Membrane-type 1 matrix metalloproteinase (MT1-MMP), a transmembrane proteinase with a short cytoplasmic domain and an extracellular catalytic domain, controls a variety of physiological and pathological processes through the proteolytic degradation of extracellular or transmembrane proteins. MT1-MMP forms a complex on the cell membrane with its physiological protein inhibitor, tissue inhibitor of metalloproteinases-2 (TIMP-2). Here we show that, in addition to extracellular proteolysis, MT1-MMP and TIMP-2 control cell proliferation and migration through a non-proteolytic mechanism. TIMP-2 binding to MT1-MMP induces activation of ERK1/2 by a mechanism that does not require the proteolytic activity and is mediated by the cytoplasmic tail of MT1-MMP. MT1-MMP-mediated activation of ERK1/2 up-regulates cell migration and proliferation in vitro independently of extracellular matrix proteolysis. Proteolytically inactive MT1-MMP promotes tumor growth in vivo, whereas proteolytically active MT1-MMP devoid of cytoplasmic tail does not have this effect. These findings illustrate a novel role for MT1-MMP-TIMP-2 interaction, which controls cell functions by a mechanism independent of extracellular matrix degradation.

Membrane-type 1 matrix metalloproteinase (MT1-MMP), the prototype member of the MT-MMP subclass of matrix metalloproteinases (MMP), is a cell membrane-bound proteinase with an extracellular catalytic site and a 20-amino acid cytoplasmic tail (1). MT1-MMP degrades a variety of extracellular matrix components, including fibrillar collagen, and activates the proenzyme form of MMP-2 (gelatinase A) (2). The analysis of the phenotype of mice genetically deficient in MT1-MMP has shown important roles of this enzyme in development, connective tissue metabolism, and angiogenesis (3–5). In addition, the expression of high levels of MT1-MMP in invasive tumors has implicated MT1-MMP as an important component of the proteolytic mechanisms of cancer invasion and metastasis (2, 6–8).

MT1-MMP forms a tri-molecular complex with MMP-2 and its physiological protein inhibitor, tissue inhibitor of metalloproteinases-2 (TIMP-2). In this complex, the N-terminal, inhibitory domain of TIMP-2 binds to the catalytic site of MT1-MMP, whereas the C-terminal domain of TIMP-2 binds the C-terminal domain of MMP-2 (9). TIMP-2 is a member of a multigene family of proteins (TIMP-1 through -4) that bind and functionally inhibit their activity (10). In addition, TIMP-1 and TIMP-2 control cell migration, proliferation, and apoptosis through MMP-independent mechanisms (11–13). TIMP-2 stimulates human HT-1080 fibrosarcoma cell and normal fibroblast proliferation (14) and inhibits angiogenic factor-induced endothelial cell proliferation in vitro, as well as tumor growth and angiogenesis in vivo by an MMP-independent mechanism (15–17). Although these inhibitory effects of TIMP-2 are mediated by α5β1 integrin, the cell membrane receptor that mediates the mitogenic activity of TIMP-2 remains unclear. The MT1-MMP capacity to act as a cell membrane binding site for TIMP-2, a protein with growth factor activity, and the presence in MT1-MMP of a cytoplasmic domain prompted us to test the hypothesis that MT1-MMP generates intracellular signaling upon binding of TIMP-2. Here we show that TIMP-2 binding to MT1-MMP rapidly activates the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, buffered saline; CT, cytoplasmic tail; BSA, bovine serum albumin; FGF-2, fibroblast growth factor-2; wt, wild type; ECM, extracellular matrix; DOX, doxycycline.
which up-regulates cell proliferation and migration by a mechanism independent of the proteolytic activity of MT1-MMP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant TIMP-2 was a kind gift from Dr. R. Fridman (Wayne State University, Detroit, MI) or was purchased from Chemicon International (Temecula, CA); human TIMP-1, proMMP-2, the recombinant soluble catalytic domain of human MT1-MMP, mouse anti-human TIMP-2 antibody (MAB13441), the TIMP-2 murine monoclonal antibody IM11L, donkey anti-rabbit and donkey anti-mouse IgG (both conjugated with horseradish peroxidase), the chromogenic TMB/E (3,3′,5,5′-tetramethylbenzidine) peroxidase substrate, and Iломastat (GM6001) were purchased from Chemicon International; rabbit antibody to the hinge region of MT1-MMP (AB815) was from Triple Point Biologics (Forest Grove, OR); rabbit antibody to total ERK2 was from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit antibodies against phospho-ERK1/2 (Thr\(^{202}/Tyr^{204}\)) and phospho-c-Raf (Ser\(^{338}\), 56A6) were from Cell Signaling Technology (Danvers, MA); horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was from GE Healthcare-Amersham Biosciences (Piscataway, NJ); UO126 was from Promega (Madison, WI); UO124 was from Calbiochem; and doxycycline (DOX) was from Sigma. Rabbit polyclonal antibody to the Escherichia coli-derived catalytic domain of MT1-MMP was generated in our laboratory and purified by protein G column (Amersham Biosciences) chromatography.

**Cells and Media**—Human MCF-7 breast carcinoma cells (Clontech, Mountain View, CA), primary embryonic fibroblasts from mice genetically deficient in MT1-MMP, or their wt counterparts (a kind gift from Dr. Suneel Apte, Cleveland Clinic) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum (FCS), 4 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

**Generation of Stable Tet-On and Tet-Off Transfectants**—MCF-7 cells stably transfected with a cDNA for the tetracycline-controlled transactivator (Tet-On and Tet-Off, Clontech, Mountain View, CA) were cotransfected with human MT1-MMP cDNA in the pTRE vector (Clontech) and with the pTRE2hyg hygromycin resistance vector (Clontech) in both Tet-On and Tet-Off MCF-7 cells. Stable cotransfectants were selected in medium containing 500 μg/ml hygromycin (Invitrogen). Pools of hygromycin-resistant cells were subcultivated overnight in the presence or absence of 1 μg/ml DOX and characterized for MT1-MMP expression by reverse transcription-PCR and Western blotting. One pool of Tet-Off cells and one pool of Tet-On cells with the highest MT1-MMP expression levels were selected and used for the experiments (Fig. 1A).

**TIMP-2 and MT1-MMP Constructs**—Catalytically inert (E240A), soluble MT1-MMP consisting of the catalytic (CAT) and hemopexin (PEX) domains was C-terminally tagged with V5 and Hisx6 (MT1-E240A-CAT-PEX). After sequencing, the construct was cloned into pET101 (Invitrogen) and transformed into E. coli BL21(DE3) Codon Plus cells (Stratagene, San Diego, CA). The transformed bacteria were induced with 1 mM isopropyl β-D-thiogalactopyranoside for 6 h at 37 °C, collected by centrifugation (5,000 X g; 15 min), and sonicated (30-s pulse, 30-s interval; 8 pulses total) on ice in 10 mM Tris-HCl, pH 8.0, 1 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 0.01% SDS. Following centrifugation (15,000 X g; 20 min), the protein was washed with 10 mM Tris-HCl, pH 8.0, containing 1 mM NaCl and 1% Triton X-100 and dissolved in 10 mM Tris-HCl, pH 8.0, containing 6 mM guanidine hydrochloride and 10 mM mercaptoethanol. The soluble material was refolded by a 50-fold dilution in 100 mM Tris-HCl, pH 8.0, supplemented with 1 mM CaCl\(_2\), 1 mM ZnCl\(_2\), 500 mM L-arginine monohydrochloride, and 20% glycerol. The refolded protein was concentrated using a 30-kDa cutoff concentrator (Millipore, Billerica, MA) and purified on a 1.6 × 10 cm Co\(^{3+}\)-chelating Sepharose Fast Flow column (Amersham Biosciences) equilibrated with PBS, 1 mM NaCl. The protein was eluted with an imidazole gradient (10–500 mM, 100 ml) in PBS, 1 mM NaCl, concentrated with a 30-kDa cutoff concentrator and dialyzed against PBS containing 0.005% Brij 35.

