Membrane-Type 1 Matrix Metalloproteinase Stimulates Cell Migration through Epidermal Growth Factor Receptor Transactivation

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Abstract

Proteolysis of extracellular matrix proteins by membrane-type 1 matrix metalloproteinase (MT1-MMP) plays a pivotal role in tumor and endothelial cell migration. In addition to its proteolytic activity, several studies indicate that the proinvasive properties of MT1-MMP also involve its short cytoplasmic domain, but the specific mechanisms mediating this function have yet to be fully elucidated. Having previously shown that the serum factor sphingosine 1-phosphate stimulates MT1-MMP promigratory function through a process that involves its cytoplasmic domain, we now extend these findings to show that this cooperative interaction is permissive to cellular migration through MT1-MMP−dependent transactivation of the epidermal growth factor receptor (EGFR). In the presence of sphingosine 1-phosphate, MT1-MMP stimulates EGFR transactivation through a process that is dependent upon the cytoplasmic domain of the enzyme but not its catalytic activity. The MT1-MMP−induced EGFR transactivation also involves G_i protein signaling and Src activities and leads to enhanced cellular migration through downstream extracellular signal-regulated kinase activation. The present study, thus, elucidates a novel role of MT1-MMP in signaling events mediating EGFR transactivation and provides the first evidence of a crucial role of this receptor activity in MT1-MMP promigratory function. Taken together, our results suggest that the inhibition of EGFR may represent a novel target to inhibit MT1-MMP−dependent processes associated with tumor cell invasion and angiogenesis. (Mol Cancer Res 2007;5(6):569−83)

Introduction

The membrane type-1 matrix metalloproteinase (MT1-MMP) is the prototypical member of a subset of the MMP family that are intrinsically associated with the plasma membrane of normal and tumor cells (1). Several lines of evidence suggest that MT1-MMP−dependent proteolysis of both extracellular matrix components (2, 3) and cell surface adhesion receptors (4-6) plays an essential role in tumor growth, invasion, and angiogenesis. The overexpression of MT1-MMP enables tumor cell growth in otherwise growth-restrictive, three-dimensional extracellular matrices (7) and promotes cell migration of a number of cancer (8-10) and endothelial cell lines (11-13). The short cytoplasmic sequence of MT1-MMP also contributes to the induction of cell migration by the enzyme (9, 10, 13) possibly through activation of the extracellular signal-regulated protein kinase (ERK) cascade (14), endocytosis and trafficking of the enzyme (15, 16), and interaction with tyrosine phosphorylated caveolin-1 (17). Additional insights into the mechanisms by which MT1-MMP induces cell migration may come from our observation that MT1-MMP cooperates with the platelet-derived bioactive lipid sphingosine 1-phosphate (S1P) to stimulate endothelial cell migration and morphogenic differentiation into capillary-like structures *in vitro* (13). The mechanisms involved in the interaction between MT1-MMP and S1P remain largely unknown but might play a significant physiologic role given the importance of the predominant S1P receptor ($S1P_1$) in cell migration (18), blood vessel maturation (19), and tumor angiogenesis (20).

The binding of S1P to its G protein−coupled receptors (GPCR) triggers activation of a number of key signaling intermediates, including ERK, and Rac and Rho small GTPases, which may account for some of the biological effects of this lipid (reviewed in ref. 21). More recently, S1P was also shown to also act via transactivation of some receptor tyrosine kinases, including the vascular endothelial growth factor receptor (22, 23), the platelet-derived growth factor β receptor (PDGFR; ref. 24), and the epidermal growth factor receptor (EGFR; refs. 24, 25), suggesting that the transactivation of these receptors may play a role in S1P-mediated cell migration.

In addition to S1P, a wide variety of signaling events elicited by GPCR ligands are dependent on the transactivation of the EGFR (26, 27). This receptor belongs to a family of four closely related receptor tyrosine kinases (EGFR, HER-2/*neu*, HER3, and HER4) that play essential roles in the control of

various biological responses, including proliferation, differentiation, migration, and modulation of apoptosis. The transactivation of EGFR by GPCR is an important signaling mechanism in the regulation of complex processes, such as cancer development (27). Although the mechanisms involved in the EGFR transactivation remain incompletely understood, numerous studies suggest that the release of membrane-associated EGFR ligands by some members of the metalloprotease-disintegrin (ADAM; ref. 28) and MMP (29) families play an important role in this process, leading to tumor growth and invasion (26, 30-34).

Our finding that S1P stimulates MT1-MMP–dependent endothelial cell migration through activation of GPCR-mediated signaling led us to investigate the role of EGFR transactivation in this process. We report that MT1-MMP induces the transactivation of EGFR in endothelial and COS-7 cells stimulated with S1P, and that this process requires the cytoplasmic domain of the enzyme but not its catalytic activity. In addition, we show that the induction of EGFR transactivation by MT1-MMP involves G_i protein signaling and Src activities, and that this transactivation of EGFR mediates enhanced cellular migration through downstream ERK activation. This study provides the first evidence that the MT1-MMP promigratory function involves a close cooperation of the enzyme with signaling events originating from GPCR and EGFR.

Results

MT1-MMP–Dependent Morphogenic Differentiation and Endothelial Cell Migration Induced by S1P Involve EGFR Activity

Our previous results indicated that MT1-MMP cooperates with the platelet-derived bioactive lipid S1P to stimulate endothelial cell migration and morphogenic differentiation into capillary-like structures *in vitro*. Both of these processes involve the activation of G protein–mediated signaling via $S1P_1$ and $S1P_3$ receptors (13). Although bovine aortic endothelial cells (BAEC), which express low levels of MT1-MMP, do not spontaneously form capillary-like structures on Matrigel (Fig. 1A), the overexpression of MT1-MMP induced the formation of a well-defined capillary network in the presence of S1P (Fig. 1A), as we have previously reported. In addition, we have shown that migration of control BAEC is significantly induced by S1P, and that the overexpression of MT1-MMP markedly increased the migratory potential of BAEC in the presence of this bioactive lipid (Fig. 1B). Although a close cooperation between the S1P- and the vascular endothelial growth factor–mediated signaling cascades has been identified in some cell systems, we have previously shown that the S1P-stimulated MT1-MMP–dependent endothelial cell migration and morphogenic differentiation do not involve vascular endothelial growth factor receptor activity (13). To determine whether the stimulatory effect of
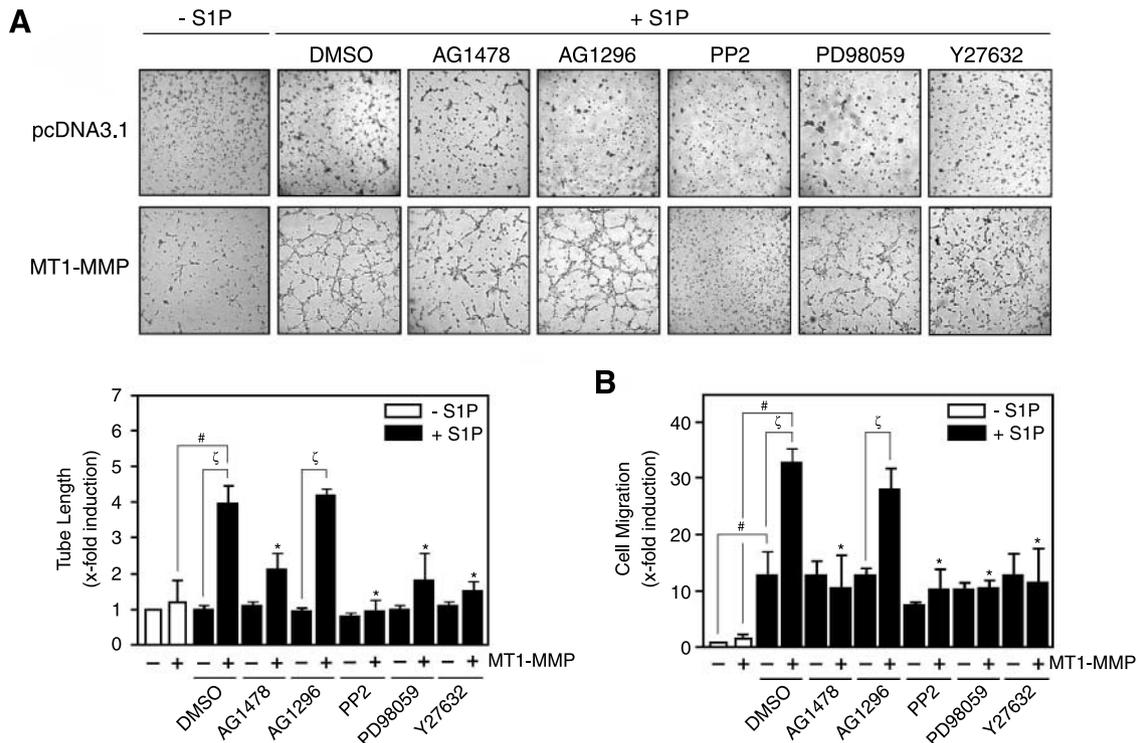

