Selective involvement of TIMP-2 in the second activational cleavage of pro-MMP-2: refinement of the pro-MMP-2 activation mechanism

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Abstract A tissue inhibitor of metalloproteinases-2 (TIMP-2)-independent mechanism for generating the first activational cleavage of pro-matrix metalloproteinase-2 (MMP-2) was identified in membrane type-1 MMP (MT1-MMP)-transfected MCF-7 cells and confirmed in TIMP-2-deficient fibroblasts. In contrast, the second MMP-2-activational step was found to be TIMP-2 dependent in both systems. MMP-2 hemopexin C-terminal domain was found to be critical for the first step processing, confirming a need for membrane tethering. We propose that the intermediate species of MMP-2 forms the well-established trimolecular complex (MT1-MMP/TIMP-2/MMP-2) for further TIMP-2-dependent autocatalytic cleavage to the fully active species. This alternate mechanism may supplement the traditional TIMP-2-mediated first step mechanism.

Key words: Membrane-type-1 matrix metalloproteinase; Matrix metalloproteinase-2; Tissue inhibitor of metalloproteinases-2; Enzyme activation

1. Introduction

Matrix metalloproteinases (MMPs) are a family of structurally related proteolytic enzymes, which collectively degrade all components of the extracellular matrix (ECM), as well as a growing number of specific cell modulators such as growth factors, cell adhesion molecules, receptors, and other proteinases [1,2]. All MMPs possess a signal peptide, a propeptide, an N-terminal catalytic domain, and most also possess a hemopexin C-terminal domain (HXCD) [2,3]. There are six membrane-type MMPs (MT-MMPs), four of which possess a putative transmembrane domain and a short cytoplasmic tail at their C-terminal region. The transmembrane domain in MT1-, MT2-, MT3-, and MT5-MMP anchors the enzyme to the cytoplasmic membrane, localizing proteolytic activity to the cell surface. An important role of several of the MT-MMPs is the cell-localized activation of pro-MMP-2. The activity of MMPs is also regulated by a family of four inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs) [4].

In the current model for MT1-MMP-mediated pro-MMP-2 activation (Fig. 4), active MT1-MMP anchored on the cell surface acts as a receptor for TIMP-2, the TIMP-2 molecule binding through its N-terminal domain to the active site of MT1-MMP. This binary complex acts as a receptor for pro-MMP-2, the TIMP-2 C-terminal domain binding to the HXCD of pro-MMP-2. A free MT1-MMP molecule in close proximity then cleaves the pro-MMP-2 propeptide at the Asn^{37}–Leu^{38} bond, generating an intermediate species [5–8]. Further autocatalytic proteolysis generates the fully active enzyme [5,9,10]. Optimum TIMP-2 levels are required for efficient pro-MMP-2 activation [6–8]. MT1-MMP dimerization via MT1-MMP's HXCD or cytoplasmic tail is also essential for pro-MMP-2 activation [11–13]. We provide data of a supplementary, TIMP-2-independent first step mechanism, and for the first time focus on the role of TIMP-2 in the second activational step.

2. Materials and methods

2.1. Recombinant proteins and reagents

Recombinant TIMP-2 was kindly provided by Dr. Chris Overall (UBC, Vancouver, BC, Canada). Recombinant full-length pro-MMP-2 expressed in a vaccinia virus system (rMMP-2) was a kind gift from Dr. Rafael Fridman (Wayne State University, Detroit, MI, USA) [14]. Recombinant ala+TIMP-2 was kindly provided by Dr. William Stetler-Stevenson (NCI, NIH, Bethesda, MD, USA) [15]. Recombinant TIMP-1 and GM6001 were purchased from Chemicon (Boronia, Australia). Concanavalin A (Con A), 4-aminophenylmercuric acetate and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (Castle Hill, Australia).

MMP-2 and MMP-2 ΔHXCD were obtained from conditioned media of MCF-7 cells stably transfected with expression vectors (pCHC6 [16]) encoding full-length MMP-2 or MMP-2 from which the HXCD (residues 469–631) was deleted.

2.2. Cell culture

ML20-MT1 cells [17] were generated by stably transfecting MT1-MMP cDNA under the control of the CMV promoter (pCNCMT1-MMP) into an MCF-7 clone previously transfected with β-galactosidase (ML20) [16]. ML20-MT1 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Life Technologies, New Zealand) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA).

TIMP-$2^{+/+}$ and TIMP-$2^{\Delta Ex2,3/\Delta Ex2,3}$ (lacking exons 2 and 3 of the TIMP-2 gene) primary mouse fibroblasts [18] were generously provided by Dr. John Caterina (NIDCR Matrix Metalloproteinase Unit NIH, Bethesda, MD, USA). Ras/myc transformed TIMP-$2^{-/-}$ mouse fibroblasts lacking the entire TIMP-2 gene [19] were kindly

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Abbreviations: Con A, concanavalin A; ECM, extracellular matrix; HXCD, hemopexin C-terminal domain; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; SFM, serum-free media; TIMP, tissue inhibitor of metalloproteinases

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provided by Dr. Paul Soloway (Roswell Park Cancer Institute, Buffalo, NY, USA). Fibroblasts were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin.

2.3. Gelatin zymography

Zymography was performed using a 10% polyacrylamide resolving gel co-polymerized with 1 mg/ml gelatin (BDH Laboratory Supplies, Poole, UK) in the large gel format (15 cm×12 cm) as previously described [20].

