

Differential Regulation of Membrane Type 1-Matrix Metalloproteinase Activity by ERK 1/2- and p38 MAPK-modulated Tissue Inhibitor of Metalloproteinases 2 Expression Controls Transforming Growth Factor- β 1-induced Pericellular Collagenolysis*

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Acquisition of matrix metalloproteinase-2 (MMP-2) activity is temporally associated with increased migration and invasiveness of cancer cells. ProMMP-2 activation requires multimolecular complex assembly involving proMMP-2, membrane type 1-MMP (MT1-MMP, MMP-14), and tissue inhibitor of metalloproteinases-2 (TIMP-2). Because transforming growth factor- β 1 (TGF- β 1) promotes tumor invasion in advanced squamous cell carcinomas, the role of TGF- β 1 in the regulation of MMP activity in a cellular model of invasive oral squamous cell carcinoma was examined. Treatment of oral squamous cell carcinoma cells with TGF- β 1 promoted MMP-dependent cell scattering and collagen invasion, increased expression of MMP-2 and MT1-MMP, and enhanced MMP-2 activation. TGF- β 1 induced concomitant activation of ERK1/2 and p38 MAPK, and kinase inhibition studies revealed a negative regulatory role for ERK1/2 in modulating acquisition of MMP-2 activity. Thus, a reciprocal effect on proMMP-2 activation was observed whereupon blocking ERK1/2 phosphorylation promoted proMMP-2 activation and MT1-MMP activity, whereas inhibiting p38 MAPK activity decreased proteolytic potential. The cellular mechanism for the control of MT1-MMP catalytic activity involved concurrent reciprocal modulation of TIMP-2 expression by ERK1/2 and p38 MAPKs, such that inhibition of ERK1/2 phosphorylation decreased TIMP-2 production, and down-regulation of p38 MAPK activity enhanced TIMP-2 syn-

thesis. Further, p38 MAPK inhibition promoted ERK1/2 phosphorylation, providing additional evidence for cross-talk between MAPK pathways. These observations demonstrate the complex reciprocal effects of ERK1/2 and p38 MAPK in the regulation of MMP activity, which could complicate the use of MAPK-specific inhibitors as therapeutic agents to down-regulate the biologic effects of TGF- β 1 on pericellular collagen degradation and tumor invasion.

Members of the matrix metalloproteinase (MMP)¹ family have been implicated in basement membrane proteolysis, activation of growth factors, and cleavage of cell adhesion molecules (1–3). Consequently, MMPs contribute to a wide array of biological activities including wound healing, migration, differentiation, apoptosis, and angiogenesis (1–3). Moreover, MMPs are involved in tissue invasion by a number of different cancers, including oral squamous cell carcinoma (OSCC) (4–6). Immunohistochemical studies have demonstrated increased expression and activation of MMP-2 (gelatinase A, 72-kDa type IV collagenase) in invasive and metastatic cases of OSCC (4–6). Furthermore, increased expression of MMP-2 is associated with decreased staining of peritumoral extracellular matrix, suggesting that MMP-2 promotes matrix breakdown. MMP-2 is secreted from cells as a zymogen (proMMP-2) and is activated post-translationally by a trans-membrane MMP designated membrane type 1-MMP (MT1-MMP, MMP-14), the expression of which is also up-regulated in advanced, metastatic cases of OSCC (4–6). The activation of proMMP-2 is regulated by a complex mechanism involving formation of a trimolecular complex with MT1-MMP and tissue inhibitor of metalloproteinase-2 (TIMP-2) (7–9). TIMP-2 plays a dual role in the regulation of MMP-2 activation, functioning both to promote and to inhibit the activation process in a concentration-dependent manner (7–9). TIMP-2 bridges the interaction between the MMP-2 zymogen and MT1-MMP such that at low TIMP-2

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¹ The abbreviations used are: MMP, matrix metalloproteinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MAPKAPK-2, p38 MAPK-activated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; Me₂SO, dimethyl sulfoxide; MT1-MMP, membrane type 1-MMP; oral squamous cell carcinoma; TGF- β 1, transforming growth factor- β 1; TIMP, tissue inhibitor of metalloproteinases; MKK3, mitogen-activated protein kinase kinase.

concentration, an adjacent TIMP-2-free MT1-MMP can effectively activate proMMP-2. However, at high TIMP-2 concentration, all of the cell surface MT1-MMP undergoes complex formation with TIMP-2, thereby inhibiting proMMP-2 activation.

Transforming growth factor β 1 (TGF- β 1) regulates a wide array of cellular processes including cell proliferation, differentiation, and apoptosis (10–12). It also contributes to cell migration by promoting changes in cell morphology via reorganization of the cytoskeleton and alteration of cell-cell junctions (10–12). In addition, TGF- β 1 has been shown to promote tumorigenesis and increase metastasis in a number of different cancers including squamous cell cancer (13–15). Overexpression of TGF- β 1 in keratinocytes promotes conversion to a more malignant phenotype with increased incidence of spindle tumors and increases metastasis of skin papillomas (13–15). Consistent with the proinvasive role in cancer progression, TGF- β 1 has been shown to affect expression of MMPs. TGF- β 1 promoted expression of MMP-2 and MMP-9 in human mucosal keratinocytes and of MMP-2 and MMP-13 in human gingival fibroblasts (16–20). In addition to affecting MMP expression, TGF- β 1 can also regulate MMP activity by regulating the synthesis of TIMPs (17, 21).

TGF- β 1 signals through cell surface serine-threonine kinases to activate cellular responses (22–25). Binding of TGF- β 1 to its type II receptor (T β RII) promotes T β RII association with and phosphorylation of type I receptor (T β RI), which then phosphorylates receptor-associated Smads (R-Smads), Smad2 and Smad3. The R-Smads then bind to Smad4 and translocate to the nucleus, wherein the complex can associate with transcription factors and activate target genes. In addition to the Smad-mediated TGF- β 1 signaling pathway, recent data demonstrate that TGF- β 1 can signal through distinct pathways involving mitogen-activated protein kinases (MAPKs) (26–30). TGF- β 1 can activate extracellular signal-regulated kinases 1/2 (ERK1/2) and p38 MAPKs with varying kinetics and magnitude in a number of different cells (26–30). Recently, p38 MAPK was shown to regulate TGF- β 1-induced epithelial to mesenchymal transdifferentiation and cell migration in NMuMG mouse mammary epithelial cells (29) as well as TGF- β 1-induced MMP-13 expression both in transformed keratinocytes and in human gingival fibroblasts (18, 19).

In this report, we have examined the role of TGF- β 1 in the regulation of MMP activity in a cellular model of invasive OSCC and have evaluated the contribution of MAPK signaling to TGF- β 1-induced MMP activity. Treatment of OSCC cells with TGF- β 1 promoted MMP-dependent cell scattering and collagen invasion, increased expression of MMP-2 and MT1-MMP, and enhanced MMP-2 activation. Mechanistic analyses of individual components of the MT1-MMP-TIMP-2-proMMP-2 trimeric activation complex demonstrated that these effects were mediated by cellular modulation of TIMP-2 levels. TGF- β 1 also activated MAPK signaling, resulting in increased activation of both ERK1/2 and p38 MAPKs. Kinase inhibition studies revealed reciprocal regulatory pathways because inhibition of ERK1/2 phosphorylation promoted TGF- β 1-induced pericellular proteolytic potential, whereas inhibition p38 MAPK activity led to down-regulation of proteolysis. Further, p38 MAPK inhibition promoted ERK1/2 activation, providing evidence for cross-talk between TGF- β 1-activated MAPK pathways. These observations highlight the complex reciprocal effects of ERK1/2 and p38 MAPK in the regulation of MT1-MMP activity, which could complicate the use of MAPK-specific inhibitors as therapeutic approaches to down-regulate the biologic effects of TGF- β 1 on pericellular collagen degradation and tumor invasion in OSCC.

