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Activation of the extracellular signal-regulated protein kinase (ERK) cascade by membrane-type-1 matrix metalloproteinase (MT1-MMP)

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Abstract The mechanisms underlying membrane-type-1 matrix metalloproteinase (MT1-MMP)-dependent induction of cell migration were investigated. Overexpression of MT1-MMP induced a marked increase in cell migration, this increase being dependent on the presence of the cytoplasmic domain of the protein. MT1-MMP-dependent migration was inhibited by a mitogen-activated protein kinase kinase 1 inhibitor, suggesting the involvement of the extracellular signal-regulated protein kinase (ERK) cascade in the induction of migration. Accordingly, MT1-MMP overexpression induced the activation of ERK, this process being also dependent on the presence of its cytoplasmic domain. MT1-MMP-induced activation of both migration and ERK required the catalytic activity of the enzyme as well as attachment of the cells to matrix proteins. The MT1-MMP-dependent activation of ERK was correlated with the activation of transcription through the serum response element, whereas other promoters were unaffected. Taken together, these results indicate that MT1-MMP trigger important changes in cellular signal transduction events, leading to cell migration and to gene transcription, and that these signals possibly originate from the cytoplasmic domain of the protein.

Key words: Membrane-type-1 matrix metalloproteinase; Migration; Extracellular signal-regulated protein kinase; Signal transduction

1. Introduction

Cell migration represents an essential process for a variety of biological events, including embryonic development, inflammatory responses, angiogenesis and tumor metastasis [1]. In this latter case, it is well established that cell invasion requires remodeling of the extracellular matrix through activation of both serine proteases and matrix metalloproteinases (MMPs) [2]. The localization of these proteases at the cellular surface appears to be a general strategy essential for the focusing of the degradative processes underlying tumor invasion [3]. While this association can occur through binding to specific receptors or integrins [3,4], the identification of a subfamily of MMP, the membrane-type MMPs (MT-MMP), that are intrinsically anchored to the cell membrane, has driven considerable interest [5]. MT-MMPs are unique among the members of the MMP family in that they contain a transmembrane domain that allows their localization to the cell surface as well as a short cytoplasmic tail of still unknown function. Six members of this family have been described to date, MT1-MMP being the most studied so far (reviewed in [6,7]). Expression of MT1-MMP at the cell surface promotes the activation of proMMP-2, leading to the hypothesis that the function of MT1-MMP is to regulate the activity of MMP-2 and the resulting tumor invasion [6,7]. There is however increasing genetic and biochemical evidences that MT1-MMP plays additional roles in both normal cell function as well as in tumor cell migration and invasion. Gene disruption of MT1-MMP results in severe abnormalities in bone formation, angiogenesis and collagen turnover, leading to the development of dwarfism, arthritis and premature death [8,9]. Since MMP-2 null mice do not show these defects [10], these observations suggest that MT1-MMP has other biological functions in addition to its role in proMMP-2 activation. Such a role was exemplified by studies showing an essential role for MT1-MMP in fibrinolysis [11]. More recently, overexpression of MT1-MMP was shown to potentiate invasion of collagen basement membranes by epithelial cells [12] as well as the migratory potential of glioma [13], melanoma [14], breast [15] and colon cancer cells [16].

We have recently shown that MT1-MMP was predominantly localized to caveolae [17], small plasma membrane domains that contain a very high proportion of signal-transducing proteins, suggesting that MT1-MMP-stimulated cellular processes may involve the activation of intracellular signaling events. Indeed, the present report presents evidence that MT1-MMP-dependent cell migration is correlated with an induction of the extracellular signal-regulated protein kinase (ERK) cascade, leading to an increase in gene transcription under the control of the serum response element (SRE). These results strengthen the hypothesis that the role of MT1-MMP in tumor progression is not strictly restrained to its classical role in activation of proMMP-2 but also to the induction of intracellular signaling pathways.

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Abbreviations: ERK, extracellular signal-regulated protein kinase; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase

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2. Materials and methods

2.1. Materials

COS-7 cells were cultured under an air:CO_2 (19:1) atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 4 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The conditioned media (CM) from U87 glioma cells, containing proMMP-2, was prepared as described [18]. Transwells (8-μm pore size) used for cell migration were from Costar. All products for electrophoresis and zymography were purchased from Bio-Rad. The various inhibitors used in the study were from Biomol. Human recombinant tissue inhibitor of MMP1 (TIMP-1) and TIMP-2 were kindly provided by R. Fridman (Detroit, MI, USA). The anti-MT1-MMP polyclonal antibodies (raised against the hinge region) were from Chemicon. Antibodies against phospho-ERK (Thr202/Tyr204) and ERK were purchased from Cell Signaling Technology, New England Biolabs. Horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG were obtained from Jackson Immunoresearch Laboratories.