The soluble MT1-MMP hemopexin domain-cyttoplasmic tail construct (MT1-PEX-CT) consisted of the Leu\(^{563}\)–Ser\(^{538}\) sequence of the PEX domain and the hinge region of MT1-MMP spliced to the Arg\(^{563}\)–Val\(^{582}\) sequence of the cytoplasmic tail (CT). The construct was expressed in Pichia pastoris as described (18, 19), and the secreted protein was purified by Mono S fast-protein liquid chromatography. The recombinant catalytic domain of human MT1-MMP (MT1-CAT) was expressed in E. coli, purified from inclusion bodies, and refolded to restore the catalytic activity as described previously (20).

The cDNA for the MT1-MMP mutant with deletion of the pro-domain (Apro) (21) was a kind gift from Drs. S. Zucker and J. Cao (State University of New York at Stony Brook, School of Medicine). The cDNAs for the MT1-MMP mutants with partial deletions of the prodomain (Δ573, Δ575, and Δ567) (22) were a kind gift from Dr. J. Keski-Oja (University of Helsinki, Finland). The ΔTM mutant containing a deletion of the prodomain and transmembrane domains, the Y573, Y574D, and C574A mutants, and the Ala + TIMP-2 mutant have been described before (23, 24). Ala + TIMP-2 was produced in P. pastoris and purified to homogeneity by Mono-Q fast-protein liquid chromatography.

**Transient Transfections**—Wild-type and mutant MT1-MMP cDNAs cloned in pcDNA3 were transfected into subconfluent MCF-7 cells in 24-well plates using 1 μg of the constructs and 3 μl of FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). Twenty-four hours after transfection, the cells were incubated with Dulbecco’s modified Eagle’s medium containing 0.5% FCS for additional 24 h and immediately used for the experiments.

**Western Blotting**—Western blotting was performed as described (25).

**Immunoprecipitation**—Triton X-100 cell extracts were immunoprecipitated as described (25) with antibody A8B15.

**Ras Activation Assay**—Ras activation was measured by the EZ-Detect Ras Activation Kit (Pierce).

**TIMP-2-MT1-MMP Binding Assay**—To measure TIMP-2 and Ala + TIMP-2 binding to MT1-MMP, the wells of a 96-well plate (Nunc, Rochester, NY) were coated with either purified MT1-E240A-CAT-PEX or MT1-CAT or MT1-PEx-CT, or
with BSA as a control (3 μg/ml of each protein in 125 μl of 15 mM bicarbonate buffer, pH 9.6) at 4 °C for 18 h. The wells were then blocked with 3% BSA in PBS, 0.01% Tween 20 for 90 min at 37 °C. TIMP-2 or Ala+TIMP-2 (2 μg/ml in 75 μl of PBS, 3% BSA, 0.01% Tween 20; ~ 100 nM) were added for 2 h at room temperature. An equivalent amount of BSA was added as a control instead of TIMP-2 or Ala+TIMP-2. After washing five times with PBS, 0.005% Tween 20, murine antibody to TIMP-2 (IM11L, 0.5 μg/ml in 75 μl of PBS, 3% BSA, 0.01% Tween 20) was added for 2 h at room temperature. The unbound antibody was removed by washing with PBS-0.005% Tween 20, and donkey anti-mouse IgG conjugated with horseradish peroxidase (1:10,000 in 75 μl of PBS, 3% BSA, 0.01% Tween 20) was added for 1 h. Following extensive washing with PBS, 3% BSA, 0.01% Tween 20, and with H2O, TMB/E substrate (100 μl) was added to the wells. The reaction was stopped by adding 0.1 ml of 1 M H2SO4, and A450 was measured with a plate reader. Each sample was analyzed in triplicate. To measure TIMP-2 and Ala+TIMP-2 binding to the MT1-MMP catalytic domain (MT1-CAT) the wells of a 96-well plate (Nunc) were coated at 4 °C for 18 h with TIMP-2 or Ala+TIMP-2, or with BSA as a control (2 μg/ml each in 125 μl of 15 mM bicarbonate buffer, pH 9.6). After blocking with 3% BSA in PBS, 0.01% Tween 20 for 90 min at 37 °C, purified MT1-CAT (2.5 μg/ml in 75 μl of PBS, 3% BSA, 0.01% Tween 20; ~ 125 nM) was added into the wells for 2 h at room temperature. An equivalent amount of BSA was added as a control instead of MT1-CAT. The wells were washed five times with PBS-0.005% Tween 20, and rabbit antibody against the catalytic domain of MT1-MMP (0.5 μg/ml in 75 μl PBS, 3% BSA, 0.01% Tween 20) was added for 2 h at room temperature. The unbound antibody was removed by washing with PBS, 0.005% Tween 20. Horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:10,000 in PBS, 3% BSA, 0.01% Tween 20) was added, and incubation was continued for 1 h. Following extensive washing with PBS, 3% BSA, 0.01% Tween 20, and with H2O, TMB/E substrate (100 μl) was added to the wells. The reaction was stopped by adding 0.1 ml 1 M H2SO4, and A450 was measured with a plate reader. Each sample was analyzed in triplicate.

Cell Migration Assays—MCF-7 cells grown overnight in 0.5% FCS in the presence or absence of 1 μg/ml DOX were seeded in the upper compartment of modified blind wells with 8-μm pore polycarbonate filters (2.5 × 104 cells/200 μl of Dulbecco’s modified Eagle’s medium containing 0.5% FCS). Purified TIMP-2 or TIMP-1 (100 ng/ml) and the MEK1/2 inhibitor UO126, or UO124 as a control (10 μM), were added to the upper compartment. After 6-h incubation at 37 °C, the cells on the lower part of the membrane were fixed-stained with Diff-Quick (Dade-Behring, Newark, DE) and counted under a light microscope. The data are presented as the mean number of migrated cells per five 20× fields. To measure the migration of MT1-MMP+/+ or MT1-MMP−/− fibroblasts, confluent cells grown in 6-well plates and incubated overnight with medium containing 0.5% FCS were scraped with the edge of a sterile razor blade in one to three areas measuring ~1.5 × 0.3 cm. Following wounding, the cells were incubated for 24 h with either control medium (Control) or TIMP-2 (100 ng/ml) or, as positive controls, FGF-2 (10 ng/ml) or FCS (10%). Fixed cells were stained with crystal violet, and cells migrated beyond 0.8 mm into the denuded areas of triplicate wells were counted in a blinded fashion using an ocular grid (26).

Cell Proliferation Assay—Cells grown overnight in 0.5% FCS in the presence or absence of 1 μg/ml DOX were seeded into 96-well plates (5,000 cells/well) in the same medium. After 24-h incubation TIMP-2 (100 ng/ml) and/or GM6001 (50 μM) were added to the medium, and the incubation was continued. Doxycycline, TIMP-2, GM6001, and UO126 were added again every 48 h. Cell number was measured daily by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining method using the Cell Titer 96 Non-radioactive Cell Proliferation Assay kit (Promega). Each sample was assayed in triplicate. To measure MT1-MMP+/+ and MT1-MMP−/− fibroblast proliferation bromodeoxyuridine (10 μM) was added to the culture medium of cells incubated overnight in 0.5% FCS. Following 20 min incubation at 37 °C, bromodeoxyuridine incorporation was detected by immunocytochemistry according to the manufacturer’s instructions (Roche Applied Science).