2.4. Western analysis

For MMP-2 Western analysis, MMP-2 from the conditioned media of ML20-MT1 cells was concentrated using gelatin–agarose beads (Sigma-Aldrich) overnight at 4°C. The beads were then washed and eluted with 4× sodium dodecyl sulfate sample buffer. All samples (for MMP-2 or TIMP-2 Western analysis) were prepared with 100 mM dithiothreitol as reducing agent and separated using a 10% polyacrylamide resolving gel. After electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane for 2 h at 100 V. Membranes were then blocked in 5% (w/v) skim milk in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 (TBS–Tween 20) overnight. Membranes were probed either with a mouse anti-human TIMP-2 antibody (clone #67-4H11, Chemicon) or a rabbit anti-human polyclonal MMP-2 antibody (PAB 753) (kindly provided by Dr. Jack Windsor, University of Alabama at Birmingham) for 1 h then washed in TBS–Tween 20. Membranes were then probed with a horseradish peroxidase-conjugated goat anti-mouse (Pierce, Darra, Australia) secondary antibody (for TIMP-2) or a goat anti-rabbit (Pierce) secondary antibody (for MMP-2) for 1 h, then washed in TBS–Tween 20. Detection was performed using the SuperSignal West Pico Chemiluminescent Substrate system (Pierce).

3. Results

3.1. TIMP-2 potentiation of the second step of pro-MMP-2 activation in ML20-MT1 cells

ML20-MT1 cells do not produce MMP-2, therefore exogenous recombinant pro-MMP-2 was added to these cultures. TIMP-2 production by these cells was low and only detectable by Western blot after concentration of the conditioned medium (approximately 200 ng/ml TIMP-2 produced by 1×10^4 cells in 100 μl for 24 h as determined by Western analysis using a standard curve of known TIMP-2 concentration, data not shown). Transfection of MT1-MMP caused weak pro-MMP-2 activation generating both intermediate and fully active species, and this was greatly increased in response to Con A (Fig. 1a). Addition of recombinant TIMP-2 preferentially increased the second step of pro-MMP-2 activation at the lower concentrations, although a slight increase in the first step product was also seen (Fig. 1a). In order to confirm the results obtained by zymography in Fig. 1a, we also analyzed some of these samples by Western analysis for MMP-2. Fig. 1b demonstrates that similar results were obtained either by zymography or Western analysis for MMP-2 with a biphasic response to TIMP-2. Because zymography is more sensitive than Western analysis, MMP-2 from the conditioned media was concentrated using gelatin–agarose beads for Western analysis. The levels of the intermediate form of MMP-2 were too low to detect by Western analysis using the ML20-MT1 cells. The relative levels of pro- and active MMP-2 observed by Western blot were determined by scanning densitometry and plotted as a percentage of the fully active enzyme.

In order to verify that TIMP-2 was indeed preferentially affecting the second step, and not simply increasing the first step thereby generating more intermediate product that would be rapidly processed to the fully active species, we added excess TIMP-1 to selectively block the second step. We used concentrations of TIMP-1 that have been shown previously not to block MT-MMPs, as TIMP-1 is a very poor inhibitor of MT1-MMP [21–23]. TIMP-2 alone selectively increased the second step, with only a minor accumulation of the intermediate form observed with co-addition of TIMP-1 (Fig. 1c), confirming that the effects seen in the second step are not due to an enhanced first step.

3.2. TIMP-2-insensitive first step activation of pro-MMP-2 by TIMP-2-deficient fibroblasts

To further study these observations in a TIMP-2-free environment, we used TIMP-$2^{\Delta Ex2,3/\Delta Ex2,3}$ (lacking exons 2 and 3 and thus lacking the MMP inhibitory region at the N-terminus of the protein [18]) and TIMP-$2^{+/+}$ primary mouse fibroblasts and a ras/myc transformed TIMP-$2^{-/-}$ (lacking the entire TIMP-2 gene [19]) mouse fibroblast cell line. In the TIMP-$2^{+/+}$ fibroblasts, pro-MMP-2 activation proceeded mostly to the fully active species. In TIMP-$2^{\Delta Ex2,3/\Delta Ex2,3}$ fibroblasts, pro-MMP-2 activation occurred in response to Con A (but not PMA), proceeding mostly to the intermediate form, with only very minor amounts of fully active MMP-2 (Fig. 2a). We tested the possibility that this small amount of fully active MMP-2 was due to residual TIMP-2 from the serum. TIMP-$2^{+/+}$ and TIMP-$2^{\Delta Ex2,3/\Delta Ex2,3}$ fibroblasts were cultured in serum-containing media with or without excess GM6001 (a synthetic MMP inhibitor) for several days, in order to block potential TIMP-2 binding sites on the cell surface as previously described [6]. Cells were then washed extensively in serum-free media (SFM) without GM6001, and assessed for pro-MMP-2 activation. Under these conditions, we observed similar amounts of MMP-2 processing to the intermediate form in the TIMP-$2^{\Delta Ex2,3/\Delta Ex2,3}$ cells in response to Con A, however, noticeably less fully active enzyme was observed (Fig. 2a). Upon concentration of the conditioned media from these cells, some TIMP-2 was found in the conditioned media of TIMP-$2^{\Delta Ex2,3/\Delta Ex2,3}$ and TIMP-$2^{-/-}$ cells but none was detected when these cells were previously cultured with GM6001 (Fig. 2b). This indicates that some serum-derived TIMP-2 bound the cells during previous culture of the non-GM6001-treated cells in serum, and that this was responsible for the low levels of MMP-2 activation seen in the TIMP-$2^{-/-}$ and the TIMP-$2^{\Delta Ex2,3/\Delta Ex2,3}$ cell cultures. No cell-associated TIMP-2 was detected by Western blot analysis, however, we believe that these TIMP-2 levels are below the detection limit of our assay system. These data confirm that the first step of pro-MMP-2 activation can proceed in the absence of TIMP-2.