FIGURE 1. Induction of endothelial cell morphogenic differentiation and migration by MT1-MMP and S1P involve EGFR activity. BAEC overexpressing MT1-MMP (or empty vector pcDNA3.1) were treated (or not) with AG1478 (0.5 μmol/L), AG1296 (1 μmol/L), PP2 (10 μmol/L), PD98059 (10 μmol/L), Y27632 (20 μmol/L), or DMSO (*Ctl*) for 30 min before the addition of S1P and subjected to morphogenic differentiation, cell migration, and cell viability assays. Columns, mean x-fold induction of non-stimulated empty vector–transfected cells (pcDNA3.1) from at least three independent experiments; bars, SD. #, $P \leq 0.01$ versus non-stimulation; ζ, $P \leq 0.01$ versus S1P-stimulated empty vector–transfected cells (pcDNA3.1); *, $P \leq 0.01$ versus S1P-stimulated MT1-MMP–transfected cells treated with DMSO. **A.** Formation of capillary-like structures. **B.** Cell migration.
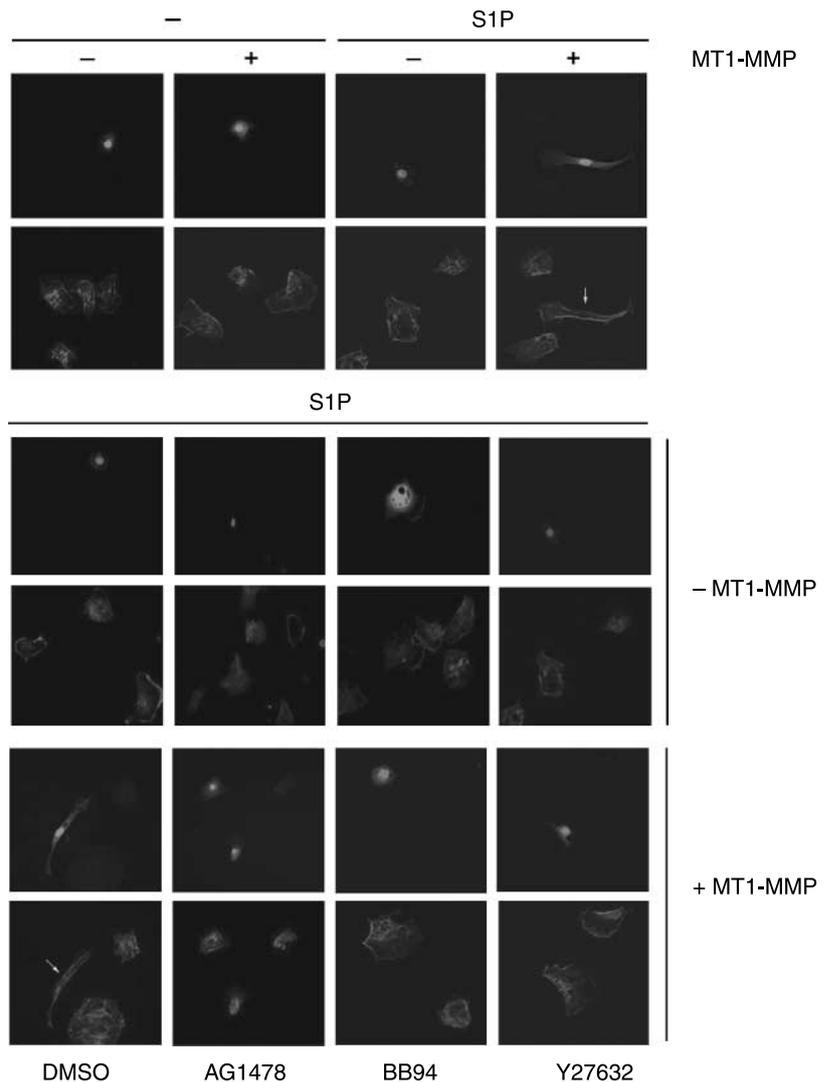
FIGURE 2. Morphological changes induced by MT1-MMP in the presence of S1P involve EGFR activity. pIRES2-GFP-MT1-MMP–transfected (or control vector pIRES2-GFP) cells were pretreated for 30 min and then stimulated (or not) with 1 μmol/L S1P for 5 min. Images of GFP fluorescence of transfected cells (*green*) and staining with phalloidin-TRITC (*red*). Arrows indicate cells that overexpress MT1-MMP, which are elongated following stimulation with S1P. Representative of three independent experiments.

S1P involves the transactivation of other receptor tyrosine kinases, such as PDGFR (24), and EGFR (24, 25), we tested AG1478 and AG1296, two selective inhibitors of EGFR and PDGFR tyrosine kinase activities, respectively. As shown in Fig. 1A and B, AG1478, but not AG1296, markedly decreased S1P-stimulated MT1-MMP–dependent morphogenic differentiation ($\sim$ 50% inhibition, $P \leq 0.01$) and completely abrogated MT1-MMP–dependent endothelial cell migration ($P \leq 0.01$). These data suggest that EGFR is involved in both processes.

Because GPCR-mediated EGFR transactivation has been shown to involve Src and to induce ERK activation (29, 35, 36), we then evaluated the effect of Src (PP2) and ERK (PD98059) inhibitors on S1P-stimulated MT1-MMP–dependent endothelial cell morphogenic differentiation and cell migration. Both processes were inhibited by PP2 and PD98059 (Fig. 1A and B), indicating that S1P-stimulated MT1-MMP–dependent migration and morphogenic differentiation also involve Src and ERK activities. We and others have previously reported that the induction of endothelial cell migration by S1P involves Rho kinase (ROCK; refs. 37-39).

We thus tested the effect of the ROCK inhibitor Y27632 on S1P-stimulated MT1-MMP–dependent endothelial cell morphogenic differentiation and cell migration. As expected, both processes were significantly inhibited by Y27632, suggesting that ROCK signaling is involved in the induction of endothelial cell morphogenic differentiation and cell migration by MT1-MMP.

During migration, endothelial cells acquire a migratory phenotype with an expansion of lamellipodia and cytoplasmic projections in contrast to their quiescent polygonal shape found in confluent monolayers (11). To visualize whether endothelial cells overexpressing MT1-MMP acquire a migratory phenotype upon stimulation with S1P, BAEC were transfected with the bicistronic vector MT1-MMP-pIRES2-GFP or with pIRES2-GFP as control. Transfected cells were incubated in the absence or presence of S1P, and filamentous actin was stained with Texas red-phalloidin. In the absence of S1P, $\sim$ 75% of both control and MT1-MMP–transfected BAEC kept their round shape (Fig. 2). Following stimulation with S1P, control cells transfected with the empty vector kept their round or polygonal

shape [~70% of the green fluorescent protein (GFP)–positive cells], whereas overexpression of MT1-MMP induced an elongated endothelial cell shape into ~70% of the GFP-positive cells, as indicated by arrows in Fig. 2. AG1478 inhibited the morphologic changes acquired by MT1-MMP–transfected BAEC (35% of the GFP-positive cells showed an elongated phenotype; Fig. 2), again suggesting a role for EGFR in S1P-stimulated MT1-MMP–dependent migratory function. These morphologic changes were also inhibited by BB94 (30% of the GFP-positive cells showed a migratory phenotype; Fig. 2), a broad-spectrum metalloprotease inhibitor, which is in keeping with our previous study showing that MT1-MMP–dependent BAEC migration involves the catalytic activity of the enzyme (13). In addition, overexpression of a catalytically inactive MT1-MMP mutant (E240A) and treatment with MT1-MMP physiologic tissue inhibitors of metalloproteinase (TIMP-2 and TIMP-3) inhibited the ability of MT1-MMP to induce BAEC migratory phenotype (data not shown). As expected, no migratory phenotype was induced by MT1-MMP in the presence of Y27632 (Fig. 2). As a control, we verified that none of the pharmacologic inhibitors tested affected BAEC viability (data not shown). Taken together, these results suggest that MT1-MMP–dependent morphogenic differentiation and endothelial cell migration induced by S1P involve EGFR, Src, and ERK activities.

MT1-MMP Induces the Transactivation of EGFR in S1P-Stimulated Cells, which Correlates with an Enhancement of Cell Migration

The inhibition of MT1-MMP–dependent morphogenic differentiation and endothelial cell migration by AG1478 suggests that in the presence of S1P, MT1-MMP stimulates the tyrosine kinase activity of EGFR. To confirm this hypothesis, BAEC overexpressing MT1-MMP were stimulated with 1 μmol/L S1P, cell lysates were subjected to immunoprecipitation with anti-EGFR antibodies, and the immune complexes were then immunoblotted using a monoclonal antibody against phosphotyrosine. S1P did not significantly induce tyrosine phosphorylation of endogenous EGFR in control-transfected cells (pcDNA3.1; Fig. 3A), whereas it induced a time-dependent stimulation of EGFR phosphorylation in MT1-MMP–transfected BAEC, reaching a maximal response after 5 min of S1P stimulation (4.4 ± 0.4-fold induction, $P \leq 0.01$). As a result of the poor transfection efficiency of BAEC, we further investigated the mechanisms involved in the MT1-MMP–dependent transactivation of EGFR using COS-7 cells. This cell type has been used in number of studies to determine the signaling mechanisms involved in GPCR-mediated trans-activation of EGFR (32, 40-44). These cells contain detectable amounts of the S1P receptor $S1P_1$ (45), but no detectable MT1-MMP, and thus constitute an interesting and appropriate cell model to study the mechanisms involved in S1P-stimulated MT1-MMP–dependent EGFR transactivation. MT1-MMP, along with an epitope-tagged EGFR construct (FLAG-EGFR), was overexpressed in COS-7 cells, and following stimulation with S1P, the FLAG-EGFR was immunoprecipitated to monitor its phosphotyrosine content. Consistent with the results obtained in BAEC, a strong stimulation of EGFR trans-activation was observed in cells cotransfected with MT1-MMP

and FLAG-EGFR ($P \leq 0.01$; Fig. 3B). Although not significant, there was a trend towards an induction of FLAG-EGFR tyrosine phosphorylation in control cells stimulated by S1P. The stimulatory effect of MT1-MMP on EGFR phosphorylation is not restricted to the overexpressed form of the receptor because the stimulation of COS-7 cells with S1P also leads to an MT1-MMP–dependent phosphorylation of the endogenous EGFR (2.3 ± 0.5-fold, $P \leq 0.01$; Fig. 3C). Consistent with other studies showing that MMPs are involved in the transactivation of EGFR, but not in the direct ligand-dependent activation of the receptor (33, 34, 42, 46-49), the overexpression of MT1-MMP had no inducible effect on the tyrosine phosphorylation of either endogenous or exogenous EGFR in the presence of EGF (Fig. 3B and C).

The inhibitory effect of AG1478 on S1P-stimulated MT1-MMP–dependent endothelial cell migration suggests that the kinase activity of EGFR is involved in this process. Because MT1-MMP induces EGFR transactivation in cells stimulated with S1P, we next determined whether or not this stimulatory effect of MT1-MMP translates into an increase in COS-7 cell migration. As we previously reported (45), S1P induced a slight increase in basal cell migration (1.5 ± 0.1-fold, $P \leq 0.05$), which was further augmented by MT1-MMP overexpression (2.6 ± 0.1-fold, $P \leq 0.05$; Fig. 3D). The overexpression of FLAG-EGFR led to an enhancement of cell migration in the presence of S1P (2.7 ± 0.2-fold, $P \leq 0.05$) but also increased the MT1-MMP–dependent cell migration induced by this lipid (3.8 ± 0.1-fold, $P \leq 0.05$; Fig. 3D). By contrast, EGF did not increase basal nor MT1-MMP–dependent cell migration (Fig. 3D), in keeping with our previous study showing that this growth factor does not cooperate with MT1-MMP (13). Overall, these results show that overexpression of MT1-MMP induces EGFR transactivation in the presence of S1P in both BAEC and COS-7 cells, and that this enhancement of EGFR transactivation is correlated with an augmentation of cell migration.