Tumor Growth in Vivo—Confluent MCF-7 cells stably transfected with the indicated MT1-MMP mutants or empty vector (27) were suspended at 1.0 × 106 cells/ml in Dulbecco’s modified Eagle’s medium. Fifty microliters of cell suspension (5.0 × 106 cells) were injected subcutaneously in the mammary fat pad of 4-week-old female athymic BALB/c nu/nu mice (Benton & Kingman, San Francisco, CA). Five to six animals per group were used. Tumor growth was monitored every 6–8 days by caliper measurements of two perpendicular diameters (D1 and D2) of the xenografts. Tumor volume was calculated by the formula: π/6(D1 × D2)3/2.

Immunohistochemistry—Five-micrometer frozen sections were immunostained with anti-Ki67 (Dako MIB-1), anti-cleaved caspase-3 (Cell Signaling Technology #9664S), or anti-CD31 (Dako JC70A) antibodies using the M.O.M. Vectastain Elite ABC and Vectastain Elite Kits (Vector Laboratories, Burlingame, CA) as described (28, 29). Sections stained in the absence of primary antibodies were used as negative controls. Peroxidase-positive cells were counted in 20 random 400× microscopic fields.

Densitometry—Quantitative analysis of immunoreactive bands was performed by scanning densitometry with Kodak 1D Image Analysis software (Kodak, Rochester, NY) and ImageJ 10.2 (National Institutes of Health). The results are presented as the ratio between the readings of the sample and that of the corresponding loading control.

Statistical Analysis—Statistical analysis was performed by the Student’s t test with SigmaStat and Stat A, and data were interpolated using SigmaPlot software (Jandel Corp., Corte Madera, CA). p values < 0.05 were considered significant.

RESULTS

MT1-MMP Expression and Exogenous TIMP-2 Induce ERK1/2 Activation with a Dose-dependent Effect—To test the hypothesis that TIMP-2 binding to MT1-MMP triggers intracellular signaling, we used MCF-7 cells transfected with MT1-MMP cDNA under control by the tetracycline-controlled transactivator. Parental MCF-7 cells express no, or very low....
levels of MT1-MMP, MMP-2, and TIMP-2 (27). Addition to, or removal of DOX (1 μg/ml) from the culture medium of Tet-On or Tet-Off transfectants, respectively, resulted in strong induction of MT1-MMP expression (27). The recombinant MT1-MMP expressed by the transfected cells was active and was inhibited by human TIMP-2 (100 ng/ml) or by the hydroxamate inhibitor Ilosmatat (27) (supplemental Fig. S1). TIMP-2 (100 ng/ml) was then added to the medium of Tet-On transfectants in the presence or absence of DOX, and the cells were analyzed for ERK1/2 activation after 5–30 min of incubation (Fig. 1, C and D). Consistent with previous reports (30), MT1-MMP expression resulted in constitutive ERK1/2 activation. Addition of TIMP-2 to MT1-MMP-expressing cells induced rapid (5–15 min) up-regulation of ERK1/2 activation but had a much lower, non-significant effect on cells that did not express MT1-MMP. Comparable results were obtained with either Tet-On or Tet-Off transfectants (Fig. 1, A and B), showing that ERK1/2 activation did not result from a nonspecific effect of DOX. In addition, TIMP-2 induced ERK1/2 activation in human HT1080 fibrosarcoma cells and MDA-MB-231 breast carcinoma cells that constitutively express MT1-MMP, as well as in primary wild-type (wt) mouse fibroblasts but had a dramatically lower, non-significant effect in fibroblasts from mice genetically deficient in MT1-MMP (Fig. 2). These results indicated that TIMP-2 up-regulates ERK1/2 activation in MT1-MMP-expressing cells.

To investigate the mechanism of ERK1/2 activation we analyzed the effect of TIMP-2 on phosphorylation of Ras and c-Raf, upstream activators of the ERK1/2 pathway. The results (Fig. 3) showed that TIMP-2 addition to MT1-MMP-expressing cells induced rapid (5 min) up-regulation of Ras and c-Raf activation but had no such effect on cells devoid of MT1-MMP. Thus, TIMP-2 interaction with MT1-MMP induces ERK1/2 phosphorylation through the Ras-Raf-1-MEK1/2 pathway.

To characterize the relative contribution of MT1-MMP and TIMP-2 to ERK1/2 activation, we incubated MT1-MMP
Tet-On transfectants with increasing DOX concentrations (0.1 to 1 μg/ml). This treatment induced MT1-MMP expression and ERK1/2 activation in a dose-dependent manner. Addition of TIMP-2 (100 ng/ml) to the culture medium dramatically up-regulated ERK1/2 activation (Fig. 4, A and B). In a second set of experiments (Fig. 4, C and D) increasing concentrations of TIMP-2 (0 to 300 ng/ml) were added to the culture medium of cells that either expressed or did not express MT1-MMP, and the levels of both cell-associated TIMP-2 and active ERK1/2 were characterized. With cells that expressed MT1-MMP the levels of both cell-associated TIMP-2 and active ERK1/2 increased in a dose-dependent manner. In contrast, with cells that did not express MT1-MMP the levels of cell-associated TIMP-2 and active ERK1/2 did not increase significantly.

To characterize the cellular binding site of TIMP-2 MT1-MMP Tet-Off transfectants grown in the presence or absence of DOX were incubated with 100 ng/ml TIMP-2. Cell extracts were immunoprecipitated with MT1-MMP antibody, and the precipitates were analyzed by Western blotting with antibodies to TIMP-2. Induction of MT1-MMP expression directly correlated with the amount of TIMP-2 that coimmunoprecipitated with MT1-MMP, and caused a concomitant decrease of soluble TIMP-2 in the culture medium. No MT1-MMP or TIMP-2 was detected in the immunoprecipitation supernatant, showing that all TIMP-2 in the cell extract was associated with MT1-MMP (supplemental Fig. S2). Thus, in our transfected cells MT1-MMP is the only, or at least the major, binding site for TIMP-2. This conclusion is consistent with previous reports showing the existence on several cell types of one TIMP-2 binding site identified as MT1-MMP (9, 31).

To test the specificity of TIMP-2 in inducing ERK1/2 activation, we analyzed the effect of TIMP-1, whose binding constant for MT1-MMP ($k_{on} = 0.000503$ (m$^{-1}$) $\times 10^{-6}$) is five orders of magnitude lower than that of TIMP-2 ($k_{on} = 4.2 \pm 0.22$ (m$^{-1}$) $\times 10^{-6}$) (32). TIMP-1 did not activate ERK1/2 in MT1-MMP-expressing cells (Fig. 5, A and B; compare the intensity of the band in the MT1-MMP plus TIMP-1 sample, right-most lane, to that of its control, MT1-MMP minus TIMP, second lane from left). Thus, altogether these results indicated that TIMP-2 binding to MT1-MMP up-regulates ERK1/2 activation.