EXPERIMENTAL PROCEDURES

Materials—Type I collagen, cell culture reagents, TGF- β 1, peroxidase-conjugated secondary antibodies, and the MT1-MMP antibody directed against the hinge region were purchased from Sigma. The kinase inhibitors U0126, PD98059, SB202190, and SB203580 were obtained from Calbiochem. U0126 and PD98059 inhibit MEK, the upstream kinase that catalyzes phosphorylation of ERK1/2, whereas SB202190 and SB203580 are direct inhibitors of p38 MAPK activity. The vacuolar ATPase inhibitor bafilomycin A1 was also obtained from Calbiochem. Dulbecco's modified Eagle medium, Ham's F-12 and keratinocyte-SFM were purchased from Invitrogen. Rabbit polyclonal TIMP-2 antibody and the broad spectrum MMP inhibitor GM6001 were purchased from Chemicon (Temecula, CA). Anti-phosphorylated p42/p44 (MAPK/ERK), anti-phosphorylated p38 MAPK, and anti-p38 MAPK were obtained from Cell Signaling Technology (Beverly, MA). Anti-ERK1/2 (anti-p42/p44) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 594 phalloidin was from Molecular Probes (Portland, OR). Paraformaldehyde was purchased from Electron Microscopy Sciences (Fort Washington, PA). SuperSignal enhanced chemiluminescence (ECL) reagent, EZ-Link Sulfo-NHS-LC-Biotin, and UltraLink immobilized streptavidin gel were obtained from Pierce. Microcon 10 microconcentrators and polyvinylidene difluoride membranes were purchased from Millipore. TransIT keratinocyte transfection reagent was purchased from Mirus (Madison, WI). A nucleofactor electroporation kit specifically designed for keratinocytes was obtained from Amaxa (Gaithersburg, MD). A luciferase reporter assay system was from Promega (Madison, WI). Protease inhibitor mixture was purchased from Roche Applied Science.

Cell Cultures—SCC9, SCC25, and SCC68 cells are derived from squamous cell carcinoma of the oral cavity. SCC25 and SCC9 cells were obtained from American Type Culture Collection (ATCC). Tert-immortalized normal oral keratinocytes (OKF6 cells) and SCC68 cells were kindly provided by Dr. J. Rheinwald (Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston) (31, 32). SCC25 and SCC9 cells were routinely maintained in Dulbecco's modified Eagle medium and Ham's F-12 medium (1:1) containing 10% fetal calf serum and supplemented with 100 units/ml penicillin and 100 μ g/ml streptomycin. OKF6 and SCC68 cells were maintained in keratinocyte-SFM supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, 25 μ g/ml bovine pituitary extract (supplied with the medium), 0.2 ng/ml epidermal growth factor, and 0.31 mM CaCl₂. The cells were plated in serum-containing medium and serum starved overnight prior to treatment with TGF- β 1. In additional experiments, inhibitors or other chemical reagents were added 30 min prior to TGF- β 1 treatment.

Analysis of Cell Scattering and Collagen Invasion—SCC25 cells were plated overnight on glass coverslips coated with type I collagen and treated with TGF- β 1 in the presence or absence of the MMP inhibitor GM6001 for an additional 24 h. The cells were then washed with phosphate-buffered saline, fixed with 4% paraformaldehyde solution for 10 min, blocked with 1% bovine serum albumin for 20 min, permeabilized with 0.1% Triton X-100 for 3 min, and stained with Alexa Fluor 594 phalloidin, after which the cells were washed, mounted, and observed using a Zeiss LSM 510 confocal laser microscope.

Invasive activity was quantified using a Boyden chamber (8- μ m pore size) coated with 10 μ g of type I collagen (33). 2×10^5 SCC25 cells were added to the upper chamber in 500 μ l of serum-free medium in the presence or absence of TGF- β 1. In addition, 500 μ l of serum-containing medium was added to the lower well to promote invasion. In selected experiments, the proteinase dependence of invasion was determined by quantifying invasion in the presence of the MMP inhibitor GM6001. Nonmigrating cells were removed from the upper chamber with a cotton swab, filters were fixed and stained with Diff-Quik Stain, and migrating cells adherent to the underside of the filter were enumerated using an ocular micrometer and counting a minimum of five high-powered fields. Data are expressed as relative migration (number of cells/field).

Analysis of MMP-2 and TIMP-2 Expression—Gelatinase activities in 24-h serum-free conditioned medium were determined using SDS-PAGE gelatin zymography as described previously (33). Briefly, SDS-polyacrylamide gels (9% acrylamide) were copolymerized with 0.1% gelatin, and samples were electrophoresed without reduction or boiling using 5 \times Laemmli sample buffer (34). SDS was removed through a 30-min incubation in 2.5% Triton X-100, and gels were incubated in 20 mM glycine, pH 8.3, 10 mM CaCl₂, 1 μ M ZnCl₂ at 37 $^{\circ}$ C for 24–36 h. The gels were stained with Coomassie Blue to visualize zones of gelatinolytic activity. The conditioned media were concentrated 15–20-fold using Microcon 10 microconcentrators, boiled in Laemmli sample dilution

buffer, analyzed for TIMP-2 by SDS-PAGE (15% gels), and immunoblotted with rabbit polyclonal antibody (Chemicon).

MT1-MMP, MMP-2, and TIMP-2 Promoter Activities—pGL luciferase reporter vector (Promega, Madison, WI) containing the MMP-2 promoter (−1659 to +57 from the start site) was obtained from Dr. Y. Sun (Parke-Davis Pharmaceutical Research, Ann Arbor, MI) (35). pGL3 luciferase reporter vector (Promega) containing 3.3 kb of the MT1-MMP promoter was provided by Dr. J. A. Madri (Yale University) (36). The full-length 2243-bp TIMP-2 promoter expressed in pGL2 Luciferase Reporter vector (Promega) was obtained from Dr. Y. A. DeClerck (University of Southern California) (37). The dominant negative mutant of MKK3 was obtained from Dr. R. J. Davis (University of Massachusetts Medical School, Worcester) (38). The constitutively active and dominant negative mutants of MEK1 were kindly provided by Dr. D. Boyd (M. D. Anderson Cancer Center, Houston, TX) (39, 40). SCC25 cells were transiently transfected with the various promoter luciferase reporter constructs in serum-free condition using TransIT keratinocyte transfection reagent or the Nucleofactor Amaxa kit specifically designed for keratinocytes using program T18. In additional experiments, 0.5 μ g of the dominant negative mutant form of MEK1 or MKK3, or the constitutively active mutant form of MEK1 was cotransfected with the MMP-luciferase constructs. 6 h later, the cells were washed with serum-free medium to remove the unbound DNA-lipid complexes and treated with TGF- β 1 for 24 h. The cells were then lysed and luciferase activities determined by luminometry. The results were normalized to protein concentration and expressed relative to control untreated cells.

p38 MAPK Activity Assay—SCC25 cells were serum starved overnight and treated with 5 μ M SB202190 for 30 min prior to treatment with TGF- β 1. The cells were lysed in phosphorylation lysis buffer (0.5% Triton X-100, 150 mM NaCl, 200 μ M sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM EDTA, 50 mM Hepes, 1.5 mM magnesium chloride, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml aprotinin) for 60 min at 4 $^{\circ}$ C, and lysates were immunoprecipitated with antibodies to MapKap kinase-2 as detailed previously (41, 42). The immunoprecipitated proteins were washed three times in phosphorylation lysis buffer and twice in kinase buffer (25 mM Hepes, pH 7.4, 25 mM MgCl₂, 25 mM β -glycerophosphate, 100 μ M sodium orthovanadate, 2 mM dithiothreitol, 20 μ M ATP), and the immunocomplex kinase assays were initiated by the addition of 30 μ l of kinase buffer containing 5 μ g of Hsp25 protein (Stress Gen Laboratories) as a substrate and 25 μ Ci of [γ -³²P]ATP. The reaction was incubated for 30 min at room temperature and was terminated by the addition of SDS sample buffer. Proteins were subsequently analyzed by 10% SDS-PAGE, and the phosphorylated form of Hsp25 was detected by autoradiography.