MT1-MMP–Dependent Cell Migration Induced by S1P Involves EGFR Transactivation, Src, and ERK Activities

Our initial experiments showed that inhibition of EGFR, Src, and ERK activities markedly reduce endothelial cell migration and morphogenic differentiation (Fig. 1). To determine whether or not these effects could be related to the inhibition of MT1-MMP–dependent EGFR transactivation, COS-7 cells coexpressing FLAG-EGFR along with MT1-MMP were preincubated with EGFR, Src, and ERK inhibitors before stimulation with S1P, and the extent of EGFR transactivaton and cell migration were monitored (Fig. 4A and B). Cotransfections along with FLAG-tagged EGFR were used in all further experiments to obtain a stronger and more sensitive detection of EGFR tyrosine phosphorylation. As expected, AG1478 (0.5 ± 0.5-fold, $P \leq 0.05$), but not AG1296, significantly reduced the S1P-stimulated MT1-MMP–mediated EGFR tyrosine phosphorylation (2.7 ± 0.5-fold, $P \leq 0.05$), showing the efficiency of this compound at inhibiting EGFR kinase activity (Fig. 4A). Inhibition of EGFR activity using AG1478 abolished the induction of cell migration by MT1-MMP (Fig. 4B), confirming the pivotal role of EGFR in this process. Both processes were also sensitive to inhibition of

G$_i$-mediated signaling cascade by pertussis toxin (Fig. 4A and B). MT1-MMP–dependent EGFR transactivation was abrogated by PP2 (1.4 $\pm$ 0.6-fold, $P \leq 0.05$), but not by PD98059, whereas both compounds strongly inhibited MT1-MMP–dependent cell migration (Fig. 4A and B). These pharmacologic inhibitors were used in conditions known to efficiently inhibit their respective targets (EGFR, Fig. 4A; Src, ref. 50; or ERK, refs. 14, 50, 51 and Fig. 7), without affecting cell viability (data not shown). In keeping with the results obtained using BAEC, these data suggest that the induction of COS-7 cell migration by MT1-MMP in the presence of S1P involves EGFR, Src, and ERK activities. Altogether, these results suggest that the MT1-MMP–dependent transactivation of

EGFR leads to a stimulation of cell migration in the presence of S1P, and that Src family kinases play a role in the transactivation of EGFR, whereas ERK is involved downstream of the receptor.

MT1-MMP–Dependent EGFR Transactivation Involves the Cytoplasmic Domain of the Enzyme but not Its Catalytic Activity

To better understand the mechanisms by which MT1-MMP stimulates EGFR transactivation, we next examined the role of the catalytic and cytoplasmic domains of MT1-MMP in this process by overexpressing cytoplasmic domain-deleted (CΔ20) and catalytically inactive (E240A) MT1-MMP
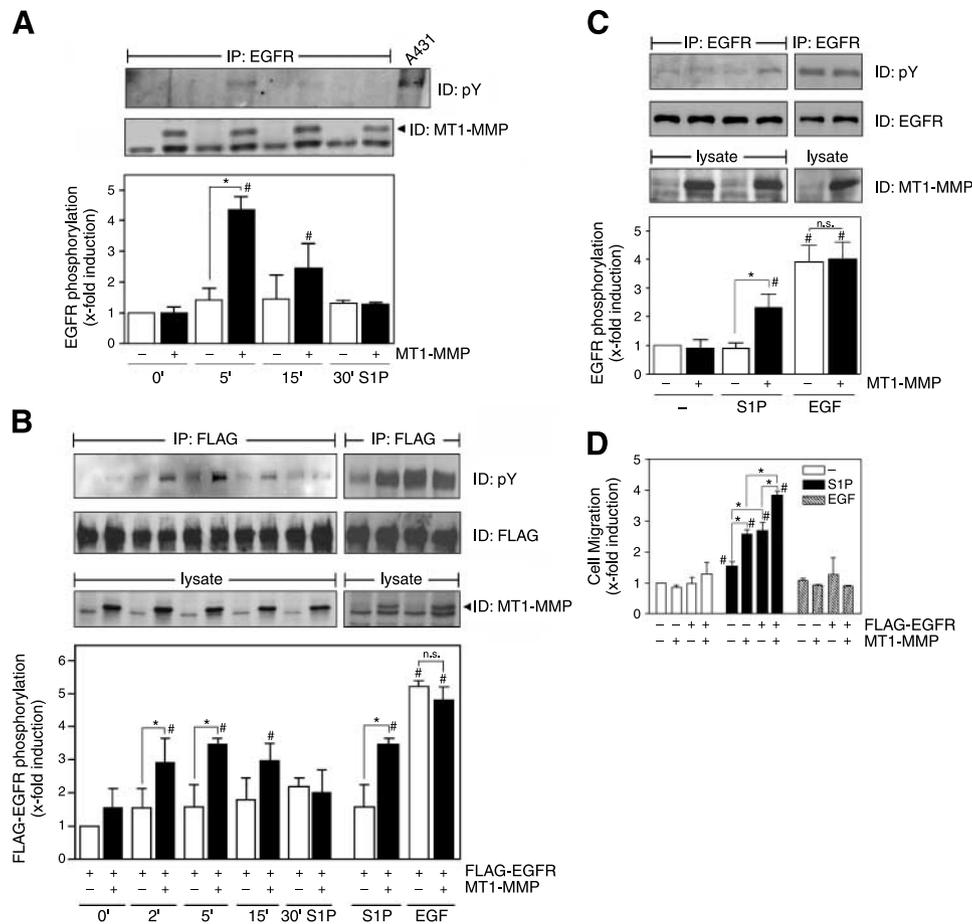

FIGURE 3. MT1-MMP promotes S1P-dependent transactivation of the EGFR. **A.** Serum-starved MT1-MMP–transfected BAEC (or control pcDNA3.1) were treated for the indicated times with 1 μmol/L S1P. After lysis, EGFR was immunoprecipitated, and tyrosine-phosphorylated EGFR was detected by immunoblotting with a monoclonal anti-phosphotyrosine (pY). Western blots of isolated crude membrane fractions were probed with anti-MT1-MMP antibodies. The extent of EGFR tyrosine phosphorylation was quantified by densitometry and normalized to the expression of MT1-MMP. Columns, mean x-fold induction of non-stimulated control cells (pcDNA3.1) from three independent experiments; bars, SD. *, $P \leq 0.01$ versus stimulated empty vector–transfected cells (pcDNA3.1); #, $P \leq 0.01$ versus non-stimulated MT1-MMP–transfected cells. **B.** COS-7 cells were cotransfected with MT1-MMP (or pcDNA3.1) along with FLAG-EGFR construct, serum starved, and treated for the indicated times with 1 μmol/L S1P (*left*) or for 5 min with 1 μmol/L S1P or 30 ng/mL EGF (*right*). After lysis, FLAG-EGFR was immunoprecipitated, and the extent of phosphorylation was monitored (*top*). The EGFR tyrosine phosphorylation was quantified by densitometry and normalized to the FLAG content. Columns, means x-fold induction of non-stimulated control transfected cells from four independent experiments; bars, SD. *, $P \leq 0.01$ versus stimulated control transfected cells; #, $P \leq 0.01$ versus non-stimulated MT1-MMP–transfected cells. n.s., not significant. **C.** Serum-starved COS-7 cells overexpressing MT1-MMP (or pcDNA3.1) were treated for 5 min with (or without) 1 μmol/L S1P (*left*), or with 30 ng/mL EGF (*right*). The extent of endogenous EGFR tyrosine phosphorylation was quantified by densitometry and normalized to the EGFR content. Columns, means x-fold induction of non-stimulated control transfected cells from three independent experiments; bars, SD. *, $P \leq 0.05$ versus stimulated control transfected cells; #, $P \leq 0.01$ versus non-stimulated MT1-MMP–transfected cells. **D.** COS-7 cells were cotransfected as described in (**B**) and were allowed to migrate for 3 h in media containing (or not) either 1 μmol/L S1P or 30 ng/mL EGF. Columns, mean x-fold induction of non-stimulated control from three independent experiments; bars, SD. *, $P \leq 0.05$; #, $P \leq 0.05$ versus respective non-stimulated transfected cells.
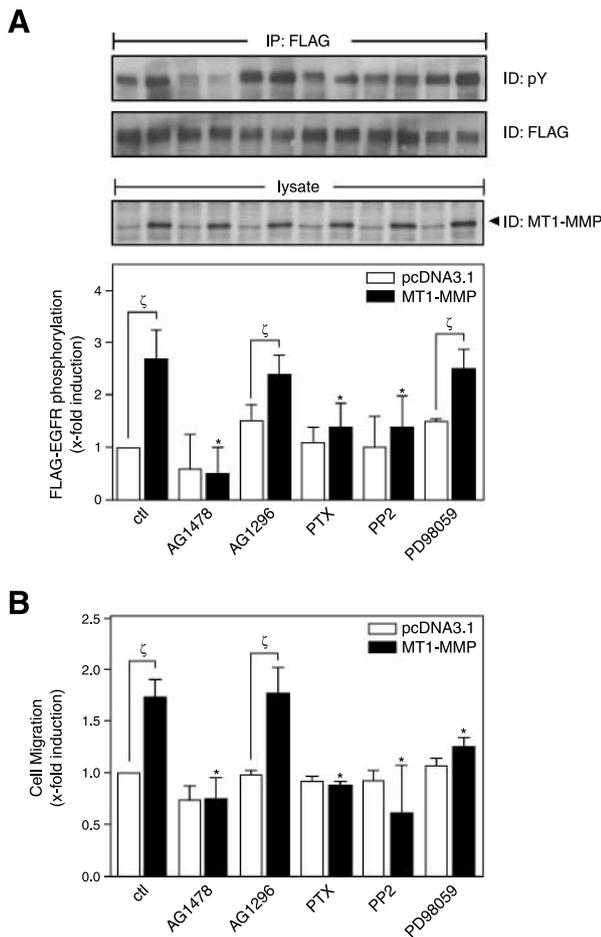
FIGURE 4. MT1-MMP–dependent COS-7 cell migration induced by S1P involve EGFR transactivation, Src, and ERK activities. **A.** COS-7 cells were cotransfected with the FLAG-EGFR construct along with pcDNA3.1 or with MT1-MMP. Serum-starved transfected COS-7 cells were pretreated with AG1478 (0.5 μmol/L), AG1296 (1 μmol/L), pertussis toxin (*PTX*; 10 ng/mL), PP2 (10 μmol/L), PD98059 (10 μmol/L), or DMSO (*Ctl*) for 30 min and then stimulated with 1 μmol/L S1P for 5 min. FLAG-EGFR was immunoprecipitated, and phosphorylation was monitored as described in Fig. 3 legend and was quantified by densitometry and normalized to the FLAG content. Columns, mean *x*-fold induction of non-stimulated control cells from three independent experiments; bars, SD. ζ, $P \leq 0.05$ versus empty vector–transfected cells (pcDNA3.1); *, $P \leq 0.05$ versus the DMSO-treated MT1-MMP–transfected cells. **B.** COS-7 cells were cotransfected and pretreated with the inhibitors described in (**A**) or with BB94 (5 μmol/L) for 30 min. Cells were subjected to migration assay in the presence of 1 μmol/L S1P (with or without the inhibitors). Cell migration was quantified. Columns, mean *x*-fold induction of DMSO-treated control transfected cells (pcDNA.3.1) from three independent experiments; bars, SD. ζ, $P \leq 0.01$ versus empty vector–transfected cells (pcDNA3.1); *, $P \leq 0.05$ versus control (DMSO) MT1-MMP–transfected cells.