In the TIMP-$2^{\Delta Ex2,3/\Delta Ex2,3}$ cells, pro-MMP-2 activation was rescued by the addition of wild-type TIMP-2 (Fig. 2c). We observed the expected biphasic response whereby increasing TIMP-2 concentrations initially facilitated pro-MMP-2 activation, but became inhibitory at higher concentrations. Of note, it was the second step that was preferentially stimulated in response to TIMP-2. We also analyzed immortalized ras/myc transformed TIMP-$2^{-/-}$ fibroblasts which behaved similarly to the TIMP-$2^{\Delta Ex2,3/\Delta Ex2,3}$ cells. These TIMP-$2^{-/-}$ cells also activated pro-MMP-2 in response to Con A, but only to the intermediate form (Fig. 2d), as reported previously [24]. Upon titration of recombinant TIMP-2, pro-MMP-2 activation was stimulated, and although it was the second step that was preferentially potentiated, some effect on the first step was

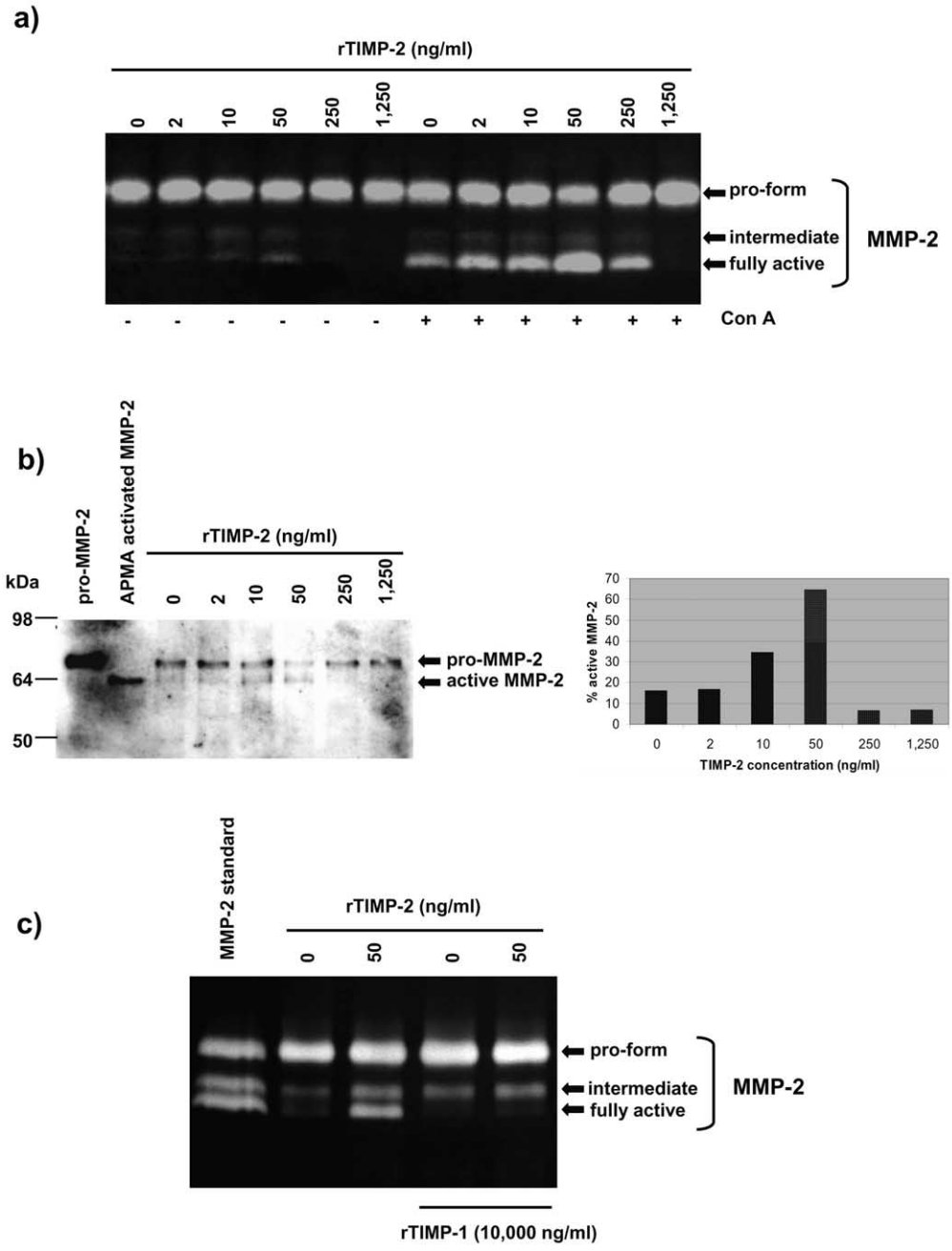

Fig. 1. a: ML20-MT1 cells were plated in a 96-well plate and washed 3× in SFM. The cells were then incubated in fresh SFM containing 100 ng/ml rMMP-2, without or with Con A (20 μg/ml) stimulation, and without or with TIMP-2 as indicated. After 24 h, the conditioned media were analyzed by zymography. b: ML20-MT1 cells were treated as in a, except that the cells were plated in a 24-well plate and all treated with Con A and with the indicated concentrations of TIMP-2. MMP-2 was then concentrated from the conditioned media using gelatin–agarose beads and the bound fractions analyzed by Western analysis for MMP-2. The relative levels of pro- and active MMP-2 were determined by scanning densitometry using molecular dynamics software and plotted as a percentage of the fully active enzyme. c: ML20-MT1 cells were treated as in a, except that the cells were treated with Con A and with TIMP-1 and/or TIMP-2 as indicated. The relative migrations of pro-, intermediate, and fully mature MMP-2 are indicated at 72, 64, and 62 kDa, respectively.

evidenced by loss of intensity in the pro-MMP-2 band (Fig. 2d). This effect is specific for TIMP-2 as TIMP-1 did not potentiate or inhibit pro-MMP-2 activation (Fig. 2e).