Cell Surface Biotinylation—To label cell surface proteins, SCC25 cells were grown to confluence in a 6-well plate, washed with ice-cold phosphate-buffered saline, and incubated at 4 $^{\circ}$ C with gentle shaking for 30 min with 0.5 mg/ml cell-impermeable Sulfo-NHS-LC-Biotin in ice-cold phosphate-buffered saline, followed by washing with 100 mM glycine to quench free biotin (43). Cells were detached by scraping, lysed in modified radioimmune precipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.1% SDS) with protease inhibitor mixture and clarified by centrifugation. To isolate biotinylated cell surface proteins, equal amounts of protein from each sample were incubated with streptavidin beads at 4 $^{\circ}$ C for 14 h followed by centrifugation. After boiling in Laemmli sample dilution buffer to dissociate streptavidin bead-biotin complexes, the biotin-labeled samples were analyzed by SDS-PAGE (9% gels) and immunoblotted for MT1-MMP.

RESULTS

TGF- β 1 Promotes MMP-mediated Cell Scattering and Type I Collagen Invasion—Because TGF- β 1 expression is correlated with invasive behavior in advanced squamous cell cancers (13–15), the effects of TGF- β 1 on cell scattering and collagen invasion were examined in a cellular model of invasive OSCC. SCC25 cells were plated on type I collagen and treated with TGF- β 1 in the presence or absence of a broad spectrum MMP inhibitor (GM6001). Treatment of SCC25 cells with TGF- β 1 promoted MMP-mediated cell scattering (Fig. 1A), as evidenced by inhibition of colony dispersion in the presence of GM6001. To examine the effect of TGF- β 1 on invasion, SCC25 cells were plated in Boyden chambers overlaid with type I collagen to provide a three-dimensional barrier to invasion. Control exper-

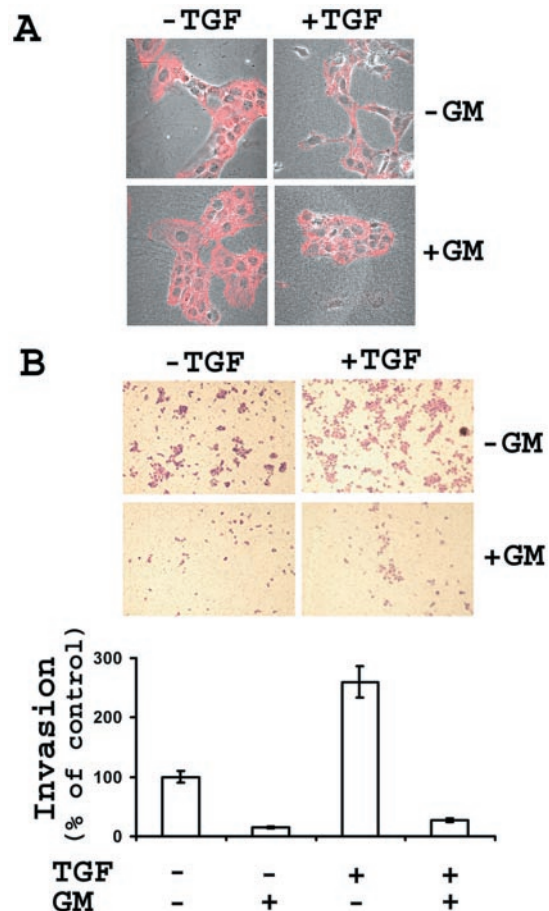


FIG. 1. TGF- β 1 promotes MMP-mediated cell scattering and type I collagen invasion. A, SCC25 cells were plated overnight on glass coverslips coated with type I collagen and treated with TGF- β 1 in the presence or absence of the MMP inhibitor GM6001 (GM) (5 μ M) for an additional 24 h. The cells were then fixed, permeabilized, and stained with Alexa Fluor 594 phalloidin, after which the cells were observed using confocal laser microscopy. B, SCC25 cells were added to porous polycarbonate filters (8- μ m pore) coated with 10 μ g of type I collagen and treated with 10 ng/ml TGF- β 1 in the presence or absence of 5 μ M GM6001. Nonmigrating cells were removed from the upper chamber, filters were fixed and stained, and invading cells were enumerated using an ocular micrometer. The results represent the mean \pm S.E. of three different experiments.

iments included the inhibitor GM6001 to evaluate the contribution of MMPs to collagen invasion. As shown in Fig. 1B, GM6001 blocked collagen invasion in untreated cells, indicating that collagen invasion by SCC25 cells requires MMP activity. TGF- β 1 promoted collagen invasion by 2.5-fold, and the addition of GM6001 abrogated this effect, indicating that the TGF- β 1-enhanced invasion was mediated by increased pericellular MMP activity.

TGF- β 1 Induces ProMMP-2 and MT1-MMP Expression, Enhances MT1-MMP Activity, and Promotes MMP-2 Activation—Because TGF- β 1 promoted MMP-dependent cell scattering and collagen invasion, the effect of TGF- β 1 on MMP expression and activation was examined in four oral keratinocyte cell lines. In three of four cell lines (SCC25, OKF6, SCC68; Fig. 2A), a dose-dependent increase in activation of proMMP-2 was observed after TGF- β 1 treatment, but with no effect on SCC9. As we have characterized the MT1-MMP-dependent activation of proMMP-2 in SCC25 cells previously (43), the effect of TGF- β 1 on MT1-MMP expression was also examined by Western blotting. To prevent MT1-MMP autolytic degradation, GM6001 was added in the indicated samples at the time of TGF- β 1 treatment. Moreover, to capture the 43-kDa MT1-MMP prod-