mutants. In the presence of S1P, the expression of the CΔ20 MT1-MMP mutant abolished the stimulatory effect of MT1-MMP on cell migration (Fig. 5B) and on EGFR transactivation (1.9 ± 0.4-fold, $P \leq 0.05$), compared with the wild-type enzyme (3.3 ± 0.7-fold, $P \leq 0.05$; Fig. 5A). Interestingly, the overexpression of the E240A MT1-MMP mutant stimulated EGFR tyrosine phosphorylation to a similar extent than the wild-type enzyme (Fig. 5A) but failed to enhance cell migration (Fig. 5B). To confirm these data, we used TIMP-2 and TIMP-3, which are both potent physiologic inhibitors of MT1-MMP activity (52). In keeping with the results obtained using the E240A mutant, neither TIMP-2 nor TIMP-3 affected the S1P-stimulated MT1-MMP–dependent EGFR transactivation (Fig. 5A). Similar to the catalytically inactive MT1-MMP mutant, TIMP-2 and TIMP-3 inhibited the S1P-stimulated MT1-MMP–dependent cell migration (Fig. 5B), reflecting a requirement for MT1-MMP–mediated degradation of extracellular matrix proteins in the migratory process.

Although TIMP-2 and TIMP-3 are known inhibitors of MT1-MMP catalytic activity, they also inhibit other enzymes of the MMP and ADAM families. To further determine whether or not another metalloproteinase is involved in the MT1-MMP–dependent transactivation of EGFR, the effect of BB94 was investigated. BB94 reduced the S1P-induced MT1-MMP–dependent EGFR transactivation (1.7 ± 0.8-fold, $P \leq 0.05$), compared with DMSO-treated MT1-MMP–transfected cells (3.3 ± 0.7-fold, $P \leq 0.05$; Fig. 5A). These data suggested that although this effect could not be attributed to the intrinsic catalytic activity of MT1-MMP, this process, nevertheless, requires the activity of a TIMP-2– and TIMP-3–insensitive MMP or ADAM. BB94 abolished the S1P-stimulated MT1-MMP–induced cell migration (Fig. 5B) possibly as a result of its inhibitory effect on EGFR transactivation and/or through the inhibition of matrix-degrading activity of MT1-MMP and/or other MMPs and ADAMs. These data indicate that the S1P-stimulated MT1-MMP–dependent EGFR transactivation involves the cytoplasmic domain of MT1-MMP and the catalytic activity of an unidentified metalloproteinase. Although BB94 inhibits MT1-MMP–dependent proMMP-2 activation (data not shown), the MMP involved is unlikely to be MMP-2 because the MT1-MMP CΔ20 mutant, which failed to induce EGFR transactivation, can activate pro-MMP-2 in COS-7 cells (14).