2.2. Cloning and transfection of MT1-MMP constructs

The cDNA encoding full-length human MT1-MMP was generated as previously described [17,18]. Transmembrane domain-deleted as well as cytoplasmic deletion mutants of MT1-MMP were generated by polymerase chain reaction (PCR), using wild-type MT1-MMP as the template and anti-sense primers annealing to the desired sequences. Three different cytoplasmic mutants were synthesized: MT1-MMP(CΔ20), in which the entire cytoplasmic domain is deleted, MT1-MMP(CΔ16), containing the sequence RRHV and MT1-MMP(CΔ10), containing the sequence RRHGTPRRLV. The C-terminal valine residue was included in the PCR primers in order to allow trafficking of the MT1-MMP mutants to the plasma membrane [19]. The catalytically inactive mutant MT1-MMP(E240A) was generated by PCR, using the overlap extension method [20]. The PCR products were cloned into pcDNA3.1 TOPO (Invitrogen) and the identity of all constructs was confirmed by DNA sequencing. The transient transfection of the plasmids in subconfluent COS-7 cells was performed using the non-liposomal formulation FUGENE-6 transfection reagent (Roche Molecular Biochemicals). The expression levels of the various MT1-MMP constructs were monitored by immunoblotting and activation of proMMP-2 to its active form by COS-7 cells transfected with the various MT1-MMP cDNAs was monitored by gelatin zymography [17,18].

2.3. Cell migration assays

COS-7 cell migration was performed using transwells (Costar; 8-μm pore size) precoated with 0.5% gelatin/phosphate-buffered saline, as described [17]. The transwells were assembled in 24-well plates (Falcon 3097) and the lower chambers filled with 600 μl of DMEM supplemented with 10% fetal calf serum. For the study of the effect of MT1-MMP on cell migration, cells were transfected with the various MT1-MMP constructs and allowed to recover for 48 h. Cells were harvested by trypsinization, spun down, resuspended in 100 μl fresh DMEM media at 7.5×10^5 cells/ml, and inoculated into the upper chamber of each transwell. For inhibition studies, cells were preincubated for 2 h at 37°C with the inhibitors, spun down to remove the inhibitor from the media, and resuspended to the same cell density. The plates were then placed at 37°C in 5% CO_2/95% air for 2 h. Cells that had migrated to the lower surface of the filters were fixed, stained with 0.1% crystal violet/20% MeOH, and counted. Data are presented as the average number of migrated cells per five fields (×100).

2.4. Activation of ERK by MT1-MMP

COS-7 cells were transfected with either empty vector (pcDNA3.1), wild-type or mutated versions of MT1-MMP as described above and the cells were allowed to recover for 48 h. Cells were then trypsinized, washed and incubated with gentle agitation for 1 h in the presence of 0.1% bovine serum albumin (BSA). The resulting cells were then either kept in suspension or plated on dishes coated with gelatin or poly-L-lysine and incubated for an additional 2 h at 37°C.

For the preparation of whole cell lysates, cells were resuspended in a buffer containing 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 100 μM sodium vanadate, 10 mM NaF and 20 mM Tris–HCl (pH 7.4). The mixture was incubated on ice for 30 min and insoluble matter was removed by centrifugation. Equal amounts of proteins from control and treated cells were resuspended in sample buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 9% acrylamide gels. After electrophoresis, proteins were electrotransferred to a 0.45-μm pore size polyvinylidene difluoride membrane using a Milliblot graphite electroblotter (Millipore), blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 and incubated with the anti-phospho-specific ERK (Thr202/Tyr204) antibodies. Blots were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1/10 000 dilution) and immunoreactive material was visualized by enhanced chemiluminescence.

2.5. Gene reporter assays

The Mercury Pathway Profiling System, in which a secreted form of alkaline phosphatase (SEAP) is fused to promoters activated by different responsive elements, was used. Cells were transfected with the various constructs and aliquots of the CM were collected at different times. SEAP activity was measured by the hydrolysis of *p*-nitrophenyl phosphate (pNPP), as described by the manufacturer (Clontech).