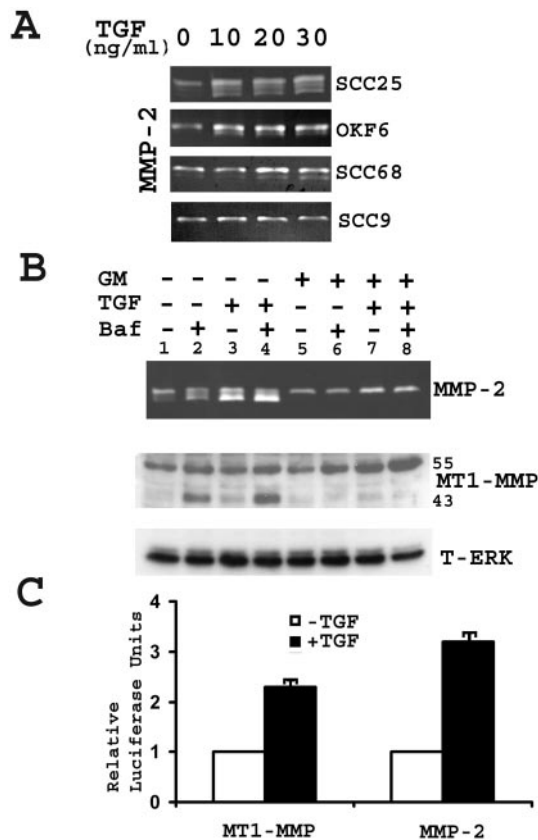


FIG. 2. TGF- β 1 induces proMMP-2 and MT1-MMP expression and promotes MMP-2 activation. *A*, SCC25, OKF6, SCC68, and SCC9 cells were treated with increasing concentrations of TGF- β 1 for 24 h and the conditioned media analyzed for MMP activity by gelatin zymography. *B*, SCC25 cells were treated with 10 ng/ml TGF- β 1 in the presence of Me₂SO (vehicle control), 5 μ M MMP inhibitor GM6001 (GM), or 50 nM bafilomycin A1 (Baf). At 24 h the conditioned media were analyzed for MMP activity by gelatin zymography and the cell lysates for MT1-MMP and for total ERK1/2 (as loading control) by Western blotting. *C*, SCC25 cells were transiently transfected with either the MT1-MMP or the MMP-2 promoter/reporter constructs as detailed under "Experimental Procedures." 6 h later, the cells were treated with TGF- β 1 for an additional 24 h. The cells were then lysed, and luciferase activities were determined by luminometry. Results are normalized for transfection efficiency and expressed relative to control cells, which was set as 1.0. The results represent the mean \pm S.E. of four different experiments.

uct that is endocytosed rapidly from the cell surface and degraded (43, 44), bafilomycin A1 was also added in the indicated samples at the time of TGF- β 1 treatment. TGF- β 1 treatment of SCC25 cells promoted MMP-2 expression and activation (Fig. 2*B*, top panel, compare lanes 1 and 3). Consistent with the recently published report (44), bafilomycin A1 enhanced MMP-2 activation (Fig. 2*B*, top panel, compare lanes 1 and 2) without affecting MMP-2 expression (Fig. 2*B*, top panel, compare lanes 5 and 6). In the presence of bafilomycin A1, TGF- β 1 enhanced MMP-2 activation further (compare lanes 2 and 4). The TGF- β 1-mediated increase in MMP-2 activation was blocked by GM6001 (Fig. 2*B*, top panel, compare lanes 3 and 4 with lanes 7 and 8), indicating that proMMP-2 activation requires MMP catalytic activity. TGF- β 1 also enhanced MT1-MMP expression in SCC25 cells (Fig. 2*B*, middle panel, compare lanes 2 and 4), with the effect demonstrated more clearly when MT1-MMP autolytic processing was blocked using GM6001 (Fig. 2*B*, middle panel, compare lanes 3 and 4, and compare lanes 6 and 8), resulting in accumulation of the 55-kDa MT1-MMP protein. TGF- β 1 also enhanced generation of 43-kDa MT1-MMP protein (Fig. 2*B*, middle panel, compare

lanes 2 and 4), an effect that was only apparent in the presence of bafilomycin. Thus, the enhanced preservation of the 43-kDa MT1-MMP protein, captured with bafilomycin A1, may be interpreted as evidence of increased MT1-MMP activity. Reprobing the blots for ERK1/2 demonstrated that equal amounts of protein were loaded (Fig. 2*B*, bottom panel). To determine whether the increased MMP-2 activation was the result of changes in proteinase gene expression, the effect of TGF- β 1 on MT1-MMP and MMP-2 promoter activities was examined using promoter/luciferase reporter constructs. Treatment of SCC25 cells with TGF- β 1 increased the promoter activity of MT1-MMP and MMP-2 by \sim 2- and 3-fold, respectively (Fig. 2*C*), demonstrating transcriptional activation by TGF- β 1.

TGF- β 1 Promotes ERK1/2 and p38 MAPK Phosphorylation—Because recent reports indicate that TGF- β 1 signals through MAPK pathways to regulate MMP-1 and MMP-13 expression (18, 19, 45), we first examined whether TGF- β 1 influenced the ERK1/2 and p38 MAPK signaling pathways in SCC25 cells. Cells were treated with TGF- β 1 in the presence or absence of highly specific inhibitors of MEK1/2 and p38 MAPK (U0126 and SB202190, respectively) or Me₂SO (control), and lysates were analyzed for phosphorylated (active) forms of ERK1/2 and p38 MAPK by Western blotting with phospho-specific antibodies. The membranes were then reprobed to evaluate either total ERK1/2 or total p38 MAPK expression. As shown in Fig. 3*A* (upper panel), TGF- β 1 transiently increased ERK1/2 phosphorylation with maximal activity at 1 h. The addition of the MEK1/2 inhibitor U0126 completely blocked ERK1/2 phosphorylation (Fig. 3*A*, right panel). Similarly, TGF- β 1 increased p38 MAPK phosphorylation with maximal activity at 2 h (Fig. 3*B*, upper panel). In contrast to the effect of MEK1/2 inhibitor on ERK1/2 phosphorylation, blocking p38 MAPK activity with SB202190 enhanced p38 MAPK phosphorylation, suggesting a negative feedback regulation of p38 MAPK phosphorylation (Fig. 3*B*, right panel). To demonstrate that TGF- β 1-enhanced p38 MAPK activity was blocked after treatment with SB202190, we examined the ability of SB202190 to block phosphorylation of a downstream target substrate (p38 MAPK-activated kinase or MAPKAPK-2) using an immunocomplex kinase assay (Fig. 3*C*). SCC25 cells were treated with TGF- β 1 in the presence or absence of SB202190, and the cell lysates were subjected to immunoprecipitation with anti-MAPKAPK-2 or control IgG as detailed under "Experimental Procedures." The immunoprecipitates were utilized to catalyze *in vitro* phosphorylation of the MAPKAPK-2 substrate Hsp25 using radiolabeled [γ -³²P]ATP. A time-dependent increase in MAPKAPK-2 activity was observed with TGF- β 1 treatment as evidenced by increased phosphorylation of Hsp25 (Fig. 3*C*). Treatment with the p38 MAPK inhibitor SB202190 blocked TGF- β 1-induced Hsp25 phosphorylation (Fig. 3*C*), demonstrating the efficacy of this compound as an inhibitor of p38 MAPK activity. No Hsp25 phosphorylation was observed in samples precipitated with control IgG (Fig. 3*C*). To examine further the effect of TGF- β 1 on the p38 MAPK signaling pathway, we examined the effect of the dominant negative (AlaMKK3) mutant of the immediate upstream p38 MAPK activator MKK3 (Fig. 3*D*). Transfection of dominant negative MKK3 mutant (AlaMKK3) in SCC25 cells decreased basal p38 MAPK phosphorylation and blocked TGF- β 1-induced p38 MAPK phosphorylation (Fig. 3*D*).