We have previously shown, using the constitutively active Gα$_{i2}$ mutant (G$_{i2}$-Q205L), that the stimulatory effect of S1P on MT1-MMP–dependent endothelial cell migration and morphogenic differentiation involves G protein α$_i$ subunits. To verify if S1P also acts through Gα$_i$-dependent signaling to induce MT1-MMP–dependent EGFR transactivation, a constitutively active Gα$_{i2}$ mutant (G$_{i2}$-Q205L) or the wild-type α$_{i2}$ protein was overexpressed in COS-7 cells, and the extent of EGFR transactivation was monitored without prior stimulation with S1P. The overexpression of the mutant Gα$_{i2}$ protein did not induce EGFR tyrosine phosphorylation in control-transfected cells (Fig. 5C), although it was sufficient to induce EGFR tyrosine phosphorylation in cells that overexpress MT1-MMP (3.3 ± 0.1-fold, $P \leq 0.05$). These results are in agreement with the stimulatory effect of S1P on EGFR transactivation in cells overexpressing MT1-MMP as well as with the inhibitory effect of pertussis toxin on this process. Also in keeping with our previous results, cotransfection of the Gα$_{i2}$ mutant (G$_{i2}$-Q205L) with the various MT1-MMP constructs along with FLAG-EGFR showed that removal of the cytoplasmic domain of the enzyme resulted in the loss of its ability to induce EGFR tyrosine phosphorylation (1.7 ± 0.6-fold versus 3.0 ± 0.1-fold for the wild-type enzyme, $P \leq 0.05$), whereas the inactivation of its catalytic activity had no effect (3.0 ± 0.5-fold; Fig. 5D). Taken together, these results suggest that the G$_i$-mediated
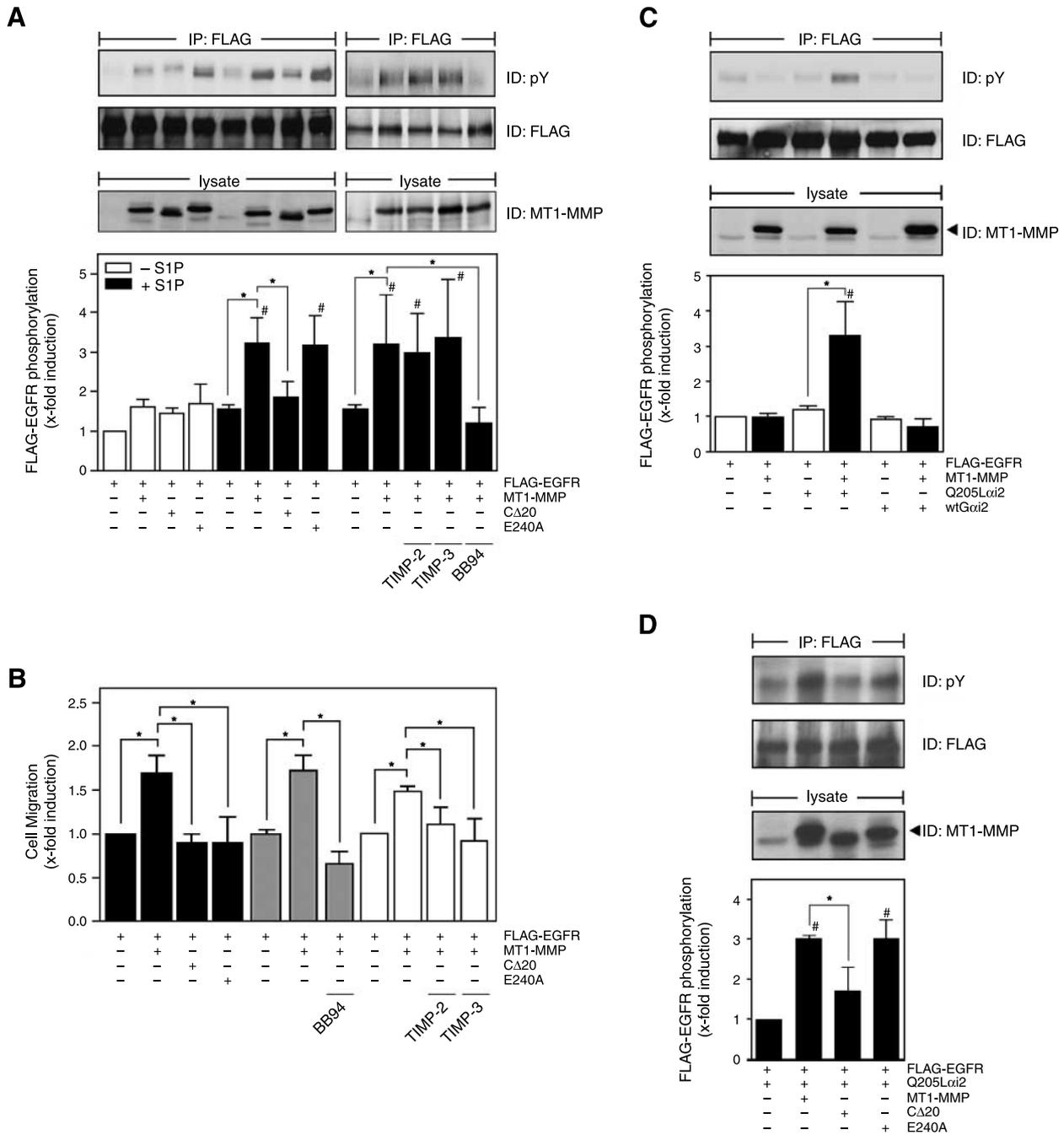
FIGURE 5. S1P-stimulated MT1-MMP−dependent EGFR transactivation involves its cytoplasmic domain but not its catalytic activity. **A.** COS-7 cells were cotransfected with the FLAG-EGFR construct along with pcDNA3.1 or with the various MT1-MMP constructs. Serum-starved transfected cells were stimulated (or not) with 1 μmol/L S1P (*left*) for 5 min and lysed. Where indicated, cells were pretreated with TIMP-2 (50 nmol/L), TIMP-3 (50 nmol/L), BB94 (5 μmol/L), or DMSO (Ctl) for 30 min before S1P stimulation. The extent of tyrosine phosphorylation of FLAG-EGFR was monitored and quantified as described in Fig. 3 legend. Columns, means *x*-fold induction of non-stimulated transfected cells from three independent experiments; bars, SD. *, $P \leq 0.05$ versus stimulated MT1-MMP−transfected cells; #, $P \leq 0.01$ versus respective non-stimulated cells. **B.** COS-7 cells were cotransfected as described in (**A**) and then pretreated or not with TIMP-2 (50 nmol/L), TIMP-3 (50 nmol/L), BB94 (5 μmol/L), or DMSO for 30 min. Cells were subjected to migration assay in the presence of 1 μmol/L S1P with or without the various inhibitors (or DMSO). Columns, mean *x*-fold induction of stimulated control from three independent experiments; bars, SD. *, $P \leq 0.05$. **C.** COS-7 cells were cotransfected with FLAG-EGFR along with either pcDNA3.1, MT1-MMP and with either pcDNA3.1 or plasmids encoding a constitutively active $G\alpha_{i2}$ mutant (G_{i2}-Q205L) or wild-type $G\alpha_{i2}$. Transfected cells were serum starved and lysed without any stimulation. Immunoprecipitation and immunoblotting procedures were done as described in (**A**). The extent of EGFR tyrosine phosphorylation was quantified by densitometry and normalized to the FLAG content. Columns, means *x*-fold induction of non-stimulated control transfected cells from three independent experiments; bars, SD. *, $P \leq 0.05$; #, $P \leq 0.05$ versus MT1-MMP−expressing cells cotransfected along with pcDNA3.1 or with wild-type $G\alpha_{i2}$. **D.** COS-7 cells were cotransfected with FLAG-EGFR and the constitutively active $G_{\alpha i2}$ mutant (G_{i2}-Q205L) with either pcDNA3.1 or the various MT1-MMP constructs. The extent of EGFR tyrosine phosphorylation was monitored as described in (**C**). Columns, means *x*-fold induction of non-stimulated control transfected cells from three independent experiments; bars, SD. #, $P \leq 0.05$ versus control transfected cells (pcDNA3.1); *, $P \leq 0.05$ versus MT1-MMP−overexpressing cells.
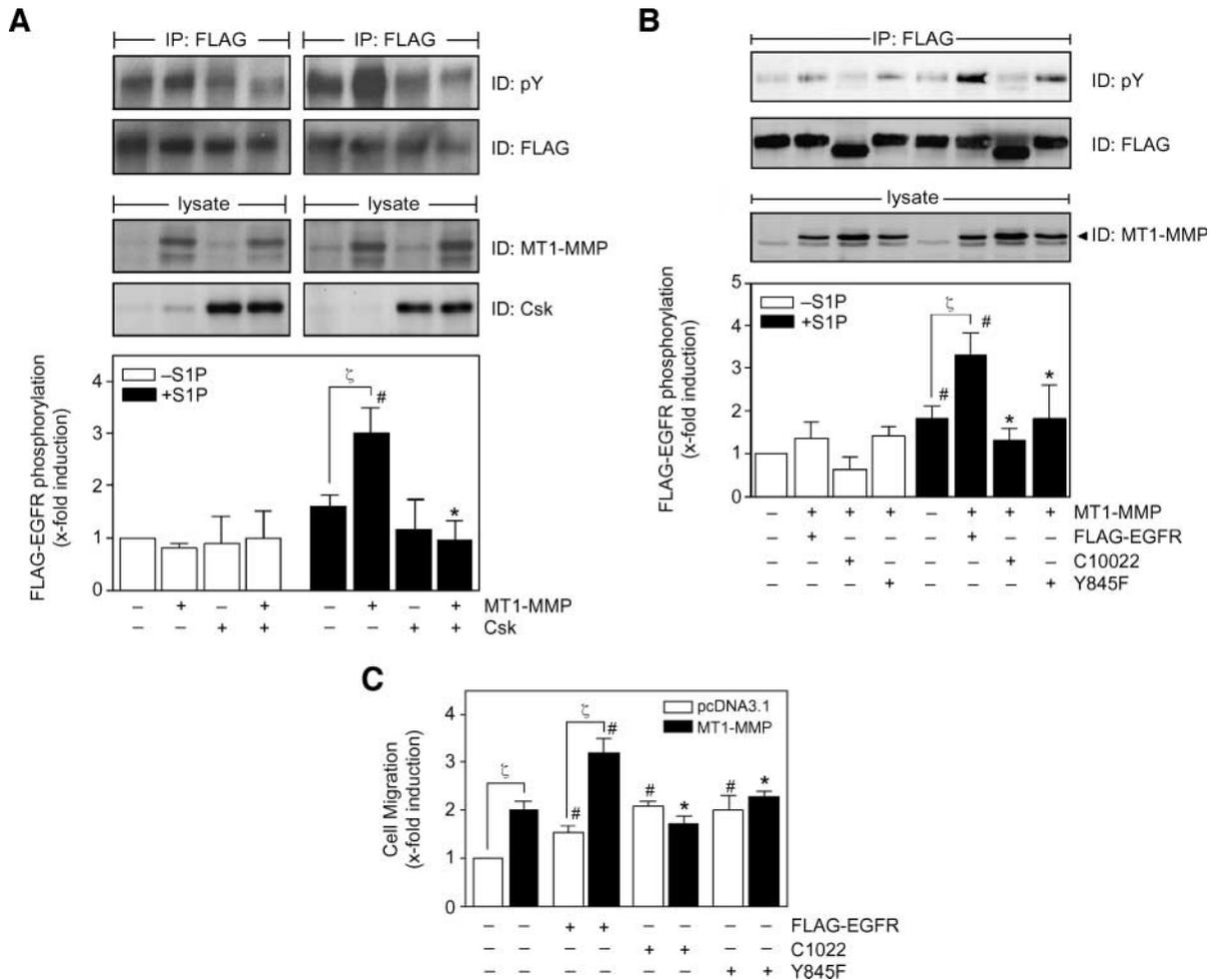
FIGURE 6. MT1-MMP−dependent EGFR transactivation and migration involve Src. **A.** COS-7 cells were cotransfected with FLAG-EGFR along with either pcDNA3.1, MT1-MMP, and with or without a plasmid encoding Csk. Cells were stimulated or not with 1 μmol/L S1P for 5 min and lysed. The extent of tyrosine phosphorylation of FLAG-EGFR was monitored as described in Fig. 3 legend. Columns, means x-fold induction of non-stimulated control transfected cells from three independent experiments; bars, SD. #, $P \le 0.05$ versus respective non-stimulated cells; ζ, $P \le 0.05$ versus empty vector−transfected cells (pcDNA3.1); *, $P \le 0.05$ versus S1P-stimulated MT1-MMP−transfected cells without Csk. **B.** COS-7 cells were cotransfected with pcDNA3.1 or with MT1-MMP construct along with the various FLAG-EGFR constructs, serum starved, and stimulated (or not) with 1 μmol/L S1P for 5 min. Tyrosine phosphorylation of FLAG-EGFR was assayed by immunoprecipitation and was quantified as described in (**A**). Columns, means x-fold induction of non-stimulated control transfected cells from four independent experiments; bars, SD. #, $P \le 0.05$ versus respective non-stimulated cells; ζ, $P \le 0.05$ versus empty vector−transfected cells (pcDNA3.1); *, $P \le 0.05$ versus S1P-stimulated cells overexpressing MT1-MMP along with FLAG-EGFR. **C.** COS-7 cells were cotransfected with FLAG-EGFR constructs along with either pcDNA3.1 or MT1-MMP and subjected to migration assay in the presence of 1 μmol/L S1P. Cell migration was quantified. Columns, mean x-fold induction of their respective cotransfected control from three independent experiments; bars, SD. #, $P \le 0.05$ versus control transfected cells without EGFR construct; ζ, $P \le 0.05$ versus empty vector−transfected cells (pcDNA3.1); *, $P \le 0.05$ versus cells cotransfected with MT1-MMP and FLAG-EGFR.

signaling induced by S1P triggers the MT1-MMP−dependent transactivation of EGFR via a mechanism that requires the cytoplasmic domain of the enzyme.

MT1-MMP−Dependent EGFR Transactivation and Migration Involve Src Activity

To confirm the inhibitory effect of PP2 on S1P-stimulated MT1-MMP−dependent EGFR transactivation, we overexpressed Csk, a physiologic inhibitor of Src (53). Consistent with our previous results, the overexpression of Csk inhibited the S1P-stimulated MT1-MMP−dependent EGFR transactivation (1.0 ± 0.4-fold versus 3.0 ± 0.5-fold without Csk, $P \le 0.05$; Fig. 6A). We also further confirmed the role of EGFR transactivation in MT1-MMP−induced cell migration and the

role of Src in these processes by examining the effects of two tyrosine phosphorylation-defective EGFR mutants: C1022 and Y845F. The C1022 mutant lacks three tyrosine residues (Tyr[1068], Tyr[1148], and Tyr[1173]) that are autophosphorylated following stimulation of EGFR (54). Tyr[845] is a unique phosphorylation residue within the cytoplasmic portion of EGFR known to be phosphorylated by Src both *in vitro* and *in vivo* (54, 55). The removal of these residues abrogated the transactivation of EGFR mediated by MT1-MMP in the presence of S1P (1.3 ± 0.3-fold versus 3.3 ± 0.5-fold with FLAG-EGFR, $P \le 0.05$; Fig. 6B) and abolished the stimulation of MT1-MMP−dependent cell migration, compared with the wild-type receptor (Fig. 6C). These results confirmed that EGFR activity plays a primary role in MT1-MMP migratory function. MT1-MMP−dependent

transactivation of the Y845F EGFR mutant was also markedly reduced (1.8 ± 0.8-fold, $P \leq 0.05$), resulting in inhibition of MT1-MMP–dependent cell migration, compared with the wild-type receptor (Fig. 6B and C). Overall, these results confirm the crucial role of EGFR in MT1-MMP migratory function and suggest a role for Src kinase activity in this process, involving EGFR phosphorylation on the Tyr[845] residue.