**TIMP-2 Induction of ERK1/2 Activation Does Not Require the MMP Inhibitory Activity and Occurs in the Absence or Presence of MMP-2**—To investigate whether TIMP-2 activation of ERK1/2 results from inhibition of MT1-MMP (or other MMPs) we characterized the effect on ERK1/2 activation of Ala+TIMP-2, a mutant TIMP-2 devoid of MMP inhibitory activity (23). The results (Fig. 5, C and D) showed that Ala+TIMP-2 induced ERK1/2 activation as effi-
FIGURE 5. Specificity of TIMP-2 binding to MT1-MMP and ERK1/2 activation. A, MT1-MMP Tet-Off MCF-7 cells grown for 24 h in the presence (MT1-MMP−) or absence (MT1-MMP+) of 1 μg/ml DOX in medium containing 0.5% FCS were treated with 100 ng/ml purified human TIMP-2 or TIMP-1 for 15 min. Cell extract protein was characterized for active ERK1/2 (pERK1/2) and for ERK2 as a loading control. B, densitometric analysis. C, Western blotting analysis of ERK1/2 activation in MT1-MMP Tet-Off transfectants incubated for 15 min with 100 ng/ml TIMP-2 or AlaMAp+TIMP-2. ERK2, loading control. D, densitometric analysis. B and D show mean ± S.E. of densitometric readings normalized to the corresponding loading control; *, p < 0.05 (MT1-MMP+ versus MT1-MMP−). These experiments were repeated three times with comparable results.

FIGURE 6. Non-proteolytic interaction of AlaTIMP-2 with MT1-MMP. A, the wells of a 96-well plate were coated with TIMP-2 or AlaTIMP-2 or with control BSA and then incubated with the MT1-MMP catalytic domain (MT1-CAT). B, the wells of a 96-well plate were coated with a construct consisting of the MT1-MMP hemopexin domain and hinge region spliced to the cytoplasmic tail (MT1-PEX-CT) or with a construct consisting of the catalytic domain (CAT) of the proteolytically inactive E240A MT1-MMP mutant spliced to the hemopexin (PEX) domain (MT1-E240A-CAT-PEX), or with control BSA and then incubated with TIMP-2 or AlaTIMP-2. Bound proteins were measured as described under "Experimental Procedures." Mean ± S.E. of triplicate samples are shown. These experiments were repeated three times with comparable results; *, p < 0.05 (sample + versus BSA).

wt TIMP-2 and AlaTIMP-2 bound to a construct consisting of the MT1-MMP hemopexin domain and hinge region spliced to the cytoplasmic tail (MT1-PEX-CT). The results showed that both wt TIMP-2 and AlaTIMP-2 bound significantly to this construct, indicating that the signaling effect of TIMP-2 can be mediated by interaction of its C-terminal domain with the PEX or hinge domain of MT1-MMP.

On the cell surface MT1-MMP forms a tri-molecular complex with TIMP-2 and MMP-2. In this complex, the N-terminal, inhibitory domain of TIMP-2 binds to the catalytic site of MT1-MMP, whereas the C-terminal domain of TIMP-2 binds the C-terminal domain of MMP-2 (9). To investigate the potential role of this complex in intracellular signaling we characterized the effect of proMMP-2-TIMP-2 complex on ERK1/2 activation. Addition of a pre-formed proMMP-2-TIMP-2 complex to cells expressing MT1-MMP activated ERK1/2 as efficiently as TIMP-2 alone. In contrast, addition of proMMP-2 alone to MT1-MMP-expressing cells, or addition of proMMP-2-TIMP-2 complex to cells devoid of MT1-MMP did not induce ERK1/2 activation (supplemental Fig. S3). These results showed that MT1-MMP mediates ERK1/2 activation when it interacts with either TIMP-2 or proMMP-2-TIMP-2 complex.

Inhibition of TIMP-2 Binding to Cell Membrane-bound MT1-MMP Abolishes ERK1/2 Activation—To confirm that TIMP-2 binding to MT1-MMP is necessary for ERK1/2 activation, we tested the effect of reagents that compete with TIMP-2 for binding to the MMP catalytic site. For this purpose, we characterized ERK1/2 activation in MT1-MMP-expressing cells treated with TIMP-2 in the presence or absence of the recombinant, soluble catalytic domain of MT1-MMP (Fig. 7, A and B), or Ilomastat, a low molecular weight inhibitor that binds to the catalytic domain of MMPs (Fig. 7, C and D). Both the soluble MT1-MMP catalytic domain and Ilomastat, as well as other hydroxamic acid-based MMP inhibitors, compete with membrane-bound MT1-MMP for TIMP-2 binding, and thus remove TIMP-2 from cell-bound MT1-MMP (31). Both treatments induced a dose-dependent decrease of cell-associated TIMP-2 and ERK1/2 activation. The highest concentrations of Ilomastat or soluble MT1-MMP catalytic domain completely abrogated cell-associated TIMP-2 and ERK1/2 activation, showing that TIMP-2 binding to cell surface-associated MMP up-regulates ERK1/2 activation (Fig. 7). This finding also suggested that the low level of ERK1/2 activation observed in MT1-MMP-expressing cells in the absence of exogenous TIMP-2 may be induced by low amounts of endogenous TIMP-2. Thus, TIMP-2 interaction with membrane-bound MT1-MMP stimulates ERK1/2 activation.
ERK1/2 Activation Requires the Cytoplasmic Tail but Not the Proteolytic Activity of MT1-MMP—To determine the relative contribution of the catalytic and cytoplasmic domains of MT1-MMP to ERK1/2 activation, we characterized the effect of mutations in the catalytic site and cytoplasmic tail of MT1-MMP. For this purpose we used a mutant with one amino acid substitution (E240A) in the catalytic domain that abrogates the proteolytic activity, and a mutant lacking the entire cytoplasmic domain (27). The mutant devoid of proteolytic activity (E240A) bound TIMP-2 and activated ERK1/2 with an effect comparable to that of wt MT1-MMP (Figs. 9 and 10, A and B). In contrast, cells transfected with mutant MT1-MMP lacking the cytoplasmic tail did not activate ERK1/2, although their level of MT1-MMP-bound TIMP-2 was comparable to that of wt or E240A MT-MMP transfectants (Fig. 9). Comparable levels of wt and MT1-MMP mutants were exposed on the cell membrane (supplemental Figs. S4 and S5), showing that the mutants' inability to induce intracellular signaling did not result from reduced cell membrane expression. Thus, TIMP-2 binding to MT1-MMP generates ERK1/2 activation with a proteolysis-independent mechanism that requires the cytoplasmic domain of MT1-MMP. This conclusion is also supported by our finding that the soluble MT1-MMP catalytic domain, or MT1-MMP devoid of the transmembrane and cytoplasmic domains (ATM), does not mediate ERK1/2 activation (Figs. 7A, 7B, 10A, and 10B).

The $^{573}$YCQR$^{576}$ Sequence of the MT1-MMP Cytoplasmic Tail Mediates TIMP-2 Induction of ERK1/2 Activation—We then analyzed the role of the MT1-MMP cytoplasmic tail in ERK1/2 activation. For this purpose we used mutants lacking either 6 or 10 or 16 C-terminal amino acid residues that include three putative phosphorylation sites, Ser$^{577}$, Tyr$^{573}$, and Thr$^{567}$, respectively. The cell membrane expression of these mutants is comparable to that of wt MT1-MMP (22). Deletion of the 6 C-terminal amino acids ($\Delta$577) did not abolish ERK1/2 activation upon addition of TIMP-2. In contrast, MT1-MMP mutants lacking the 10 or 16 C-terminal amino acids ($\Delta$573 and $\Delta$567) did not mediate ERK1/2 activation (Fig. 10, A and B).