TGF- β 1 Induction of MT1-MMP and MMP-2 Promoters Does Not Involve ERK1/2 or p38 MAPK—To examine whether the TGF- β 1-mediated increase in MMP-2 and MT1-MMP expression was regulated by ERK1/2 or p38 MAPK, the effect of specific kinase inhibitors on MMP promoter activity was examined. As shown previously in Fig. 1, treatment of SCC25 cells with TGF- β 1 increased MT1-MMP and MMP-2 promoter activ-

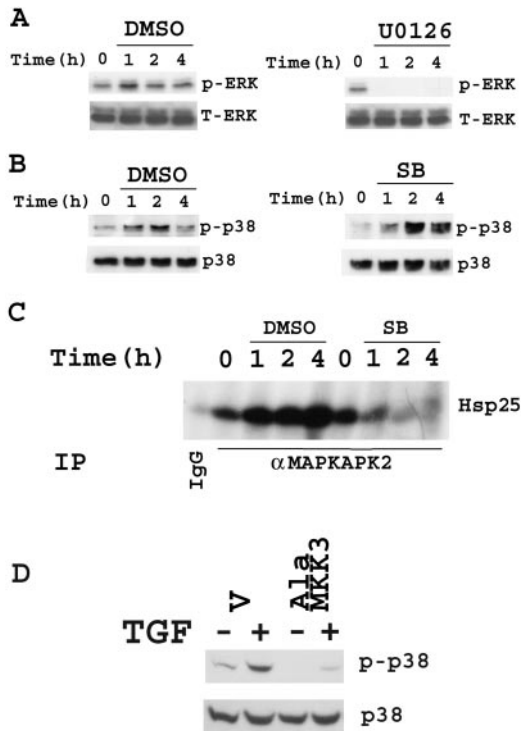


FIG. 3. TGF- β 1 promotes ERK1/2 and p38 MAPK phosphorylation. SCC25 cells were pretreated with Me₂SO (DMSO; vehicle control), 5 μ M MEK1/2 inhibitor U0126, or 5 μ M p38 MAPK inhibitor SB202190 (SB) for 30 min, treated with 10 ng/ml TGF- β 1, and lysed at the indicated times. *A*, the lysates were probed with anti-phospho-ERK1/2 antibody to detect the phosphorylated, active form of ERK1/2 (*p*-ERK) or with anti-ERK1/2 antibody to detect total ERK1/2 (*T*-ERK) expression. *B*, the lysates were also probed with anti-phospho-p38 antibody to detect the phosphorylated, active form of p38 MAPK or with anti-p38 MAPK antibody to detect total p38 MAPK expression. *C*, SCC25 cells were serum starved for 24 h and then treated with TGF- β 1 in the presence or absence of 5 μ M SB202190 as indicated. Cells were lysed, immunoprecipitated (IP) with an antibody against MAPKAPK-2, and *in vitro* kinase assays were carried out using Hsp25 as an exogenous substrate as detailed under "Experimental Procedures." Phosphorylated Hsp25 was detected by autoradiography. *D*, SCC25 cells were transfected with vector (V) or dominant negative (AlaMKK3) mutant using a Nucleofactor Amaxa kit. After serum starvation for 24 h, the cells were treated with TGF- β 1 for 1 h, and the lysates were probed with anti-phospho-p38 antibody to detect the phosphorylated, active form of p38 MAPK or with anti-p38 MAPK antibody to detect total p38 MAPK expression.

ities (Fig. 4). Treatment with U0126 or SB202190 did not significantly affect the basal promoter activity nor block the TGF- β 1-enhanced activation of either the MT1-MMP (Fig. 4A) or the MMP-2 promoter (Fig. 4C). Similar results were obtained with a different MEK inhibitor PD98059 and with a different p38 MAPK inhibitor SB203580 (data not shown). To confirm our findings with the pharmacologic inhibitors, the dominant negative mutants of the immediate upstream ERK1/2 (DNMEK1) and p38 MAPK (AlaMKK3) activators were utilized. SCC25 cells were transfected with either the MT1-MMP or the MMP-2 promoter and cotransfected with DNMEK1 or AlaMKK3 (Fig. 4, B and D). Consistent with the results obtained with the pharmacologic inhibitors, the dominant negative catalytically inactive MEK1 and MKK3 failed to abrogate TGF- β 1 enhanced activation of either the MT1-MMP (Fig. 4B) or the MMP-2 promoter (Fig. 4D). These data indicate that ERK1/2 and p38 MAPK are not involved in the regulation of TGF- β 1-induced MT1-MMP and MMP-2 transcription.

MMP-2 Activation and MT1-MMP Autolytic Processing Are Modulated Differentially by ERK1/2 and p38 MAPK—Because MMPs are subjected to extensive post-transcriptional control

(2), the effects of MAPK inhibitors on MMP expression and activity were examined. As shown previously in Fig. 1, treatment with TGF- β 1 increased MMP-2 activation in SCC25 and OKF6 cells (Fig. 5A, lane 4). Interestingly, inhibition of MEK1/2 activity with U0126 increased TGF- β 1-mediated MMP-2 activation (Fig. 5A, upper panel, compare lanes 4 and 5), whereas inhibiting p38 MAPK activity with SB202190 blocked the TGF- β 1-mediated increase in MMP-2 activation (Fig. 5A, upper panel, compare lanes 4 and 6). Similar results were also obtained with a different MEK inhibitor PD98059 and with a different p38 MAPK inhibitor SB203580 (data not shown). Moreover, treatment with either inhibitor alone did not affect total proMMP-2 protein levels (data not shown). These data support results obtained in the promoter/reporter assays that these MAPKs do not regulate the expression of proMMP-2 (Fig. 4), and suggest a post-transcriptional mechanism for TGF- β 1-induced proMMP-2 activation.

Because the data indicate regulation by TGF- β 1 signaling at the level of MT1-MMP activity, the relative conversion of the 55-kDa MT1-MMP active protein to the 43-kDa autolysis product was assessed to provide a qualitative indicator of MT1-MMP activity. Because bafilomycin A1 facilitates detection of MT1-MMP activity by preserving the 43-kDa autolysis product, this reagent was used as a tool to examine further the effect of MAPK inhibitors on MT1-MMP activity. As shown previously in Fig. 2B, treatment with TGF- β 1 promoted MMP-2 activation and enhanced generation of 43-kDa MT1-MMP protein (Fig. 5B, compare lanes 1 and 4). Inhibition of ERK1/2 activation with U0126 enhanced MMP-2 activation and MT1-MMP activity, as evidenced by enhanced conversion of the 55-kDa active MT1-MMP protein to the 43-kDa MT1-MMP autolysis product (Fig. 5B, compare lanes 4 and 5). In contrast, inhibition of p38 MAPK blocked MT1-MMP activity (Fig. 5B, compare lanes 4 and 6). In control experiments, addition of GM6001 abrogated autolytic degradation and demonstrated that effects observed with MAPK inhibitors reflect changes in MT1-MMP activity rather than expression levels (Fig. 5C).

p38 MAPK Inhibitor Blocks TGF- β 1-mediated MMP-2 Activation by Increasing ERK1/2 Activity—Because our data suggest reciprocal regulation of TGF- β 1-induced MMP-2 activation via differential activation of the ERK1/2 and p38 MAPK pathways, the potential for signaling cross-talk between these pathways was evaluated. After serum starvation of SCC25 cells, cells were pretreated with SB202190 for 30 min and then treated with TGF- β 1 for varying times (Fig. 6A). As shown previously in Fig. 3A, treatment with TGF- β 1 transiently enhanced ERK1/2 phosphorylation (Fig. 6A, left panel). Interestingly, treatment with SB202190 dramatically enhanced TGF- β 1-induced ERK1/2 phosphorylation (Fig. 6A, middle panel). The effect of SB202190 was mediated by MEK because concomitant treatment with the MEK inhibitor U0126 abrogated the effect (Fig. 6A, right panel).