EGFR Transactivation Mediated by MT1-MMP Induces ERK Activation

The inhibitory effect of PD98059 on S1P-stimulated MT1-MMP–dependent BAEC and COS-7 cell migration together

with a lack of effect on EGFR tyrosine phosphorylation suggest that ERK activation plays a role downstream of the trans-activated receptor. Therefore, we verified whether or not MT1-MMP–induced EGFR transactivation leads to stimulation of ERK activation in COS-7 cells. In the presence of S1P, both MT1-MMP and FLAG-EGFR independently induced the activation of ERK (Fig. 7A). The coexpression of both proteins led to a heightened response (Fig. 7A), in keeping with the enhanced induction of cell migration observed under these conditions (Fig. 3D). AG1478 (1.2 ± 0.5-fold, $P \leq 0.05$), but not AG1296, prevented ERK activation in cells overexpressing MT1-MMP along with FLAG-EGFR (4.1 ± 0.8-fold; Fig. 7A),
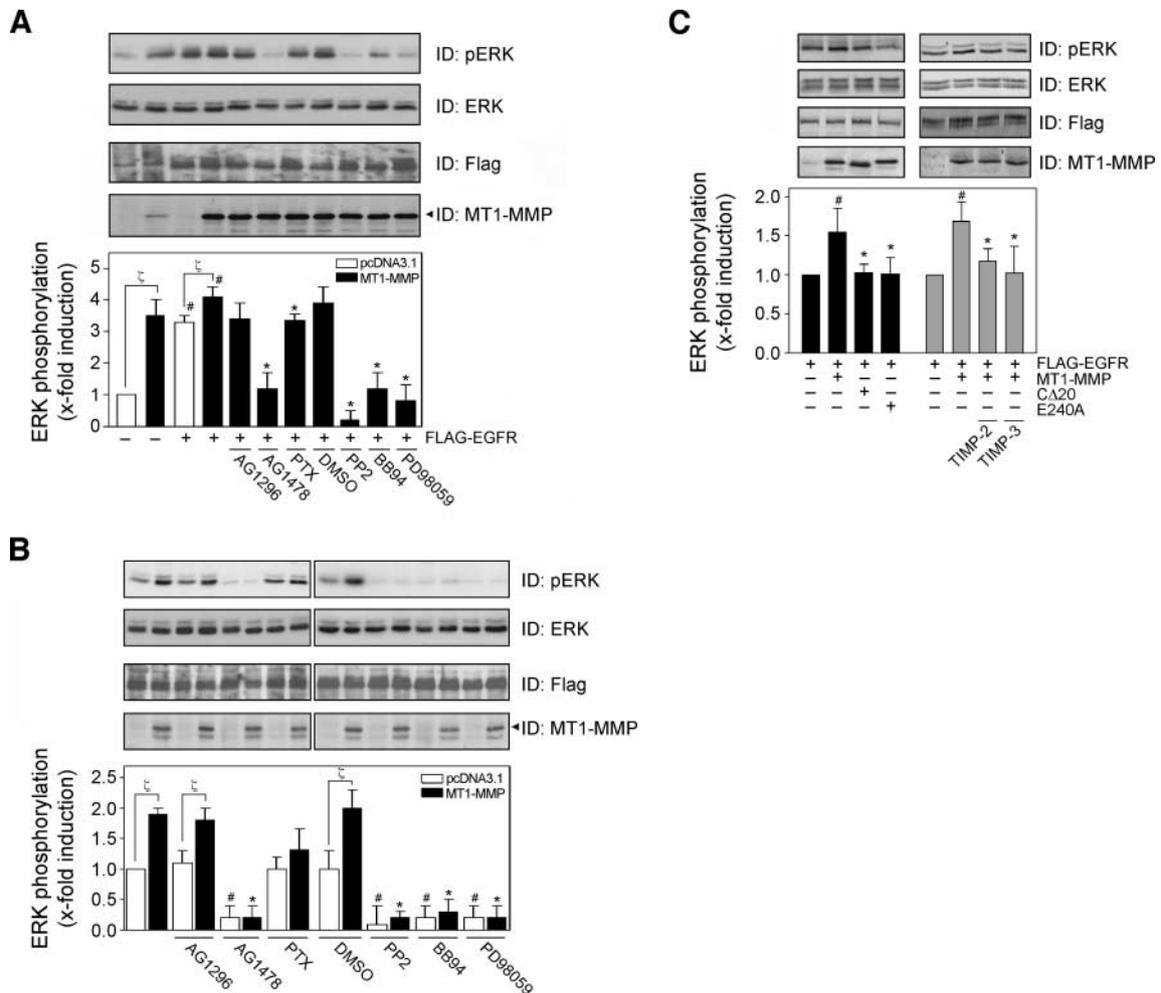

FIGURE 7. EGFR transactivation mediated by MT1-MMP induces ERK activation. **A.** COS-7 cells were cotransfected with the FLAG-EGFR construct along with pcDNA3.1 or with MT1-MMP, harvested by trypsinization, and kept in suspension for 1 h at 37°C. Cells were then plated on fibronectin, pretreated as described in Fig. 5A for 30 min, and then stimulated with 1 μmol/L S1P for 5 min. Cells were lysed, and expression levels of phosphorylated ERK (*pERK*), ERK, FLAG-EGFR, and MT1-MMP were monitored by Western blotting. The extent of ERK phosphorylation was quantified by densitometry and normalized to the expression of ERK. Columns, means *x*-fold induction of control transfected cells (empty vector without FLAG-EGFR) from three independent experiments; bars, SD. #, $P \leq 0.05$ versus their control transfected cells (without FLAG-EGFR); ζ, $P \leq 0.05$ versus FLAG-EGFR–transfected cells; *, $P \leq 0.05$ versus DMSO-treated cells overexpressing MT1-MMP along with FLAG-EGFR. **B.** COS-7 cells were cotransfected with FLAG-EGFR and Gα$_{i2}$ mutant (G$_{i2}$-Q205L) along with either pcDNA3.1 or MT1-MMP construct. Cells were harvested without any prior stimulation with S1P, and the extent of ERK phosphorylation was assessed and quantified as described in (**A**). Columns, means *x*-fold induction of control transfected cells (empty vector) from three independent experiments; bars, SD. #, $P \leq 0.05$ versus DMSO-treated control transfected cells (empty vector); ζ, $P \leq 0.05$ versus MT1-MMP overexpressing cells; *, $P \leq 0.05$ versus DMSO-treated MT1-MMP overexpressing cells. **C.** COS-7 cells were cotransfected with the FLAG-EGFR construct along with pcDNA3.1 or with the various MT1-MMP constructs. Serum-starved transfected cells were stimulated with 1 μmol/L S1P for 5 min and lysed. Where indicated, cells were pretreated with TIMP-2 (50 nmol/L) or TIMP-3 (50 nmol/L) for 30 min before S1P stimulation. The extent of ERK phosphorylation was monitored and quantified as described in (**A**). Columns, means *x*-fold induction of control transfected cells from three independent experiments; bars, SD. #, $P \leq 0.05$ versus control transfected cells; *, $P \leq 0.05$ versus MT1-MMP–transfected cells.

again suggesting that ERK is activated downstream of EGFR transactivation. ERK activation was slightly decreased by pertussis toxin (3.4 ± 0.2-fold, $P \leq 0.05$) but was completely abolished by PP2 (0.2 ± 0.3-fold, $P \leq 0.05$) and by BB94 (1.2 ± 0.5-fold, $P \leq 0.05$; Fig. 7A).

The involvement of EGFR, G_i protein signaling, metalloproteinase, and Src activities in S1P-stimulated MT1-MMP–dependent ERK activation was confirmed using the $G\alpha_{i2}$ mutant (G_{i2}-Q205L). As shown in Fig. 7B, the coexpression of this active mutant along with MT1-MMP and FLAG-EGFR induced a strong activation of ERK without prior S1P stimulation (1.9 ± 0.1-fold, $P \leq 0.05$). This result is in keeping with our data obtained using pertussis toxin, confirming that the MT1-MMP–dependent ERK activation involves activation of G_i protein. Pertussis toxin did not, however, show significant inhibition of ERK phosphorylation in these conditions because the $G\alpha_{i2}$ mutant (G_{i2}-Q205L) is a poor substrate for ADP-ribosylation by pertussis toxin (56). In accordance with our previous results, AG1478 (0.2 ± 0.2-fold, $P \leq 0.05$), PP2 (0.2 ± 0.1-fold, $P \leq 0.05$), and BB94 (0.3 ± 0.2-fold, $P \leq 0.05$) abrogated the MT1-MMP–dependent ERK activation induced by $G\alpha_{i2}$ (Fig. 7B). Taken together, these results suggest that the activation of G_i protein by S1P stimulates an MT1-MMP–mediated transactivation of EGFR, leading to ERK activation and to the subsequent induction of cell migration.

To determine the role of the cytoplasmic domain of MT1-MMP and catalytic in ERK activation process, we used the CΔ20 and E240A mutants of MT1-MMP. In keeping with our previous work (14), the overexpression of these MT1-MMP mutants both abolished the ability of the enzyme to activate ERK in the presence S1P (Fig. 7C). To confirm the results obtained using the E240A MT1-MMP mutant, we treated the cells with TIMP-2 and TIMP-3 before the ERK activation assay. Both TIMP-2 and TIMP-3 inhibited the ability of MT1-MMP to activate ERK in the presence of S1P. These results indicate that although the MT1-MMP–dependent EGFR transactivation occurs through the cytoplasmic domain of the enzyme independently of its catalytic activity, the stimulation of ERK activity and cell migration involve both the cytoplasmic and the catalytic domains of the enzyme.