To corroborate these findings we characterized the effect on ERK1/2 activation of a mutation in the pro-domain of MT1-MMP that abrogates the TIMP-2-binding capacity (21). MCF-7 cells were transiently transfected with wt or mutant MT1-MMP cDNA and characterized for ERK1/2 activation and cell-associated TIMP-2. Cells transfected with mutant MT1-MMP had no cell-associated TIMP-2 and no active ERK1/2 in the presence or absence of exogenous TIMP-2 (Fig. 8). Because comparable levels of wt and mutant MT1-MMP were expressed on the cell membrane (supplemental Figs. S4 and S5), these results demonstrated that TIMP-2 binding to membrane-bound MT1-MMP stimulates ERK1/2 activation.

ERK1/2 Activation by TIMP-2 Binding to MT1-MMP

FIGURE 7. Inhibition of TIMP-2 binding to MT1-MMP abrogates ERK1/2 activation. MT1-MMP Tet-Off MCF-7 cells grown for 24 h in the presence or absence of 1 $\mu$g/ml DOX were treated for 15 min at 37 °C with the indicated concentrations of the recombinant, soluble catalytic domain of MT1-MMP (A and B) or Ilomastat (C and D). TIMP-2 (100 ng/ml) was then added for 15 min, and cell extracts were characterized for cell-associated TIMP-2 and for active ERK1/2. A and C, Western blotting; B and D, densitometric analysis. The histograms show mean ± S.E. of densitometric readings normalized to the corresponding loading control; *, p < 0.05 (MT1-MMP + TIMP + MT cat + versus MT1-MMP + TIMP/MT cat 0 control). All samples in D are significantly different (p < 0.05) from the Ilomastat (50 $\mu$m) control. These experiments were repeated three times with comparable results.

FIGURE 8. Inhibition of TIMP-2 binding to MT1-MMP abrogates ERK1/2 activation. Wild-type (wt) or Δpro MT1-MMP cDNA or control empty vector (−) was transfected into MCF-7 cells. Twenty-four hours later, the cells were incubated with medium containing 0.5% FCS. After overnight incubation, 100 ng/ml purified TIMP-2 (+) or PBS (−) was added to the medium for 15 min, and cell extracts were characterized for active ERK1/2, TIMP-2, and MT1-MMP. A, Western blotting; B, densitometric analysis. Mean ± S.E. of densitometric readings normalized to the corresponding loading control; *, p < 0.05 (TIMP-2 + versus TIMP−). These experiments were repeated three times with comparable results.
that was not up-regulated by addition of TIMP-2. In contrast, in cells expressing Y573D MT1-MMP the level of active ERK1/2 was comparable to that of control, vector-transfected cells and was not increased by exogenous TIMP-2 (Fig. 10, A and B). Thus, both the Tyr573 and Cys574 residues contribute to signal transduction through the cytoplasmic tail of MT1-MMP.

**MT1-MMP-TIMP-2 Interaction Stimulates Cell Migration and Proliferation by a Proteolysis-independent Mechanism That Requires the MT1-MMP Cytoplasmic Tail and ERK1/2 Activation**—TIMP-2 modulates cell migration and proliferation, functions controlled by the ERK1/2 pathway in a variety of cell types (36). Therefore, we characterized the role of MT1-MMP/TIMP-2-mediated activation of ERK1/2 in the control of these cell functions. In blind-well assays for migration (Fig. 11A) addition of TIMP-2 to MT1-MMP-expressing cells stimulated cell migration in a dose-dependent manner but had no such effect on cells that did not express MT1-MMP. In contrast, TIMP-1, which does not induce ERK1/2 activation (Fig. 5, A and B), did not increase cell migration both in the presence and in the absence of MT1-MMP expression (Fig. 11A). Inhibition of ERK1/2 activation with the synthetic MEK1/2 inhibitor UO126 (10 μM) blocked TIMP-2-induced cell migration (Fig. 11A, C and D), showing that TIMP-2-MT1-MMP interaction up-regulates cell motility through activation of the ERK1/2 pathway. Expression of MT1-MMP devoid of proteolytic activity (E240A, Fig. 11B) had a modest effect on cell migration; however, addition of TIMP-2 up-regulated cell motility with an effect comparable to that obtained with wt MT1-MMP transfected cells. In contrast, the migration of cells transfected with MT1-MMP devoid of cytoplasmic tail (ΔCT) was similar to that of control, vector-transfected cells both in the absence and in the presence of exogenous TIMP-2 (Fig. 11B). Similarly, primary fibroblasts from mice genetically deficient in MT1-MMP (MT1-MMP−/−) showed severely impaired migration relative to MT1-MMP+/+ fibroblasts (Fig. 11C). TIMP-2 dramatically up-regulated migration in MT1-MMP+/+ cells with an effect comparable to that of FGF-2 (10 ng/ml) or serum

![FIGURE 9. ERK1/2 activation requires the cytoplasmic tail but not the proteolytic activity of MT1-MMP.](image)

MCF-7 cells transiently transfected with wt MT1-MMP, E240A, or ΔCT (Δ563–582) MT1-MMP were treated with TIMP-2 and analyzed by Western blotting for MT1-MMP expression and ERK1/2 activation, and for MT1-MMP-associated TIMP-2 by co-immunoprecipitation as described in the legend to supplemental Fig. S2. densitometric analysis. Mean ± S.E. of densitometric readings normalized to the corresponding loading control; *, p < 0.05 (TIMP-2+ versus TIMP−). This experiment was repeated three times with comparable results. Similar results were obtained with stable transfectants.

![FIGURE 10. A YCQR motif in the MT1-MMP cytoplasmic tail mediates TIMP-2 induction of ERK1/2 activation.](image)

MCF-7 cells transiently transfected with wt MT1-MMP or the indicated MT1-MMP mutant cDNAs were treated with TIMP-2 for 15 min and analyzed by Western blotting for MT1-MMP expression and ERK1/2 activation. Cells transfected with empty pcDNA3 vector (−) were used as control. wt: wild-type MT1-MMP; Δ577, Δ573, and Δ567: MT1-MMP mutants lacking either 6 or 10 or 16 C-terminal amino acid residues of the cytoplasmic tail, respectively; ΔTM: MT1-MMP mutant with deletion of the transmembrane and cytoplasmic domains; Y573A, C574A, and Y573D: alanine or aspartic acid substitutions of the Tyr573 and Tyr574 amino acid residues of the MT1-MMP cytoplasmic tail. A and C, Western blotting; B and D, densitometric analysis. Mean ± S.E. of densitometric readings normalized to the corresponding loading control; *, p < 0.05 (A, sample versus corresponding MT1-MMP− control; B, sample versus corresponding wt MT1-MMP control). This experiment was repeated three times with comparable results.

showing that the sequence Y573YCQR576 is required for ERK1/2 activation upon TIMP-2 binding to MT1-MMP.

Tyr573 is a phosphorylation site (33). Cys574 mediates MT1-MMP di- or oligomerization, interactions with the cytoskeleton (27) and phosphorylated caveolin-1 (34), and is involved in cell migration through palmitoylation, which directs MT1-MMP to lipid rafts (35). To investigate the role of Tyr573 and Cys574 in ERK1/2 activation we tested the effect of alanine substitution of Tyr573 (Y573A) or Cys574 (C574A), and of aspartic acid substitution of Tyr573 (Y573D), because aspartic acid has a negative charge similar to phosphorylated tyrosine. Alanine substitution of Tyr573 or Cys574 caused constitutive activation of ERK1/2 with TIMP-2 and analyzed by Western blotting for MT1-MMP expression and ERK1/2 activation, and for MT1-MMP-associated TIMP-2 by co-immunoprecipitation as described in the legend to supplemental Fig. S2. densitometric analysis. Mean ± S.E. of densitometric readings normalized to the corresponding loading control; *, p < 0.05 (TIMP-2+ versus TIMP−). This experiment was repeated three times with comparable results. Similar results were obtained with stable transfectants.