Because the data above indicate that there is cross-talk between p38 MAPK and ERK1/2 signaling, we examined further the potential for reciprocal MAPK signaling in MMP regulation by inhibiting the pathways individually or simultaneously. SCC25 cells were pretreated with the p38 MAPK inhibitor SB202190, the MEK inhibitor U0126, or concurrently with both compounds. As shown previously in Fig. 5A, inhibiting ERK1/2 activation with U0126 enhanced the effect of TGF- β 1 on MMP-2 activation (Fig. 6B, compare lanes 5 and 6, top and middle panels), whereas treatment with p38 MAPK inhibitor blocked this effect (Fig. 6B, compare lanes 5 and 7). Interestingly, the ability of SB202190 to down-regulate TGF- β 1-induced MMP activity was abolished completely by simultaneous inhibition of ERK activation (Fig. 6B, top and middle

FIG. 4. TGF- β 1 induction of MT1-MMP and MMP-2 promoters does not involve ERK1/2 or p38 MAPK. SCC25 cells were transiently transfected with either the MT1-MMP (A and B) or the MMP-2 (C and D) promoter/reporter construct as detailed under "Experimental Procedures." B and D, the cells were also cotransfected with dominant negative mutants of either MEK1 (DNMEK1) or MKK3 (AlaMKK3). 6 h later, SCC25 cells were pretreated with Me₂SO (vehicle control; V), 5 μ M MEK1/2 inhibitor U0126, or 5 μ M p38 MAPK inhibitor SB202190 for 30 min and then treated with 10 ng/ml TGF- β 1. 24 h later, the cells were lysed, and luciferase activities were determined by luminometry. Results are normalized relative to control cells (Me₂SO, -TGF- β 1), which was set as 1.0. The results represent the mean \pm S.E. of three different experiments. RLU, relative luciferase units.

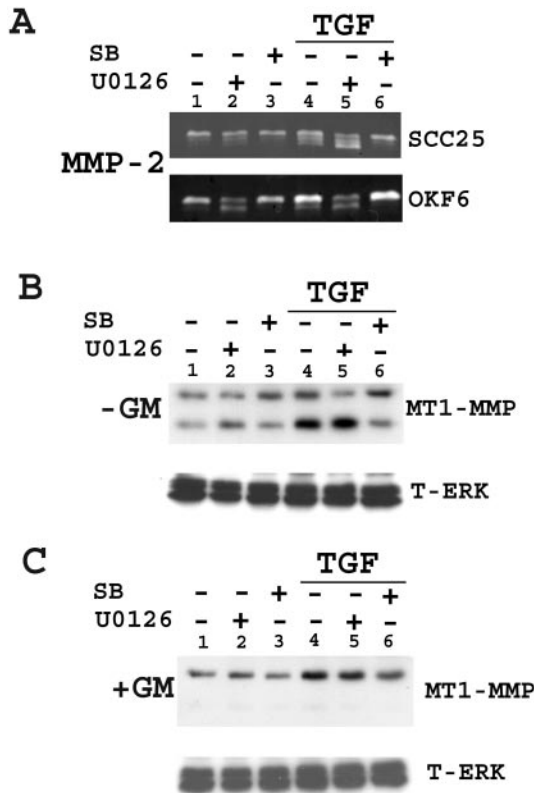
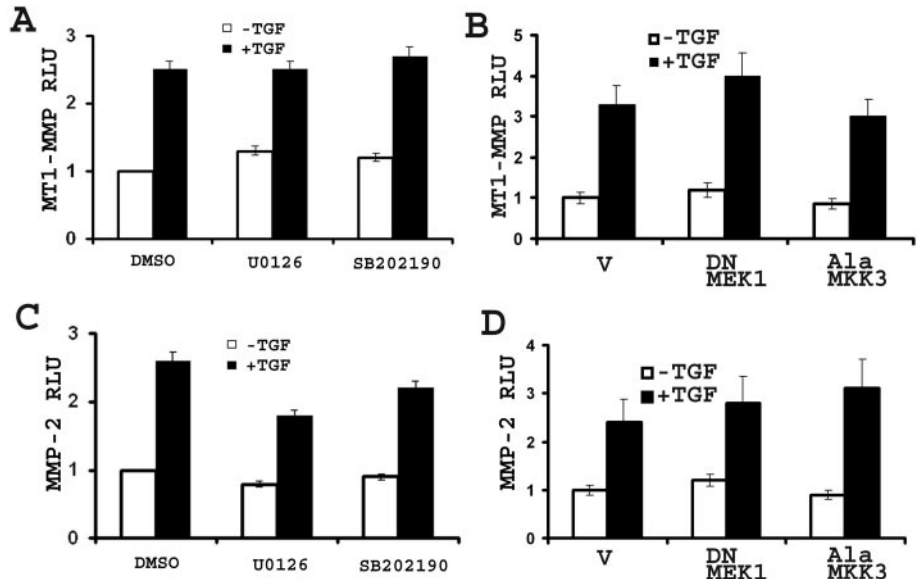


FIG. 5. MMP-2 activation and MT1-MMP autolytic processing are modulated differentially by ERK1/2 and p38 MAPK. OSCC cells were pretreated with Me₂SO (vehicle control), 5 μ M MEK1/2 inhibitor U0126, or 5 μ M p38 MAPK inhibitor SB202190 (SB) for 30 min and then treated with 10 ng/ml TGF- β 1 for 24 h. A, the conditioned media were collected and analyzed for MMP-2 activity by gelatin zymography. B and C, SCC25 cells were also treated with Me₂SO (B) or with an MMP inhibitor GM6001 (5 μ M) (C; GM) for 24 h. Cell lysates analyzed for MT1-MMP (upper panel) and total ERK1/2 (lower panel) by Western blotting.

panels, compare lanes 7 and 8). These data indicate that the predominant effect of SB202190 on MMP regulation results from reciprocal activation of ERK1/2 rather than through inhibiting phosphorylation of other p38 downstream substrates. As shown previously, these MAPK inhibitors did not affect the expression of MMP-2 (Fig. 6B, bottom panel).

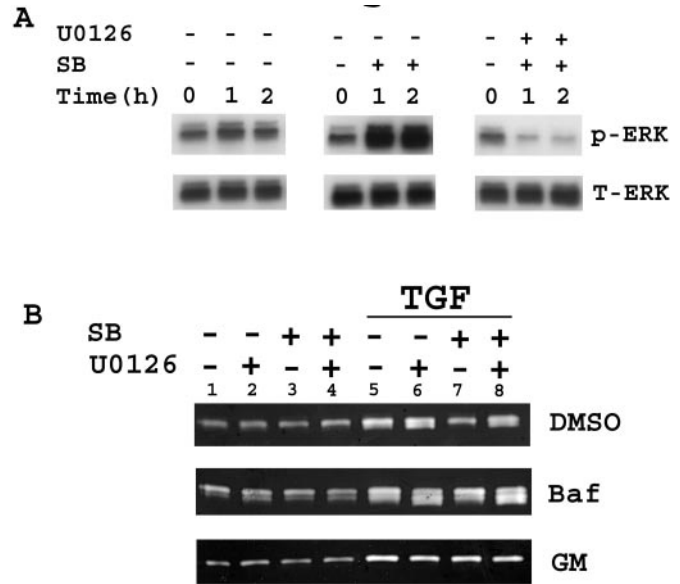


FIG. 6. p38 MAPK inhibitor blocks TGF- β 1-mediated MMP-2 activation by increasing ERK1/2 activity. A, SCC25 cells were pretreated with Me₂SO (vehicle control), 5 μ M MEK1/2 inhibitor U0126, or 5 μ M p38 MAPK inhibitor SB202190 (SB) for 30 min, treated with 10 ng/ml TGF- β 1, and lysed at the indicated times. The lysates were probed with anti-phospho-ERK1/2 antibody to detect the phosphorylated, active form of ERK1/2 (p-ERK) or with anti-ERK1/2 antibody to detect total ERK1/2 (T-ERK) expression. B, SCC25 cells were pretreated with Me₂SO (DMSO), 5 μ M U0126, 5 μ M SB202190, or both 5 μ M U0126 and 5 μ M SB202190 for 30 min and then treated with TGF- β 1. In additional experiments, the cells were also treated with either 50 nM bafilomycin A1 (Baf) or 5 μ M GM6001 (GM). The conditioned media were collected 24 h later and analyzed for MMP-2 activation by gelatin zymography.