Discussion

We have previously shown that S1P activates MT1-MMP migratory function through a mechanism involving its receptors $S1P_1$ and $S1P_3$ and activation of G_i protein, and that requires the cytoplasmic domain of MT1-MMP (13). In the present study, we further elucidate the mechanism by which S1P stimulates MT1-MMP–mediated cell migration and show a pivotal role for EGFR activation in this process. First, we showed that MT1-MMP induced the transactivation of EGFR in BAEC and COS-7 cells stimulated with S1P. This transactivation seems to play an essential role in the induction of cell migration by MT1-MMP as the inhibition of EGFR activity using AG1478, or through deleting the major autophosphorylation sites of EGFR (C1022 EGFR mutant), abolished the stimulatory effects of MT1-MMP on cell migration. Taken together, these results suggest that both the kinase activity of EGFR and its autophosphorylation play a role in the induction

of cell migration by MT1-MMP. Next, we showed that the S1P-mediated activation of G_i protein is involved in the stimulation of MT1-MMP–dependent EGFR transactivation and cell migration as these processes were both inhibited by pertussis toxin and, in the absence of S1P, could be reproduced by the overexpression of a constitutively active G_i mutant (13). We then showed that the MT1-MMP–mediated transactivation of EGFR led to the activation of ERK required for the downstream stimulation of cell migration. To our knowledge, this study illustrates a novel role for MT1-MMP in the transactivation of receptor tyrosine kinases mediated by GPCR and the first to show a role for EGFR in MT1-MMP promigratory function.

It has been recently reported that MT1-MMP can modulate signal transduction of another receptor tyrosine kinase, specifically PDGFRβ, in vascular smooth muscle cells (57). In this report, MT1-MMP was involved in the modulation of the direct stimulation of this receptor by its ligand PDGF. The regulation of PDGF-B responsiveness by the MT1-MMP/PDGFRβ signaling axis occurred through a process that involved the catalytic activity of the enzyme but not its cytoplasmic domain (57). This is in contrast to the present study in which we show that MT1-MMP stimulates EGFR transactivation by a mechanism that requires its cytoplasmic domain but not its intrinsic catalytic activity. Our data suggest that rather than acting as a matrix-degrading enzyme, MT1-MMP may function as a signaling protein to trigger EGFR transactivation. The EGFR transactivation mediated by MT1-MMP, however, most likely requires the catalytic activity of another metalloproteinase as indicated by the inhibitory effect of BB94. Because the MT1-MMP–mediated EGFR transactivation was not inhibited by TIMP-2 and TIMP-3, this indicates that the other metalloproteinase involved in this process is insensitive to these TIMPs. Participation of metalloproteinases in EGFR transactivation has been well established and classically involves the shedding of membrane-bound EGFR ligands by members of the ADAM family (32, 33, 46, 48, 49). Indeed, it has been previously suggested that both MMPs and ADAMs are implicated in the cellular invasion stimulated by S1P, and that a close cooperation may exist between these two families of enzymes (58). In addition, it has been reported that MT1-MMP induces the shedding of CD44 mediated by an unidentified ADAM-like protease (59). Thus, it is tempting to speculate that following stimulation with S1P, the cytoplasmic domain of MT1-MMP induces the activation of an ADAM-like protease, leading to the shedding of EGF-like ligands and the subsequent activation of the EGFR.

Given the promiscuous involvement of Src in the EGFR transactivation induced by GPCR ligands (27), and as we presently describe a novel role for MT1-MMP in EGFR transactivation, we explored the possibility that Src may be involved in this process as well. Indeed, we now show that Src plays a critical role in the S1P-stimulated MT1-MMP–dependent EGFR transactivation and cell migration. It has been reported that G protein α subunits, such as $G\alpha_i$, directly stimulate Src activity (60), which can lead to MMP-dependent release of EGFR ligands (61). For example, Src activation has been shown to regulate the ADAM-dependent shedding of the L1 adhesion molecule (62). Moreover, several ADAMs have proline-rich Src-homology (SH3) binding domains in their

cytoplasmic tails, which can allow interaction with Src (44). Thus, the unidentified metalloproteinase involved in the MT1-MMP–mediated EGFR transactivation may potentially interact with Src through SH3 domains. Furthermore, once EGFR is stimulated, its autophosphorylated residues can serve as ligands for Src, leading to its activation and further increased phosphorylation of the EGFR (63). Although the function of Src in MT1-MMP–dependent EGFR transactivation is likely to be considerably more complex, we provide evidence that its role involves the phosphorylation of EGFR on the Tyr[845] residue. In addition to its kinase activity and its autophosphorylation, the phosphorylation of EGFR on Tyr[845] by Src is required for the stimulation of cell migration by MT1-MMP in the presence of S1P. A close relationship between Src and MMP in EGFR transactivation has been previously suggested by the observation that the broad-spectrum MMP inhibitor GM6001 prevented EGFR phosphorylation at both Tyr[1068] and Tyr[845] residues (36). Because the phosphorylation of Tyr[845] on

the EGFR by Src is required for the induction of tumorigenesis by the receptor (64), this mechanism might contribute significantly to the migratory and invasive processes induced by MT1-MMP.

Based on these results, we propose a novel mechanism (Fig. 8) responsible for the stimulatory effect of MT1-MMP on cell migration induced by GPCR ligands, such as S1P. Stimulation of S1P receptors (S1P$_1$ and S1P$_3$; ref. 13) induces activation of the G protein α_i subunit with subsequent activation of Src (63). Activated Src would then stimulate MT1-MMP activity, either directly or through the tyrosine phosphorylation of caveolin-1, which allows the formation of an MT1-MMP-caveolin-1 complex (17). Through a process that involves its cytoplasmic domain, MT1-MMP would stimulate the activation of an unidentified TIMP-2– and TIMP-3– insensitive membrane-associated MMP or ADAM. The subsequent shedding of an endogenous EGFR ligand by this MMP would result in EGFR activation and tyrosine phosphorylation.
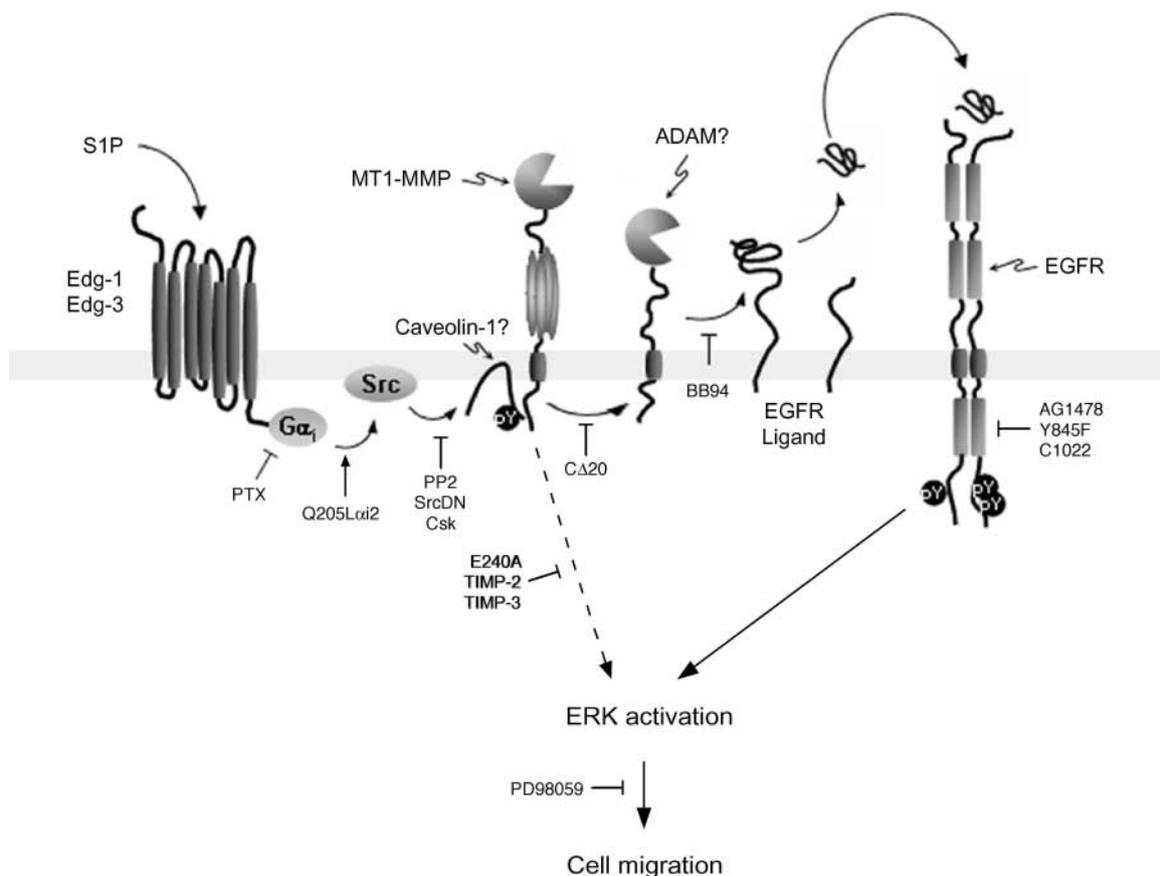

FIGURE 8. Schematic model of the MT1-MMP–dependent stimulation of cell migration through EGFR transactivation. We have previously shown that S1P activates MT1-MMP migratory function through a mechanism involving its receptors S1P$_1$ and S1P$_3$ and activation of G$_i$ protein, and that requires the cytoplasmic domain of MT1-MMP (13). Indeed, stimulation of S1P receptors induces the activation of the G protein α_i subunit with subsequent activation of Src (59). Activated Src would then stimulate MT1-MMP activity, either directly or through the tyrosine phosphorylation of caveolin-1, which allows the formation of an MT1-MMP-caveolin-1 complex (17). Through a process that involves its cytoplasmic domain, MT1-MMP stimulates the activation of an unidentified membrane-associated MMP or ADAM. The subsequent shedding of an endogenous EGFR ligand by this MMP results in EGFR activation and tyrosine phosphorylation. This process also involves Src activity as it is reduced by treatment with PP2, Csk, or by mutation of the Y845 of the receptor. The resulting EGFR activation leads to activation of ERK and to the induction of cell migration by MT1-MMP. In addition to this EGFR-dependent signaling, the MT1-MMP–mediated activation of ERK leading to stimulation of cell migration also involves an EGFR-independent pathway that requires the catalytic activity of the enzyme (*dashed arrow*). The various inhibitors or DNA constructs used to identify the signaling molecules involved in the cooperation among S1P, MT1-MMP, and EGFR leading to cell migration.