![FIGURE 9. ERK1/2 activation requires the cytoplasmic tail but not the proteolytic activity of MT1-MMP.](image)

A, MCF-7 cells transiently transfected with wt MT1-MMP, E240A, or ΔCT (Δ563–582) MT1-MMP were treated with TIMP-2 and analyzed by Western blotting for MT1-MMP expression and ERK1/2 activation, and for MT1-MMP-associated TIMP-2 by co-immunoprecipitation as described in the legend to supplemental Fig. S2. densitometric analysis. Mean ± S.E. of densitometric readings normalized to the corresponding loading control; *, p < 0.05 (TIMP-2+ versus TIMP−). This experiment was repeated three times with comparable results. Similar results were obtained with stable transfectants.
**ERK1/2 Activation by TIMP-2 Binding to MT1-MMP**

**FIGURE 11. MT1-MMP-TIMP-2 interaction stimulates cell migration through activation of the ERK1/2 pathway.** Boyden chamber assays. A, MT1-MMP Tet-Off MCF-7 cells in the absence (+MT1-MMP) or presence of 1 μg/ml DOX (-MT1-MMP). B, MCF-7 cells transiently transfected with wt or the indicated mutant MT1-MMP cDNAs or with control empty vector (zeo). Tet-Off and transient transfectants were incubated for 6 h in 0.5% FCS medium with the indicated concentrations of purified TIMP-2 or TIMP-1 in the presence or absence of the MEK1/2 inhibitor UO126 (10 μM, A), or in the absence (-TIMP-2) or presence of 100 ng/ml TIMP-2 (+TIMP-2; B). The cells were fixed, stained (Diff-Quick stain kit), and counted with a light microscope. The mean and experimental range of triplicate samples from a representative experiment are shown. These experiments were repeated three times with similar results. **C**, migration of primary MT1-MMP+/+ (black bars) or MT1-MMP−/− fibroblasts (open bars) measured as described under “Experimental Procedures.” The bars show mean ± S.D. of triplicate samples and controls (five random fields/well). **D**, Western blotting analysis of ERK1/2 activation in MT1-MMP Tet-Off MCF-7 grown in the absence of DOX and incubated for 15 min in the presence (+) or absence (−) of TIMP-2 (100 ng/ml). The cells were preincubated for 30 min with 10 μM UO126 or its inactive isomor, UO124, or vehicle alone (−). These experiments were repeated three times with similar results; *, p < 0.05 (sample versus control in A; sample versus zeo control in B; sample versus respective control in C).

In contrast, TIMP-2 had no such effect on MT1-MMP−/− fibroblasts (Fig. 11C).

Induction of MT1-MMP expression in the absence of exogenous TIMP-2, or addition of TIMP-2 to cells that did not express MT1-MMP had a modest effect on cell proliferation. However, addition of exogenous TIMP-2 to MT1-MMP-expressing cells dramatically up-regulated cell growth (Fig. 12A). The mitogenic effect of TIMP-2 binding to MT1-MMP was comparable to that of 5–10% fetal calf serum, as assessed by proliferating cell nuclear antigen expression (data not shown), and was abrogated by both the MMP inhibitor, ilomastat, and the MEK1/2 inhibitor, UO126 (Fig. 12B). Expression of MT1-MMP devoid of proteolytic activity (E240A) had an effect on cell proliferation similar to that of wt MT1-MMP (Fig. 12C). In contrast, cells expressing MT1-MMP devoid of cytoplasmic tail (∆CT) showed a growth rate comparable to that of control, vector-transfected cells both in the absence and in the presence of exogenous TIMP-2 (Fig. 12D). Primary fibroblasts from mice genetically deficient in MT1-MMP (MT1-MMP−/−) showed severely impaired proliferation relative to MT1-MMP+/+ fibroblasts (Fig. 12E). TIMP-2 strongly up-regulated MT1-MMP+/+ cell growth with an effect comparable to that of FGF-2 (10 ng/ml) or serum (10%). In contrast, TIMP-2 had no effect on MT1-MMP−/− cell proliferation. In both cell migration and proliferation experiments all the cell transfectants showed comparable adhesion to the substrate. Thus, TIMP-2 binding to MT1-MMP up-regulates cell migration and proliferation through activation of the ERK1/2 signaling pathway. These effects require the cytoplasmic tail but not the proteolytic activity of MT1-MMP.

MT1-MMP Contributes to Tumor Growth in Vivo through a Proteolysis-independent Mechanism Mediated by the Cytoplasmic Tail—To investigate the biological significance of our findings in vivo we characterized the growth of tumors expressing wt or mutant MT1-MMP. For this purpose MCF-7 cells stably transfected with wt MT1-MMP or mutant MT1-MMP devoid of proteolytic activity (E240A) or cytoplasmic tail (∆CT), or mock transfected (27) were xenografted into immunodeficient mice. Following a lag period of 80–100 days, tumors expressing wt MT1-MMP or proteolytically inactive, E240A MT1-MMP grew to a size that largely exceeded that of tumors expressing no MT1-MMP or MT1-MMP devoid of cytoplasmic tail. At day 130 post-implantation there was an over 20-fold size difference between tumors expressing wt MT1-MMP and tumors derived from mock-transfected cells. The volume of tumors expressing proteolytically inactive MT1-MMP was ~50% that of wt MT1-MMP-expressing tumors (p < 0.05). In contrast, tumors derived from cells expressing MT1-MMP devoid of cytoplasmic tail were no different in size from those derived from control, vector-transfected cells (Fig. 13A).

To confirm that the tumors derived from E240A MT1-MMP transfectants expressed the MT1-MMP mutant, tumor extracts were analyzed by Western blotting with polyclonal antibody to the hinge region (AB815) and monoclonal antibody to the catalytic domain of MT1-MMP (monoclonal antibody 5D1; generated in our laboratories). Mock-transfected cells or cells transfected with MT1-MMP devoid of cytoplasmic tail did not generate tumors large enough to be characterized by this method. The tumor extracts were compared with extracts of cells co-transfected with wt MT1-MMP and α3 integrin, which express active MT1-MMP (37). The Western blotting analysis (Fig. 13B) showed different patterns of MT1-MMP bands between wt MT1-MMP and E240A MT1-MMP tumors. wt MT1-MMP tumors expressed predominantly the 63-kDa proenzyme, as evidenced by monoclonal antibody 5D1, and the 36- to 40-kDa degradation product of MT1-MMP, as evidenced by AB815, a pattern consistent with rapid autocalytic degradation of the active proteinase. Conversely, in tumors derived from E240A MT1-MMP transfectants MT1-MMP occurred mostly in the 58-kDa form devoid of pro-peptide, with low amounts of...
ERK1/2 Activation by TIMP-2 Binding to MT1-MMP