3.3. Confirmation of MMP-2 HXCD requirement for pro-MMP-2 activation

Because TIMP-2 was not required for first step activation of pro-MMP-2, we asked whether pro-MMP-2 could be activated to the intermediate form by random interactions with MT1-MMP independently of TIMP-2. We cultured ML20-MT1 cells that lack endogenous MMP-2 with either full-length MMP-2 or MMP-2 ΔHXCD (lacking the HXCD). Although full-length pro-MMP-2 was activated by these cells in response to Con A, MMP-2 ΔHXCD was not (Fig. 3a), confirming that the HXCD of MMP-2 is essential for activation, as previously described [25–28].

We used ala+TIMP-2 to further explore the role of MMP-2 HXCD in this alternate activation mechanism. Ala+TIMP-2

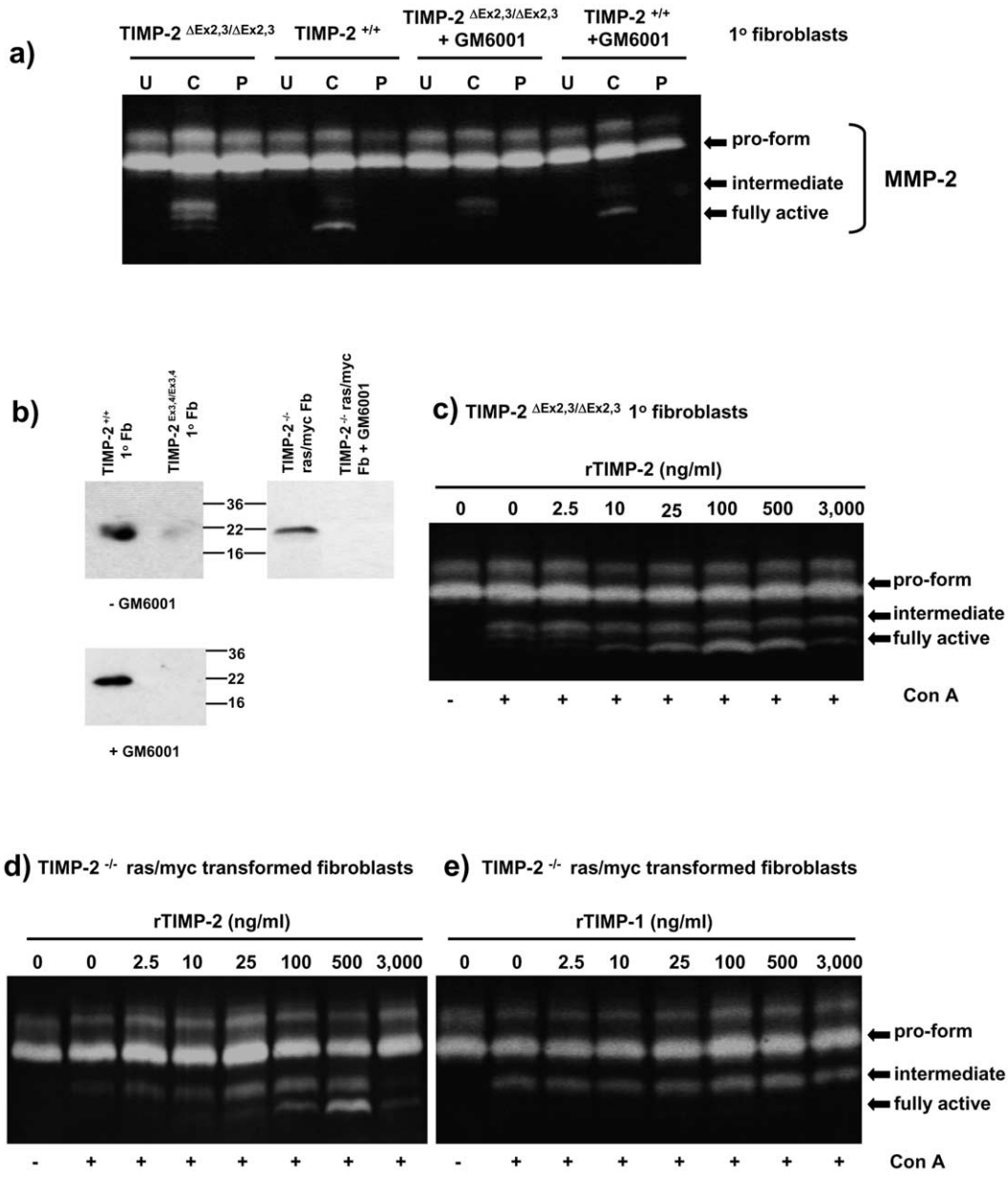

Fig. 2. a: Primary TIMP-$2^{+/+}$ or $^{\Delta Ex2,3/\Delta Ex2,3}$ mouse fibroblasts were plated in a 96-well plate without or with 1 μM GM6001. The cells were then washed 4× (including one overnight wash) in SFM, followed by incubation in fresh SFM for 48 h either unstimulated (U), or stimulated with Con A (20 μg/ml) (C) or PMA (10^{-7} M) (P). The conditioned media were analyzed by zymography. b: The conditioned media from TIMP-$2^{+/+}$ and TIMP-$2^{\Delta Ex2,3/\Delta Ex2,3}$ fibroblasts (1° Fb) (concentrated 6×) or TIMP-$2^{-/-}$ fibroblasts (concentrated 25×) cultured without or with 1 μM GM6001 were subjected to TIMP-2 Western analysis. TIMP-2 has a molecular weight of 21 kDa. c: Primary TIMP-$2^{\Delta Ex2,3/\Delta Ex2,3}$ mouse fibroblasts were treated as described in a, except with the indicated concentrations of rTIMP-2 and Con A (20 μg/ml). d: TIMP-$2^{-/-}$ fibroblasts were treated as in a, except with the indicated concentrations of rTIMP-2 and Con A (20 μg/ml). e: TIMP-$2^{-/-}$ fibroblasts were treated as in d, except that rTIMP-1 was used instead of rTIMP-2.