Regulation of TIMP-2 by TGF- β 1 and MAPK Inhibitors— TIMP-2 plays a dual role as a central regulator of proMMP-2 activation; at low concentration it facilitates activation by bridging trimolecular activation complex formation, and at higher concentrations the reaction is blocked via inhibitory interaction of TIMP-2 with the catalytically competent MT1-MMP active site (7–9). Binding of TIMP-2 to the active site of MT1-MMP also prevents autolytic processing. As the results presented above indicate that MAPK inhibitors regulate TGF- β 1-stimulated MT1-MMP activity, we examined the effect of U0126 and SB202190 on TIMP-2 levels (Fig. 7A). To prevent

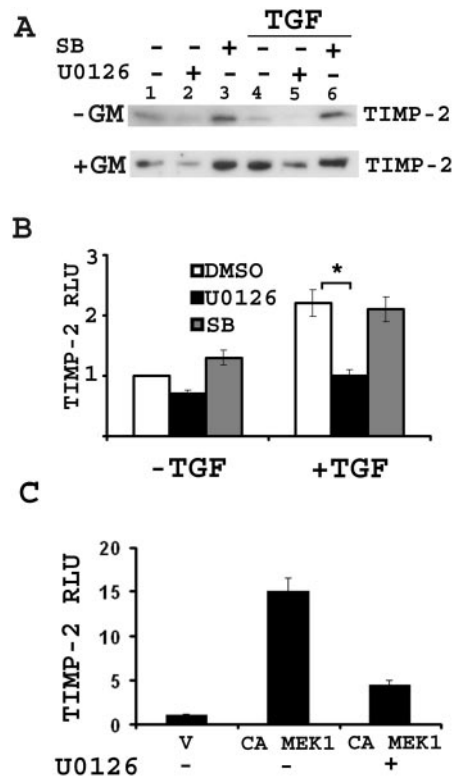


FIG. 7. Regulation of TIMP-2 by TGF- β 1 and MAPK inhibitors. A, SCC25 cells were pretreated with Me₂SO (vehicle control), 5 μ M MEK1/2 inhibitor U0126, or 5 μ M p38 MAPK inhibitor SB202190 for 30 min and then treated with 10 ng/ml TGF- β 1. The samples were also treated either with Me₂SO (*upper panel*) or with an MMP inhibitor GM6001 (*lower panel*). The conditioned media were collected at 24 h, concentrated 15–20-fold using Micron 10 microconcentrators, and analyzed for TIMP-2 by Western blotting. B and C, SCC25 cells were transiently transfected with a TIMP-2 promoter/reporter construct as detailed under “Experimental Procedures.” SCC25 cells were also cotransfected with vector or the constitutively active (CA) MEK1 mutant. 6 h later, SCC25 cells were pretreated with Me₂SO (vehicle control; DMSO), 5 μ M MEK1/2 inhibitor U0126, or 5 μ M p38 inhibitor SB202190 for 30 min and then treated with 10 ng/ml TGF- β 1 as indicated. 24 h later, the cells were lysed, and luciferase activities were determined by luminometry. Results are normalized for transfection efficiency and expressed relative to control cells, which was set as 1.0. The results represent the mean \pm S.E. of three different experiments. *, significantly different with $p < 0.05$. RLU, relative luciferase units.

MT1-MMP-mediated binding and degradation of TIMP-2, control experiments included GM6001 (Fig. 7A, *lower panel*). The conditioned media were collected, concentrated 15–20-fold, and TIMP-2 analyzed by Western blotting. A TGF- β 1-induced increase in TIMP-2 was apparent in the presence of GM6001 (Fig. 7A, *lower panel*, compare lanes 1 and 4). Inhibition of ERK1/2 activation decreased TIMP-2 levels (Fig. 7A, compare lanes 1, 2 and lanes 4, 5), whereas treatment with the p38 MAPK inhibitor sustained the TGF- β 1-induced expression of TIMP-2 protein (Fig. 7A, compare lanes 1, 3 and lanes 4, 6). Similar results were obtained by analysis of TIMP-2 transcription using a TIMP-2 promoter/luciferase reporter construct (Fig. 7B). Treatment of SCC25 cells with TGF- β 1 increased TIMP-2 promoter activity (Fig. 7B), and this effect was abrogated by U0126. To confirm our observation that TGF- β 1-induced TIMP-2 expression was mediated by ERK1/2, we examined the effect of transfecting the constitutively active MEK1 mutant (Fig. 7C). SCC25 cells were cotransfected with the TIMP-2 promoter/reporter plasmid and either vector or the constitutively active MEK1. Transfection of the constitutively active MEK1 resulted in a 15-fold increase in the TIMP-2 promoter. Treatment of the samples with U0126 partially

blocked constitutively active MEK-1-induced TIMP-2 promoter activity. These data support a model wherein TGF- β 1-mediated ERK1/2 activation is a positive signal for control of TIMP-2 expression.

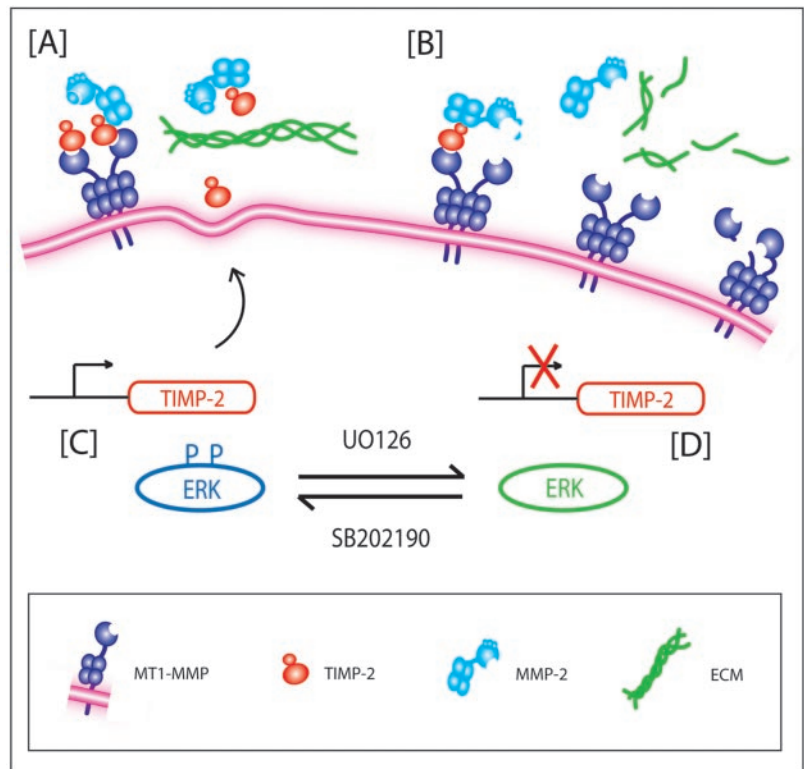
DISCUSSION

MT1-MMP plays a pivotal role in cell migration, in the formation of new blood vessels, and in normal development (46, 47). MT1-MMP-null mice have severe skeletal defects, indicating that this MMP also plays an essential role in the formation and maintenance of skeletal tissues (48, 49). In pathologic conditions MT1-MMP overexpression is seen in a number of different tumors, and this correlates with poor clinical prognosis (46, 47). In addition to its well described role in the activation of proMMP-2, MT1-MMP can also directly degrade components of the extracellular matrix, including interstitial types I and III collagen, indicating a potential mechanism for highly localized collagenolysis at sites of cell-matrix contact (50–53). Moreover, it was recently demonstrated that the ability of MT1-MMP to cleave the pericellular matrix imbues MT1-MMP-expressing tumors with the ability to overcome growth restraint imposed by three-dimensional matrices, such as collagen or fibrin (54, 55). Given the crucial role MT1-MMP plays in both physiologic and pathologic conditions, significant effort has been directed toward understanding the regulation of MT1-MMP activity at the cell-matrix interface.