FIGURE 12. MT1-MMP-TIMP-2 interaction stimulates cell proliferation through activation of the ERK1/2 pathway. Proliferation assays. A, MT1-MMP Tet-Off cells grown in medium containing 0.5% FCS with or without 1 µg/ml DOX and 100 ng/ml purified TIMP-2. B, MT1-MMP Tet-On cells grown in medium containing 0.5% FCS with or without 1 µg/ml DOX (-AT-TIMP-2). Cells grown with DOX received the addition of 100 ng/ml purified TIMP-2 without (MT+TIMP-2) or with Ilomastat 50 µM (MT+TIMP-2+Ilomastat) or UO126 10 µM (MT+TIMP-2+UO126). C and D, MCF-7 cells transiently transfected with control empty vector (zeo) or E240A MT1-MMP cDNA (C), or with wt MT1-MMP (wt), or tail-deleted MT1-MMP (ΔCT MT1-MMP) cDNAs and grown in the presence or absence of 100 ng/ml purified TIMP-2. Cell number was measured daily by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining method. Each point represents mean of triplicate samples from a representative experiment. For each point the S.D. did not exceed 5% of the mean. Similar results were obtained with stable transfectants. E, bromodeoxyuridine uptake in primary fibroblasts from MT1-MMP+/+ (black bars) or MT1-MMP−/− mice (white bars) incubated for 24 h with either control medium (Control) or TIMP-2 (100 ng/ml) or, as positive controls, FGF-2 (10 ng/ml) or fetal calf serum (FCS, 10%). Shown are mean ± S.D. of bromodeoxyuridine-positive cells determined in triplicate samples and controls (five fields/sample) from a representative experiment. These experiments were repeated three times with comparable results. *, statistical significance: A, +MT+TIMP-2 versus other samples (p < 0.05); B, MT+TIMP-2 versus other samples (p < 0.05); C, E240A+TIMP-2 versus other samples (p < 0.05); D, wt+TIMP-2 versus ΔCT ± TIMP-2 (p < 0.05); wt+TIMP-2 versus wt-TIMP-2 (p < 0.05); wt-TIMP-2 versus ΔCT ± TIMP-2 (p < 0.05). E, sample versus respective control (p < 0.05).

degradation product. Thus, the xenografts that grew to sizable tumors expressed the expected MT1-MMP forms produced by the transfected cells.

To investigate potential differences in the mechanism(s) that determine the different size of the wt MT1-MMP and E240A MT1-MMP tumors, tumor sections were characterized for cell proliferation, apoptosis, and angiogenesis. Immunohistochemical analysis showed no significant differences between the number of cells positive for Ki-67, a marker of cell proliferation (Fig. 13C, top panel) or cleaved caspase-3, an apoptosis marker (bottom panel). Similar results were obtained by staining the sections with antibody to mouse CD31, an endothelial cell marker (data not shown). Thus, tumors generated by cells expressing proteolytically active or inactive MT1-MMP have comparable rates of cell proliferation and apoptosis, and similar levels of vascularization. Altogether, these results showed that MT1-MMP cross-linking experiments that indicated the existence on different cell types of one high affinity (kD = 0.77–2.54 nM) binding site identified as MT1-MMP (9, 31). Several findings show that the signaling mechanism activated by TIMP-2 binding to MT1-MMP is independent of the proteolytic activity of MT1-MMP: 1) ERK1/2 activation is induced both by catalytically inactive MT1-MMP and by TIMP-2 devoid of MMP inhibitory activity; 2) the soluble MT1-MMP catalytic domain inhibits ERK1/2 activation as it competes with cell-associated MT1-MMP for TIMP-2 binding; and 3) TIMP-2 concentrations that inhibit MT1-MMP activation of MMP-2 induce ERK1/2 activation; in contrast, inhibition of MT1-MMP activity by Ilomastat does not have this effect. Thus, the signaling effect of TIMP-2 is not mediated by its capacity to activate MMP-2 in concert with MT1-MMP (9) or by inhibition of MMPs other than MT1-MMP.

Our finding that Ala+TIMP-2 activates ERK1/2 upon addition to MT1-MMP-expressing cells although it does not bind to

DISSCUSSION

A variety of physiological and pathological processes are controlled by MT1-MMP and TIMP-2 through direct or indirect mechanisms mediated by proteolytic interactions with extracellular, intracellular, or transmembrane proteins. Here we provide evidence that MT1-MMP binding of TIMP-2 activates intracellular signaling by a non-proteolytic mechanism that controls cell proliferation and migration. These conclusions are based on the following observations.

MT1-MMP expression and addition of TIMP-2 to the culture medium up-regulate ERK1/2 activation and the level of cell-associated TIMP-2 with a rapid, dose-dependent effect. Conversely, addition of TIMP-2 to cells devoid of MT1-MMP or that express mutant MT1-MMP devoid of TIMP-2-binding capacity does not have these effects. Cell-associated TIMP-2 co-immunoprecipitates with MT1-MMP, and MT1-MMP levels directly correlate with the amount of cell-associated TIMP-2, showing that MT1-MMP is the only, or at least the major, TIMP-2 binding site in the cells we used. This observation is consistent with 125I-TIMP-2 binding studies, Scatchard analysis, and...
ERK1/2 Activation by TIMP-2 Binding to MT1-MMP

FIGURE 13. MT1-MMP promotes tumor growth in vivo with a proteolysis-independent mechanism mediated by the cytoplasmic tail. A, growth of tumors derived from MCF-7 cells stably transfected with wt MT1-MMP or the indicated mutants, or with control empty vector. The cells were injected in the fat pad of nude mice, and tumor growth was determined as described under "Experimental Procedures." Mean ± S.E. values are shown for each point. MCF7-MOCK, empty vector transfectants; MCF7-MT-WT, wt MT1-MMP transfectants; MCF7-MT-ΔCT, tail-deleted MT1-MMP transfectants; MCF7-MT-E240A, proteolytically inactive MT1-MMP transfectants. Statistical significance: MCF7-MT-WT versus MCF7-MT-MOCK or MCF7-MT-ΔCT (p < 0.05); MCF7-MT-E240A versus MCF7-MT-MOCK or MCF7-MT-ΔCT (p < 0.05); MCF7-MT-WT versus MCF7-MT-E240A (p < 0.05). B, Western blotting analysis of MT1-MMP expression in tumors originated from wt MT1-MMP-expressing cells (wt) or E240A MT1-MMP-expressing cells (E240A). The blots were probed with antibody Ab815 to the hinge region, and with antibody MAb5D1 to the catalytic domain of MT1-MMP. Extracts of MCF-7 cells stably co-transfected with wt MT1-MMP and β3 integrin (wt/β3) are shown as positive control for MT1-MMP activation as discussed in the text. Forty micrograms of protein was loaded for each sample. C, immunohistochemical analysis of cell proliferation (Ki-67 expression, upper panel) and apoptosis (caspase-3 cleavage, lower panel) in sections of tumors originated from wt MT1-MMP-expressing cells (wt) or E240A MT1-MMP expressing cells (E240A). Peroxidase-positive cells were counted as described under "Experimental Procedures"; *, p: not significant.

the MMP catalytic site (23), indicated that the signaling effect of TIMP-2 involves TIMP-2 interactions with sites of MT1-MMP other than, or in addition to the active site cleft. Our binding assays with purified MT1-MMP domains showed that both wt TIMP-2 and Ala+TIMP-2 bind significantly to the hemopexin-like domain and/or the hinge region of MT1-MMP. These results contrast with a previous report that the MT1-MMP hemopexin domain does not bind TIMP-2 (38). Different experimental conditions can explain this discrepancy, including the use of different antibodies and procedures for the solid-phase binding assay. Therefore, our results indicate that the signaling effect of TIMP-2 can be mediated by its interaction with the PEX domain and/or the hinge region of MT1-MMP.