contains an added N-terminal alanine and thus does not bind the MMP active site but can still bind the HXCD of MMP-2 [15]. Using an MMP-2 activity-based assay (Chemicon), ala+TIMP-2 showed no inhibitory activity against the catalytic domain of MMP-2 while rTIMP-2 blocked gelatin degradation (data not shown), confirming that the ala+TIMP-2 mutant protein cannot bind the active site of MMP-2. In activation studies, ala+TIMP-2 blocked pro-MMP-2 activation at both steps in the ML20-MT1 cell system (Fig. 3b), suggesting that both mechanisms involve a region of HXCD that binds TIMP-2 (or nearby), and that ala+TIMP-2 blocks an essential requirement of HXCD binding. Although such data has been interpreted in the past to implicate TIMP-2 in the first step, the lack of TIMP-2 in our system would suggest some other binding partner for the same/similar region of the MMP-2 HXCD.

4. Discussion

Strongin and co-workers [8] were the first to report a complex comprising MT1-MMP, TIMP-2 and MMP-2 associated with the cell surface. It was therefore proposed that the MT1-MMP/TIMP-2 complex could act as a receptor for pro-MMP-2, which in turn could be activated by a TIMP-2-free MT1-

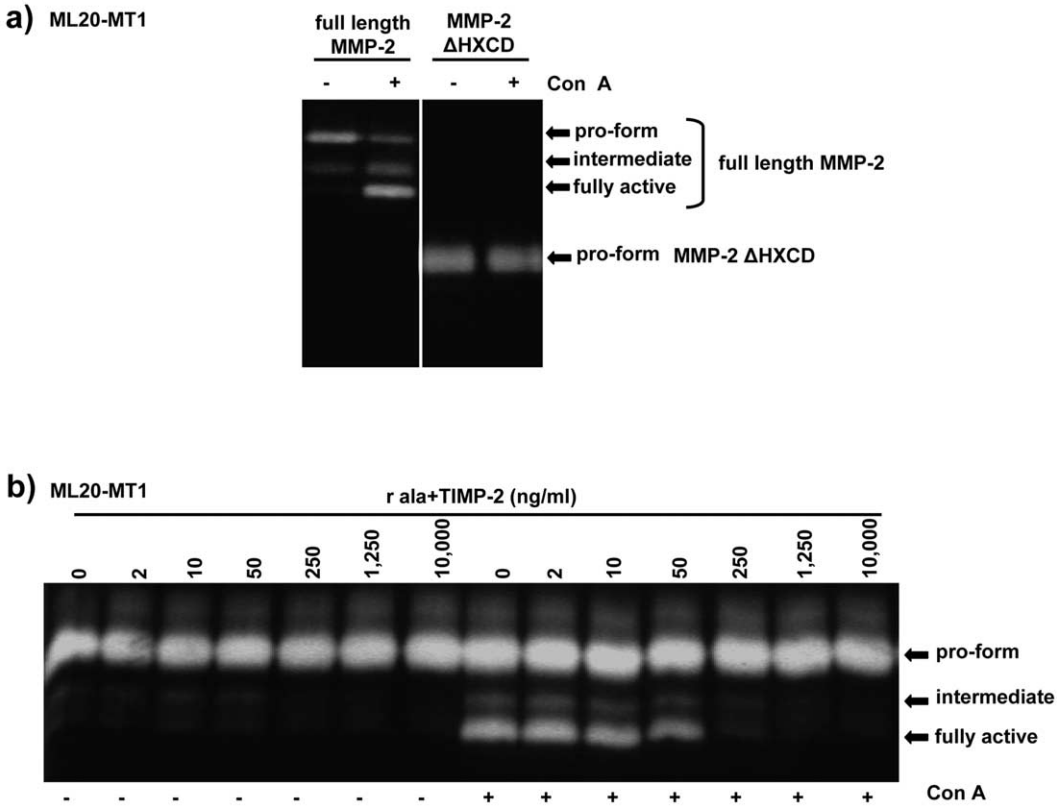

Fig. 3. a: ML20-MT1 cells were plated in a six-well plate and washed 3× in SFM. The cells were then incubated in fresh SFM (without or with Con A, 20 μg/ml) containing the conditioned media of MCF-7 cells transfected with either full-length MMP-2 or MMP-2 ΔHXCD. After 24 h, the conditioned media were analyzed by zymography. b: ML20-MT1 cells were plated in a 96-well plate and treated as in a, except rMMP-2 was used (100 ng/ml) and treated with the indicated concentrations of ala+TIMP-2 (without or with Con A, 20 μg/ml).

MMP molecule in close proximity. It was also shown that upon titration of exogenous TIMP-2, the rate of pro-MMP-2 activation was initially increased, and then fully inhibited with high amounts of TIMP-2 [8]. Further studies by Butler et al. [6] using TIMP-2-depleted HT1080 membranes demonstrated again the biphasic response of wild-type TIMP-2 on pro-MMP-2 activation. N-terminal TIMP-2 could not potentiate this activation but could still block MT1-MMP function, demonstrating the requirement for the TIMP-2 C-terminal domain in the activation process. Similar results were also reported by Kinoshita et al. [7] using MT1-MMP immobilized on agarose beads. These data, combined with the requirement of the HXCD of MMP-2 and MT1-MMP for activation [25–28], have led to the notion that the trimolecular complex (MT1-MMP/TIMP-2/MMP-2) was involved in the initial step of pro-MMP-2 activation.

