Timp-2/−/− cells, but a minimal level existed in the lysates of con A-treated cells. Bigg et al. (32) and Lafleur et al. (33) have reported that TIMP-2 deficiency results in loss of fully activated MMP-2 with the generation of detectable intermediate MMP-2 species. When TIMP-3 deficiency was superimposed on TIMP-2 loss, we observed a pattern of activation similar to TIMP-2, but the kinetics of MMP-2 processing was again accelerated. There was not only a precipitous generation of the intermediate MMP-2 form, but the fully active MMP-2 species was more pronounced in con A-treated cell lysates (6 and 24 h) as well as conditioned medium (24 h). This specific increase observed after con A stimulation may be due to cross-linking of MMP-2 and MT1-MMP at the cell surface leading to autocatalysis or inhibition of MT1-MMP constitutive endocytosis (65, 66). Thus, the extent and pattern of pro-MMP-2 activation in the double Timp-2/Timp-3 mutant MEFs reflected features of single deficiencies of TIMP-2 or TIMP-3.

In this study we also examined the effect of TIMP-4 deficiency on pro-MMP-2 processing. TIMP-3 and TIMP-4 interact with MMP-2 and MT1-MMP in a comparable fashion. Both exert inhibition after binding to the N termini of MMP-2 or MT1-MMP (26, 32, 34), and each can also interact with the C-terminal domain of MMP-2 (26, 32, 40). The latter property opens the potential of their participation in trimolecular complex assembly, although neither has been shown to do so (26, 32, 67, 68). Despite the similarities between TIMP-3 and TIMP-4, we observed accelerated pro-MMP-2 activation only in Timp-3/−/− and not Timp-4/−/− cells. This failure to accelerate pro-MMP-2 processing is not due to a compensatory increase in the levels of TIMP-2 or TIMP-3 in Timp-4/−/− cells. This highlights the need to more fully understand the role of these TIMPs beyond biochemical testing, in more biological cell-based systems.

FIGURE 7. Model of MMP-2 activation in the absence of TIMPs-2 and -3. A, this panel depicts the classical trimolecular complex and pro-MMP-2 processing in WT cells. B, in the absence of TIMP-2, pro-MMP-2 is tethered to cell surface with MMP-2 processing held at the intermediate stage. C, it functions more efficiently in the absence of TIMP-3 with a greater occurrence of fully processed MMP-2. D, the combined deficiency of TIMP-2 and TIMP-3 culminates in a precipitous elevation of intermediate MMP-2 and yields a low level of fully active MMP-2.
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