We found that ERK1/2 activation by TIMP-2 binding to MT1-MMP requires the cytoplasmic tail but not the catalytic activity of MT1-MMP. Previous reports have shown that MT1-MMP expression up-regulates activation of ERK1/2 or non-receptor Src tyrosine kinases by one or more mechanisms that require both the catalytic activity and cytoplasmic tail of MT1-MMP (30, 39). In contrast, proteolytically inactive MT1-MMP (E240A) triggers Rac-1 activation (40). Several observations can explain these discrepancies. Because autocatalysis contributes to MMP activation, cleavage of the pro-peptide is inefficient in the inactive MT1-MMP E240A mutant. The amount of pro-peptide-free, TIMP-2-binding MT1-MMP E240A in transfected cells therefore depends on the efficiency of pro-peptide cleavage by furin-like proconvertase(s) and expression level of the mutant protein. Thus, low levels of TIMP-2-binding MT1-MMP E240A may be insufficient to up-regulate ERK1/2 activation. In addition, these reports did not characterize the amount and potential effect of endogenous TIMP-2 on activation of intracellular signaling.

A recent report has shown that MT1-MMP devoid of cytoplasmic tail mediates ERK1/2 activation and vascular smooth muscle cell proliferation by a mechanism dependent on platelet-derived growth factor receptor β and blocked by MMP inhibitors, including TIMP-2 (41). The MCF-7 cells we used do not express platelet-derived growth factor receptor β (42). However, our results do not rule out the hypothesis that the effect of TIMP-2-induced activation of ERK1/2 in our cell culture model is mediated by MT1-MMP interaction with one or more growth factor receptors other than platelet-derived growth factor receptor β.

Previous studies have shown that cell migration and invasion in vitro and in vivo require the proteolytic activity but not the cytoplasmic tail of MT1-MMP and that TIMP-2 inhibits tumor cell growth and invasion in vitro and in vivo (7, 8, 41, 43, 44). These effects were obtained with TIMP-2 concentrations ranging from 2.5 to 10.0 μg/ml (100 to 400 nM) (7, 8, 43–45). These TIMP-2 concentrations are 25- to 1000-fold higher than in tissues or biological fluids (10–100 nM) (46–49) and 50- to 200-fold higher than the Kₚ of TIMP-2 for MT1-MMP (0.77–2.54 nM) (9, 31). In contrast, we found that ERK1/2 activation is elicited by TIMP-2 concentrations ranging from 50 to 100 ng/ml (2.5 to 5.0 nM). Thus, the mechanism we described is mediated by TIMP-2 concentrations similar to those present in tissues or biological fluids. Although different experimental conditions may explain these discrepancies, these reports have clearly highlighted the importance of the proteolytic activity of MT1-MMP in the extracellular matrix (ECM) degradation...
ERK1/2 Activation by TIMP-2 Binding to MT1-MMP

required for cell migration and invasion. Our data show that MT1-MMP contributes to cell migration and proliferation in vitro (in the absence of ECM) through a proteolysis-independent mechanism that requires the cytoplasmic tail of MT1-MMP. This mechanism does not rule out the requirement for proteolytic degradation of the ECM, which affords cell invasion of complex extracellular structures and promotes cell proliferation through a variety of mechanisms. On the contrary, proteolysis-independent signaling generated by MT1-MMP through its cytoplasmic tail can provide the cells with additional proliferative and migratory signals. The ECM is a biochemically complex structure whose degradation requires a wide array of proteinases (2, 50). Although MT1-MMP is required for the degradation of important ECM components, other proteinases also contribute to ECM degradation. MT1-MMP inhibition by TIMP-2 (and/or TIMP-3 and -4) can provide the cell with additional proliferative and migratory stimuli as other TIMP-2-insensitive proteinases degrade the ECM. High levels of TIMP-2, as well as MT1-MMP expression, have indeed been associated with an aggressive phenotype in a variety of tumors (51).

ERK1/2 activation upon TIMP-2 binding to MT1-MMP requires the Y573/CQR576 sequence of the cytoplasmic tail. Recent results have shown that Tyr573 is phosphorylated by a Src-dependent mechanism upon treatment of tumor or endothelial cells with sphingosine 1-phosphate, a platelet-derived chemoattractant. Tyr573 phosphorylation mediates sphingosine 1-phosphate induction of cell migration (33). We were unable to conclusively demonstrate that Tyr574 is phosphorylated in the presence or absence of TIMP-2. Therefore, we hypothesize that the effect of Ala or Asp substitution of Tyr573 on ERK1/2 activation is not caused by modification of this phospho-acceptor site but likely results from conformational changes in the cytoplasmic tail and/or altered interaction with intracellular proteins. Both alanine and aspartic acid are structurally different from tyrosine and may therefore significantly alter the conformation of the cytoplasmic tail.

A variety of tumors express high levels of MT1-MMP and TIMP-2, both of which have been associated with an aggressive phenotype (51–54). Our in vivo data show that MT1-MMP contributes to tumor cell proliferation by a cytoplasmic tail-dependent mechanism complementary to ECM degradation. Tumors expressing proteolytically inactive MT1-MMP grew to a size ~50% that of tumors expressing wt MT1-MMP. We found no significant differences between the proliferation and apoptotic rates, and the vascularity of the two tumor types. Based on previous reports (7, 8, 41, 43, 44), we speculate that the smaller volume of tumors expressing proteolytically inactive MT1-MMP reflects the lack of proteolytic activity and ECM degradation. E240A MT1-MMP possesses only marginal proteolytic activity against collagen but does not degrade fibronectin (32). Thus, it is considered a virtually inactive proteinase. Our in vivo data provide no direct evidence for a role of TIMP-2 in the growth of MT1-MMP-expressing cells. TIMP-2 is present in most tissues. However, cells expressing no MT1-MMP or MT1-MMP devoid of cytoplasmic tail originated virtually no sizable tumors, indicating that host-derived TIMP-2 had no effect on tumor growth in the absence of MT1-MMP. Further studies are required to characterize the role of TIMP-2-MT1-MMP interaction in tumor growth in vivo. This analysis is complicated by multiple interactions of MT1-MMP with a variety of extracellular proteins, including TIMP-3 and TIMP-4, which may also contribute to up-regulating intracellular signaling through MT1-MMP. Nevertheless, our data provide the first demonstration that MT1-MMP contributes to tumor growth in vivo with a proteolysis-independent mechanism mediated by its cytoplasmic tail.

The non-proteolytic effect of TIMP-2-MT1-MMP interaction on intracellular signaling can also have multiple roles in vivo in several physiological and pathological settings. The TIMP-2 concentrations that activate ERK1/2 are in the range of the K_d for TIMP-2 binding to MT1-MMP (0.77–2.5 nM) (9, 31). The genetic deficiency of MT1-MMP causes marked deceleration of postnatal growth, with severe defects in skeletal and alveolar development, and death by 3–5 weeks of age (3–5, 55). It has been proposed that this phenotype results from the lack of MT1-MMP proteolytic activity. Based on our finding of the non-proteolytic role of MT1-MMP in ERK1/2 activation, it is reasonable to hypothesize that the phenotype of MT1-MMP-deficient mice, particularly dwarfism and osteopenia, results in part from defective intracellular signaling. This hypothesis is supported by the finding that in the mouse embryo MT1-MMP is temporally and spatially coexpressed with TIMP-2, notably at the perichondral waist, the site of bone growth (56). TIMP-2−/−(null) mice do not display the severe phenotype of MT1-MMP null mice (57). However, TIMP-3 and TIMP-4, which also bind to MT1-MMP with high affinity (58), may activate intracellular signaling in TIMP-2 null mice.

In conclusion, the data reported here show a novel, non-proteolytic mechanism through which MT1-MMP and TIMP-2 activate intracellular signaling. A detailed understanding of this signaling mechanism can have important implications for our comprehension of physiological and pathological processes, and for the development of pharmacological treatments aimed to control them.

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