This process involves Src activity as it is reduced by treatment with PP2 and Csk, or by mutation of the Y845 of the receptor. The resulting EGFR activation leads to activation of ERK and to the induction of cell migration by MT1-MMP. The MT1-MMP–dependent activation of ERK leading to stimulation of cell migration involves an EGFR-dependent pathway that requires the cytoplasmic domain of the enzyme as well as an EGFR-independent pathway that involves the catalytic activity of MT1-MMP, most likely reflecting a requirement for cleavage of matrix or cell surface proteins, or molecules involved in cell-matrix interactions in these processes.

The overexpression of MT1-MMP, as well as EGFR, has been associated with tumor growth advantage and is a poor prognostic indicator in multiple tumor types (1, 65). EGFR has been shown to be expressed in tumor endothelial cells, while undetectable in their normal counterparts, and plays a role in their proliferative phenotype (66). In addition, MT1-MMP is also expressed in angiogenic endothelial cells and is well known to be an important regulator of angiogenesis (3, 11-13, 67). A relationship between MT1-MMP and EGFR has been suggested in various studies. For example, EGFR is a major stimulus regulating MT1-MMP expression, as EGFR$^{-/-}$ mice show markedly decreased MT1-MMP expression (68). In addition, EGFR mediates the invasiveness of gliomas by inducing MT1-MMP (69) More recently, MT1-MMP–dependent proteolysis of the laminin-5 γ_2 chain was shown to result in the formation of EGF ligands (70). As EGFR signaling is linked to diverse biological processes in human cancer cells, the identification of EGFR as a key component in the induction of MT1-MMP promigratory function implies a unique cooperation between pericellular proteolysis and intracellular signaling culminating in enhanced tumor growth and angiogenesis. MT1-MMP plays important roles in tumor progression and thus represents an interesting target for anticancer therapy. Broad-spectrum inhibitors of MMP catalytic activity did, however, lead to musculosketal side effects in some patients, which could involve MT1-MMP, based on the skeletal effects seen in the MT1-MMP$^{-/-}$ mice (67). Taken together, our results suggest that the inhibition of EGFR may instead represent a novel therapeutic target to inhibit MT1-MMP–dependent processes associated with tumor cell invasion and angiogenesis.

Materials and Methods

Materials

Cell culture media were obtained from Life Technologies. Matrigel was from BD Biosciences, and serum was from Hyclone Laboratories. Transwell migration chambers were purchased from Costar. S1P, pertussis toxin, TRITC-phalloidin, and anti-FLAG M2 antibody were from Sigma. EGF was from BD Biosciences Discovery Labware. Fibronectin, FUGENE-6 Transfection Reagent, and WST-1 were obtained from Roche, and Polyfect transfection reagent was from Qiagen. AG1478, AG1296, and Y27632 were from Calbiochem. PP2 and PD98059 were obtained from Biomol. TIMP-2 and TIMP-3 have been generated as previously described (71, 72). Electrophoresis products were purchased from Bio-Rad. The anti-MT1-MMP polyclonal antibodies AB815 were from

Chemicon. Antibodies against phosphorylated ERK (Thr^{202}/Tyr^{204}) and ERK were purchased from Cell Signaling Technology, and antibodies against Csk were from BD Transduction Laboratories. Antibodies against Src and against EGFR were from Upstate Biotechnology, Inc., and monoclonal antibody against phosphotyrosine (PY99) was from Santa Cruz Biotechnology. Anti-mouse and anti-rabbit IgG horseradish peroxidase–conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, and enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech.

Cell Culture

BAEC (Clonetics) and COS-7 cells (American Type Culture Collection) were cultured under an air/CO$_2$ (19:1) atmosphere. Cells were grown in DMEM supplemented with 10% (v/v) bovine calf serum, 4 mmol/L glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin and were used up to passage 12.

Plasmids and Transfection Methods

The cDNAs encoding the MT1-MMP and the Gα_{i2} protein constructs were previously described (13, 73). A cDNA encoding the human FLAG epitope-tagged EGFR was kindly provided by Dr. Y. Daaka (North Carolina University). The C1022 EGFR mutant (74) was constructed using standard PCR procedures. The Y845F mutation was generated using the QuikChange II site-directed mutagenesis kit (Stratagene). Each mutant was sequenced to verify the presence of the desired mutation. A plasmid encoding COOH-terminal Src kinase (Csk) was kindly provided by Dr. H. Hanafusa (Osaka Bioscience Institute, Japan), and the Csk coding sequence was subcloned at the EcoRI site of pcDNA3.1. The plasmids encoding the wild-type and constitutively active Gα_{i2} proteins were recently described (13, 17).

Transient transfection of plasmids in subconfluent BAE and COS-7 cells were done using the FUGENE-6 or Polyfect transfection reagents, as previously described (13).

Morphogenic Differentiation Assays

Transfected BAEC (2.5 × 10^4) were added atop the Matrigel in serum-free media; pretreated for 30 min with AG1478 (0.5 μmol/L), AG1296 (1 μmol/L), PP2 (10 μmol/L), or PD98059 (10 μmol/L); and incubated at 37°C in 5% CO$_2$/95% for 6 h in the absence or in the presence of 1 μmol/L S1P. Capillary-like structures formed by BAEC were examined microscopically after 6 h, and the extent to which these structures formed in the gel was quantified as previously described (13).

Cell Migration Assays

Migration assays of transfected BAE and COS-7 cells were done on Transwell precoated with 0.15% gelatin and 10 μg/mL fibronectin, respectively. The Transwell was assembled in 24-well plates, and the lower chambers were filled with serum-free media with or without 1 μmol/L S1P or 30 ng/mL EGF. Transfected cells were harvested, resuspended in 100 μL of fresh DMEM media at 2.5 × 10^5 per milliliter (BAEC) or at 5 × 10^5 per milliliter (COS-7), and inoculated into the upper chamber of each Transwell. For experiments using pharmacologic and

physiologic inhibitors, cells were pretreated for 30 min as described above. The plates were then placed at 37°C in 5% CO$_2$/95% air for 3 h, and the migration was quantified as previously described (13).

Fluorescence Microscopy

BAEC were transfected with pIRES2-GFP-MT1-MMP or pIRES2-GFP as described above. Transfected BAEC were seeded on glass coverslips (2.0 × 10^4) coated with 0.15% gelatin and allowed to adhere for 3 h. Cells were then pretreated for 30 min with AG1478 (0.5 μmol/L), BB94 (5 μmol/L), Y27632 (20 μmol/L), or DMSO (Ctl) and stimulated with 1 μmol/L S1P for 5 min. Cells were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. Coverslips were washed twice with PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min, washed again in PBS, and then incubated with TRITC-phalloidin for 30 min. Samples were examined in a Zeiss Axiovert S100 microscope with a ×63 oil immersion objective, and images were recorded using a Retiga 1300 camera.

Analysis of EGFR Transactivation

For monitoring S1P-induced transactivation of endogenous EGFR in BAEC, cells grown on 0.15% gelatin were transfected with MT1-MMP, serum starved for 18 h, and stimulated with 1 μmol/L S1P. In COS-7 cells, the analysis of EGFR transactivation was mainly done by overexpressing wild-type or mutated versions of a FLAG epitope-tagged EGFR construct and Csk. COS-7 cells grown on 10 μg/mL fibronectin were transfected, serum starved for 18 h, and incubated for increasing periods of time with 1 μmol/L S1P or 30 ng/mL EGF. In experiments using plasmids encoding Gα$_{i2}$ proteins, transfected COS-7 cells were not stimulated by either S1P or EGF before cell lysis. For experiments using pharmacologic inhibitors, cells were pretreated for 30 min as described above.

After stimulation, cells were immediately rinsed with PBS containing 1 mmol/L sodium orthovanadate and 1 mmol/L sodium fluoride and then lysed for 10 min on ice in lysis buffer [150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, 0.5% NP40, 1% Triton X-100, 60 mmol/L *n*-octylglucoside, 1 mmol/L sodium orthovanadate, and 1 mmol/L sodium fluoride]. Lysates were clarified by centrifugation at 8,000 × *g* for 20 min at 4°C. The extent of EGFR phosphorylation was monitored by immunoprecipitation, essentially as described (17). Precleared supernatants (500 μg of proteins) were incubated with EGFR or FLAG M2 antibodies overnight at 4°C with constant rocking. Protein G-Sepharose beads were then added to the immune complexes for 2 h at 4°C. Immunoprecipitates were washed thrice with lysis buffer; beads were resuspended in 2× Laemmli buffer and boiled for 5 min. For analysis of MT1-MMP expression in BAEC, isolated crude membrane fractions were prepared as described previously (75). Proteins from cell lysates, membrane fractions, and immune complexes were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The nonspecific binding sites in the membranes were saturated with TBS/Tween 20 containing 2% bovine serum albumin. The membranes were incubated with primary antibodies for 1 h, and the immuno-reactive bands were revealed after incubation with horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibodies. The signals were visualized using enhanced chemiluminescence detection system. Immunoblots were quantified using the software Image densitometric analysis IP Lab gel program from Scanalytics, Inc.

ERK Activation

Transfected COS-7 cells were harvested by trypsinization and kept in suspension in serum-free media for 1 h at 37°C. The cells were then plated on fibronectin (10 μg/mL) for 1 h at 37°C and stimulated with 1 μmol/L S1P for 5 min. In the experiments using pharmacologic inhibitors, cells were pre-treated for 30 min as described above. In experiments using the plasmids encoding for wild-type and constitutively active Gα$_{i2}$ proteins, transfected COS-7 cells were not stimulated by S1P or EGF before cell lysis. Cells lysates obtained were subjected to gel electrophoresis. The extent of ERK activation was monitored using phospho-specific anti-ERK antibodies, whereas total ERK was determined by reprobing using a monoclonal ERK antibody. Immunoblots were quantified as described above.

Statistical Analysis

All experiments were repeated at least thrice. Results are expressed as mean ± SD. Statistical analyses were done using one-way ANOVA followed by Bonferroni multiple comparison test. Statistical significance was accepted at $P \leq 0.05$.

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