Our observations in the ML20-MT1 and TIMP-2-deficient cell systems suggest that TIMP-2 is more critically important for the second MMP-2-activational step rather than the first. In fibroblasts from TIMP-2-deficient mice, generation of the intermediate form of MMP-2 occurred in the absence of TIMP-2. Titration of exogenous TIMP-2 caused a preferential increase in the generation of the fully active enzyme. Our results also show that this is not simply due to increased generation of the intermediate form, which is then rapidly processed to the fully active enzyme. Co-addition of TIMP-1, which arrested MMP-2 activation to the intermediate form by blocking the TIMP-2-stimulated second step, revealed that some first step increase was due to added TIMP-2, but this was much less than that seen in the second step. Thus a TIMP-2-independent first activational step appears to supplement the well-characterized TIMP-2-dependent mechanism.

In favor of the TIMP-2-independent first step processing discussed herein, others have also reported TIMP-2-independent generation of intermediate MMP-2. In isolated membranes depleted of TIMP-2, MT1-MMP was able to activate pro-MMP-2 to the intermediate form, but fully active MMP-2 was observed only upon addition of TIMP-2 [6,29]. Furthermore, Miyamori et al. discovered that pro-MMP-2 was activated in HEK 293 cells that did not express detectable levels of TIMP-2, but only to the intermediate form. Upon addition of TIMP-2, however, the fully active species was generated [30]. Incubation of HT1080 membranes with pro-MMP-2 and increasing amounts of an anti-TIMP-2 antibody blocked the generation of the fully active enzyme while the intermediate form persisted [8]. Using a reactive site-modified TIMP-2 that can only bind via C-terminal interactions with pro-MMP-2, it was demonstrated that this modified TIMP-2 could block formation of fully active MMP-2 in Con A-stimulated HT1080 cells, but the intermediate form of MMP-2 persisted [31]. Previous publications describing data from TIMP-2-deficient fibroblasts have shown defects in pro-MMP-2 activation in TIMP-2-null cells compared to wild-type control cells [18,19]. Wang et al. concluded that no intermediate or fully active MMP-2 forms were detected in their TIMP-2$^{-/-}$ cells. However, closer analysis showed first step activation in response to Con A [19] and this was confirmed by Morrison et al. [24]. Caterina et al. [18] indicated that cells derived from

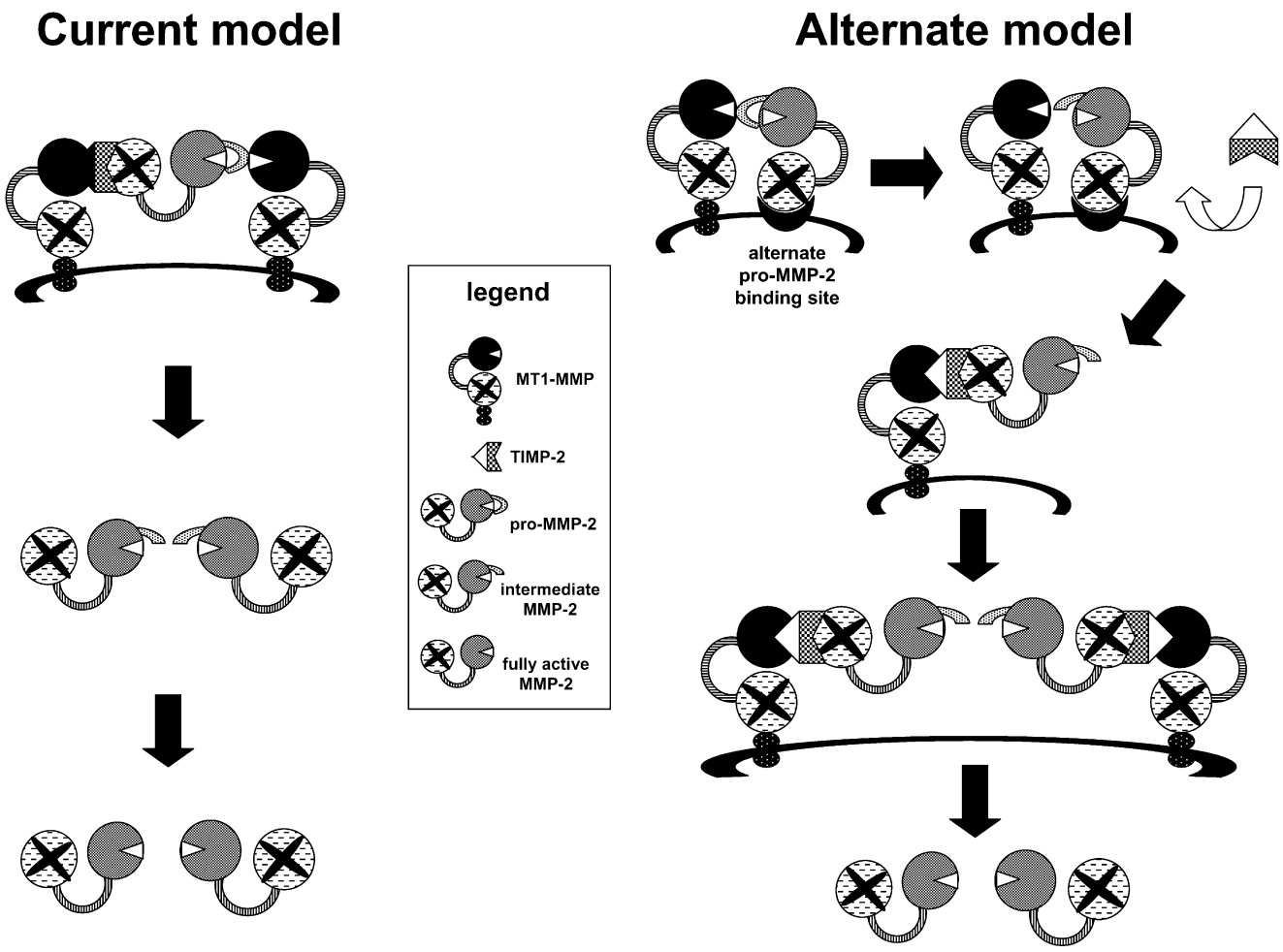

Fig. 4. Current and alternate models of pro-MMP-2 activation.

their TIMP-2$^{\Delta Ex2,3/\Delta Ex2,3}$ mice were defective in pro-MMP-2 activation. Their data also showed first step activation when stimulated with Con A, and that titration of recombinant TIMP-2 selectively enhanced the second activational step. Our data therefore consolidate observations made by others using TIMP-2-deficient cell systems where persistence of the intermediate form, but not the fully active form of MMP-2 was noted but not investigated further.