Because of its critical role in physiology and pathology, MT1-MMP activity on the cell surface is subject to multiple control mechanisms. Like other MMPs, MT1-MMP is synthesized as a proenzyme, and propeptide processing is a prerequisite for acquisition of surface-associated proteinase activity (56–58). The propeptide can be removed by the serine proteinase furin or by other related proprotein convertases in the secretory pathway (56). After zymogen activation, MT1-MMP activity is then regulated further by either autolytic processing with concurrent removal of the catalytic domain or by binding of TIMP-2 to the catalytic site (46, 47). TIMP-2 binding blocks MT1-MMP activity but also prevents autolysis, resulting in preservation of the 55-kDa MT1-MMP protein on the cell surface. Removal of active proteinase from the cell surface via diverse endocytic or shedding mechanisms has also been reported (59–62).

In this report we have demonstrated that MT1-MMP activity can also be modulated reciprocally by MAPK signaling pathways in a cellular model of OSCC. TGF- β 1 increased expression of both MMP-2 and MT1-MMP. Although ERK1/2 and p38 MAPK were activated with TGF- β 1 treatment, promoter/reporter assays and analysis of protein levels demonstrated that neither kinase affected the overall expression of proMMP-2 or MT1-MMP. In contrast to our data, ERK1/2 activation has been reported to have variable effects on MT1-MMP or MMP-2 expression. For example, constitutively active MEK increased MT1-MMP RNA expression in Madin-Darby canine kidney cells (63) and promoted MT1-MMP-dependent MMP-2 activation in HT1080 cells (64), and these effects were blocked with MEK inhibitors, whereas inhibiting ERK1/2 activation in 3RY1 rat cells decreased MMP-2 expression with no effect on MT1-MMP (65). The current data support a fine regulatory mechanism whereby the ERK1/2 and p38 MAPK pathways differentially control acquisition of pericellular proteolytic potential by affecting cell surface MT1-MMP catalytic activity as evidenced by MMP-2 activation and generation of 43-kDa MT1-MMP product. These data are in agreement with our previous observation that calcium-induced MMP-2 activation in SCC25 cells was also associated with increased generation of 43-kDa MT1-MMP product (43) and with recent reports demonstrating increased generation of the 43-kDa MT1-MMP

FIG. 8. ERK1/2 and p38 MAPK regulate TGF- β 1-mediated MT1-MMP activity differentially. Treatment of OSCC cells (A) with TGF- β 1 enhances the activity of MMP-2 and MT1-MMP against pericellular collagen (B). Inhibiting p38 MAPK with SB202190 promotes ERK1/2 phosphorylation (C), which in turn increases TIMP-2 synthesis. Increased TIMP-2 levels block TGF- β 1-induced MT1-MMP catalytic activity, thereby decreasing MMP-2 activation and matrix breakdown (A). In contrast, inhibition of ERK1/2 phosphorylation with U0126 blocks TGF- β 1-induced TIMP-2 synthesis (D), resulting in enhanced MT1-MMP catalytic activity, MMP-2 activation, and collagen cleavage (B).



product with fibronectin and phorbol ester-mediated MMP-2 activation (66–68).

In contrast to ERK1/2, p38 MAPK was a positive regulator of TGF- β 1-induced proteolysis, as evidenced by down-regulation of MMP-2 and MT1-MMP catalytic activities in the presence of p38 MAPK inhibitor SB202190. However, the effect of SB202190 on MMP-2 activation was transduced via reciprocal ERK1/2 phosphorylation because concurrent treatment with the MEK1/2 inhibitor blocked the inhibitory effect of SB202190 on MMP-2 activation. Our data support a model wherein inhibition of p38 MAPK leads to activation of ERK1/2, providing evidence for reciprocal regulation of MAPK signaling in the control of proteinase expression. Indeed, a number of recent reports have demonstrated that there is significant cross-talk between the ERK1/2 and the p38 MAPK pathways such that inhibition of p38 MAPK can promote ERK1/2 activation and vice versa (69–72).

Data in the current report highlight the essential role of TIMP-2 in the control of matrix proteolysis because this protein functions as both a stimulator and inhibitor of this process (73). This is in agreement with a previous report from our laboratory demonstrating that calcium-induced MMP-2 activation in SCC25 cells was associated with decline in soluble TIMP-2 levels with a concomitant increase in the cellular MT1-MMP-TIMP-2 complex (43). Inhibiting ERK1/2 activity in SCC25 cells decreased soluble TIMP-2 levels and enhanced proMMP-2 activation, whereas inhibition of p38 MAPK had the opposite effect. In agreement with these findings, the current data demonstrate differential activation of the TIMP-2 promoter/reporter construct as a result of ERK1/2 or p38 MAPK signaling. Moreover, expression of a constitutively active MEK1 mutant increased the promoter activity of TIMP-2, whereas expression of a constitutively active MKK3 decreased the promoter activity of TIMP-2 (data not shown). Interestingly, the TIMP-2 promoter has been shown to have AP-1, AP-2, and SP-1 binding sites (74), which can potentially be modulated by ERK1/2 and p38 MAPKs. Experiments are currently under

way to identify the regions in the TIMP-2 promoter which are regulated by these MAPKs.

In summary, our data indicate that TGF- β 1 enhances pericellular proteolysis by inducing the expression of MMP-2 and MT1-MMP and by promoting MT1-MMP activity and subsequent MMP-2 activation in SCC25 cells via a complex TIMP-2-dependent regulatory mechanism (Fig. 8, A and B). Mechanistic analyses demonstrated that differential activation of MAPK signaling pathways by TGF- β 1 altered the relative levels of TIMP-2 expression, shifting the balance between MMP activation and inhibition. Our data support a negative role for ERK1/2 in TGF- β 1-induced MMP activity (Fig. 8C), such that inhibition of ERK1/2 activation subsequently enhances pericellular proteolytic potential (Fig. 8D). Further, we provide evidence supporting complex reciprocal interactions between the ERK1/2 and p38 MAPK pathways and demonstrate that inhibition of p38 MAPK activity potentiates ERK1/2 activation. Interestingly, *in vivo* inhibition of p38 MAPK in a rat remnant kidney model of interstitial fibrosis was associated with ERK1/2 activation and worsening of the renal lesion (75). Our data, thus, highlight the potential complexity of novel therapeutic strategies that combine agents that interfere with TGF- β 1 signaling with specific MAPK inhibitors to regulate collagen degradation and tumor invasion.

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