Importantly, we confirmed a critical role of the MMP-2 HXCD in pro-MMP-2 activation as previously described [8,25–28]. MMP-2 ΔHXCD did not show any activation in our system, arguing against random interactions between MT1-MMP and pro-MMP-2 allowing first step activation. Ala+TIMP-2 also inhibited pro-MMP-2 activation, presumably due to stearic hindrance of important interactions between cell surface molecules and the MMP-2 HXCD. The inhibition seen with ala+TIMP-2 suggests that the region of the MMP-2 HXCD required for TIMP-2-independent first step processing is masked by ala+TIMP-2 binding.

We have examined possible alternative tethering of pro-MMP-2 to the cell surface for first step activation. MMP-2 has been shown to bind via its HXCD to heparin, resulting in increased MMP-2 activation [32,33], however, experiments using heparinase enzyme (data not shown) suggest that heparin-containing molecules do not act as MMP-2 receptors for first step activation. The HXCD of MMP-2 can bind fibronectin [28,33], however, addition of fibronectin to our culture systems did not perturb MMP-2 activation (data not shown), suggesting that fibronectin does not participate in pro-MMP-2 activation in this model. MMP-2 can also bind type I collagen, however, Murphy et al. [34] demonstrated that MMP-2 lacking the type II fibronectin-like repeats could not bind type I collagen, but was fully capable of pro-MMP-2 activation, suggesting that collagen is also not likely to act as an alternate MMP-2 receptor involved in this alternate activation model. Another candidate would be the integrin $\alpha_v\beta_3$, which has been shown to bind MMP-2 through the HXCD [35]. The involvement of this integrin in pro-MMP-2 activation, and specifically in MMP-2 HXCD binding, has been postulated since co-transfection of MCF-7 cells with MT1-MMP and β_3 integrin facilitated the progression of the intermediate form to the fully active form [36,37]. As with others [36,37], we find very little $\alpha_v\beta_3$ integrin in our MCF-7 cells (data not shown), such that this is also not likely to facilitate first step binding in the ML20-MT1 model. Another possibility is that the binding of MMP-2 to the cell surface is of low affinity and 'non-specific', but still mediated by the HXCD. This would seem unlikely, since Emonard et al. characterized MMP-2 binding to MCF-7 cells and found it to be relatively high affinity ($K_d = 2\times10^{-9}$ M) [38]. They further

suggested that this may be mediated by MT1-MMP-tethered TIMP-2 complexes [39], however, MT1-MMP is undetectable in MCF-7 cells in our laboratory.

In conclusion, we have documented in two independent cell systems a significant level of TIMP-2-independent first step processing of pro-MMP-2, confirming some inconsistencies observed by others in the currently accepted dogma of pro-MMP-2 activation. We therefore propose an additional model for pro-MMP-2 activation that would at least supplement the established model (Fig. 4): Pro-MMP-2 binds an alternate binding site on the cell surface through its HXCD. The intermediate form of MMP-2 is then generated following the cleavage of the propeptide by a TIMP-free MT1-MMP molecule. Following this cleavage, the intermediate form is released from its primary binding site and binds to the C-terminal domain of TIMP-2, which in turn binds MT1-MMP to form the trimolecular complex. Although we have no data for this step, it is implied by the clear requirement of TIMP-2 for the second step. A conformational change in MMP-2 may account for this event, whereby the HXCD of the intermediate form of MMP-2 would display a greater affinity for the C-terminal domain of TIMP-2 than the pro-form, however, this remains to be proven. Two trimolecular complexes are then brought in close proximity to promote the autocatalytic activation of MMP-2, thus generating the fully active enzyme.

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References

[1] Chang, C. and Werb, Z. (2001) Trends Cell Biol. 11, S37–S43.
[2] Egeblad, M. and Werb, Z. (2002) Nat. Rev. Cancer 2, 161–174.
[3] Pepper, M.S. (2001) Arterioscler. Thromb. Vasc. Biol. 21, 1104–1117.
[4] Brew, K., Dinakarpandian, D. and Nagase, H. (2000) Biochim. Biophys. Acta 1477, 267–283.
[5] Atkinson, S.J., Crabbe, T., Cowell, S., Ward, R.V., Butler, M.J., Sato, H., Seiki, M., Reynolds, J.J. and Murphy, G. (1995) J. Biol. Chem. 270, 30479–30485.
[6] Butler, G.S., Butler, M.J., Atkinson, S.J., Will, H., Tamura, T., van Westrum, S.S., Crabbe, T., Clements, J., d'Ortho, M.P. and Murphy, G. (1998) J. Biol. Chem. 273, 871–880.
[7] Kinoshita, T., Sato, H., Okada, A., Ohuchi, E., Imai, K., Okada, Y. and Seiki, M. (1998) J. Biol. Chem. 273, 16098–16103.
[8] Strongin, A.Y., Collier, I., Bannikov, G., Marmer, B.L., Grant, G.A. and Goldberg, G.I. (1995) J. Biol. Chem. 270, 5331–5338.
[9] Murphy, G., Stanton, H., Cowell, S., Butler, G., Knauper, V., Atkinson, S. and Gavrilovic, J. (1999) APMIS 107, 38–44.
[10] Sato, H., Takino, T., Kinoshita, T., Imai, K., Okada, Y., Stetler Stevenson, W.G. and Seiki, M. (1996) FEBS Lett. 385, 238–240.
[11] Itoh, Y., Takamura, A., Ito, N., Maru, Y., Sato, H., Suenaga, N., Aoki, T. and Seiki, M. (2001) EMBO J. 20, 4782–4793.
[12] Lehti, K., Lohi, J., Juntunen, M.M., Pei, D. and Keski-Oja, J. (2002) J. Biol. Chem. 277, 8440–8448.
[13] Rozanov, D.V., Deryugina, E.I., Ratnikov, B.I., Monosov, E.Z., Marchenko, G.N., Quigley, J.P. and Strongin, A.Y. (2001) J. Biol. Chem. 276, 25705–25714.
[14] Fridman, R., Fuerst, T.R., Bird, R.E., Hoyhtya, M., Oelkuct, M., Kraus, S., Komarek, D., Liotta, L.A., Berman, M.L. and Stetler-Stevenson, W.G. (1992) J. Biol. Chem. 267, 15398–15405.
[15] Wingfield, P.T., Sax, J.K., Stahl, S.J., Kaufman, J., Palmer, I., Chung, V., Corcoran, M.L., Kleiner, D.E. and Stetler-Stevenson, W.G. (1999) J. Biol. Chem. 274, 21362–21368.
[16] Miller, D.L., el-Ashry, D., Cheville, A.L., Liu, Y., McLeskey, S.W. and Kern, F.G. (1994) Cell Growth Differ. 5, 1263–1274.
[17] Gilles, C., Bassuk, J.A., Pulyaeva, H., Sage, E.H., Foidart, J.M. and Thompson, E.W. (1998) Cancer Res. 58, 5529–5536.
[18] Caterina, J.J., Yamada, S., Caterina, N.C., Longenecker, G., Holmback, K., Shi, J., Yermovsky, A.E., Engler, J.A. and Birkedal-Hansen, H. (2000) J. Biol. Chem. 275, 26416–26422.
[19] Wang, Z., Juttermann, R. and Soloway, P.D. (2000) J. Biol. Chem. 275, 26411–26415.
[20] Lafleur, M.A., Hollenberg, M.D., Atkinson, S.J., Knäuper, V., Murphy, G. and Edwards, D.R. (2001) Biochem. J. 357, 107–115.
[21] d'Ortho, M.P., Stanton, H., Butler, M., Atkinson, S.J., Murphy, G. and Hembry, R.M. (1998) FEBS Lett. 421, 159–164.
[22] Lafleur, M.A., Handsley, M.M., Knauper, V., Murphy, G. and Edwards, D.R. (2002) J. Cell Sci. 115, 3427–3438.
[23] Toth, M., Hernandez-Barrantes, S., Osenkowski, P., Bernardo, M.M., Gervasi, D.C., Shimura, Y., Meroueh, O., Kotra, L.P., Galvez, B.G., Arroyo, A.G., Mobashery, S. and Fridman, R. (2002) J. Biol. Chem. 277, 26340–26350.
[24] Morrison, C.J., Butler, G.S., Bigg, H.F., Roberts, C.R., Soloway, P.D. and Overall, C.M. (2001) J. Biol. Chem. 276, 47402–47410.
[25] Murphy, G., Willenbrock, F., Ward, R.V., Cockett, M.I., Eaton, D. and Docherty, A.J. (1992) Biochem. J. 283, 637–641.
[26] Strongin, A.Y., Marmer, B.L., Grant, G.A. and Goldberg, G.I. (1993) J. Biol. Chem. 268, 14033–14039.
[27] Ward, R.V., Atkinson, S.J., Reynolds, J.J. and Murphy, G. (1994) Biochem. J. 304, 263–269.
[28] Overall, C.M., Tam, E., McQuibban, G.A., Morrison, C., Wallon, U.M., Bigg, H.F., King, A.E. and Roberts, C.R. (2000) J. Biol. Chem. 275, 39497–39506.
[29] English, W.R., Holtz, B., Vogt, G., Knäuper, V. and Murphy, G. (2001) J. Biol. Chem. 276, 42018–42026.
[30] Miyamori, H., Takino, T., Kobayashi, Y., Tokai, H., Itoh, Y., Seiki, M. and Sato, H. (2001) J. Biol. Chem. 276, 28204–28211.
[31] Higashi, S. and Miyazaki, K. (1999) J. Biol. Chem. 274, 10497–10504.
[32] Crabbe, T., Ioannou, C. and Docherty, A.J. (1993) Eur. J. Biochem. 218, 431–438.
[33] Wallon, U.M. and Overall, C.M. (1997) J. Biol. Chem. 272, 7473–7481.
[34] Murphy, G., Nguyen, Q., Cockett, M.I., Atkinson, S.J., Allan, J.A., Knight, C.G., Willenbrock, F. and Docherty, A.J. (1994) J. Biol. Chem. 269, 6632–6636.
[35] Brooks, P.C., Stromblad, S., Sanders, L.C., von Schlascha, T.L., Aimes, R.T., Stetler-Stevenson, W.G., Quigley, J.P. and Cheresh, D.A. (1996) Cell 85, 683–693.
[36] Pfeifer, A., Kessler, T., Silletti, S., Cheresh, D.A. and Verma, I.M. (2000) Proc. Natl. Acad. Sci. USA 97, 12227–12232.
[37] Deryugina, E.I., Bourdon, M.A., Jungwirth, K., Smith, J.W. and Strongin, A.Y. (2000) Int. J. Cancer 86, 15–23.
[38] Emonard, H.P., Remacle, A.G., Noel, A.C., Grimaud, J.A., Stetler-Stevenson, W.G. and Foidart, J.M. (1992) Cancer Res. 52, 5845–5848.
[39] Emmert-Buck, M.R., Emonard, H.P., Corcoran, M.L., Krutzsch, H.C., Foidart, J.M. and Stetler-Stevenson, W.G. (1995) FEBS Lett. 364, 28–32.