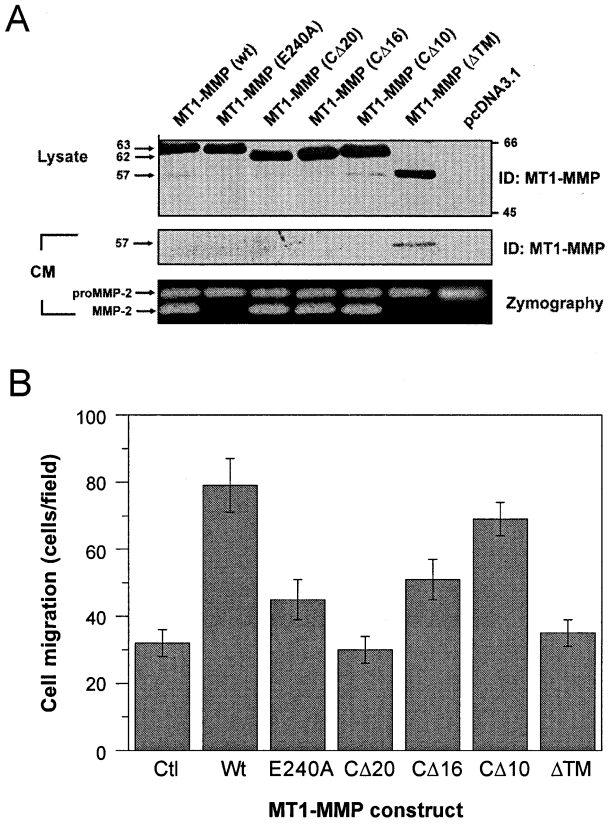

Fig. 1. The cytoplasmic domain of MT1-MMP is important for cell migration but not for proMMP-2 activation. A: COS-7 cells were transfected as described above and incubated for 24 h in the presence of an exogenous source of proMMP-2 (conditioned medium (CM) from U87 cells [17]). ProMMP-2 activation was monitored by gelatin zymography (lower panel). The expression levels of the various MT1-MMP constructs were monitored by Western blotting using anti-MT1-MMP polyclonal antibodies. B: COS-7 cells were transfected with either empty vector (Ctl) or with the various MT1-MMP constructs and allowed to recover for 48 h. Transfected cells were harvested, seeded in Boyden chambers coated with gelatin and incubated for 2 h. Cells that have migrated were visualized by staining and microscopy. Five different microscopic fields were counted for all conditions. Results are the means ± S.D. of six distinct experiments performed in triplicate.

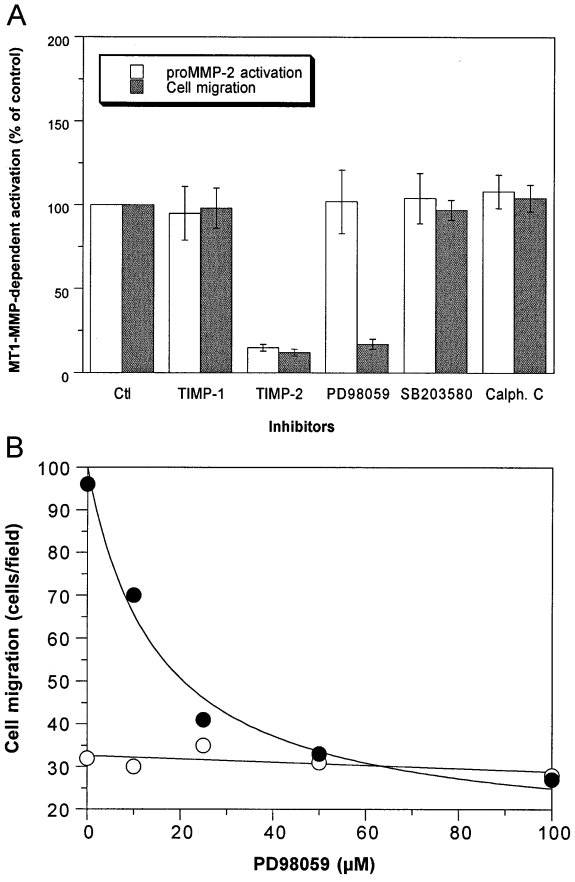

Fig. 2. MT1-MMP-dependent cell migration is inhibited by TIMP-2 and a MEK1 inhibitor. A: Effect of inhibitors on proMMP-2 activation and cell migration. COS-7 cells transfected with wild-type MT1-MMP were incubated for 24 h in the presence of an exogenous proMMP-2 in the absence (Ctl) or presence of the indicated inhibitors. These include TIMP-1, TIMP-2 (100 ng/ml each), PD98059 (50 μM), SB203580 (10 μM), and calphostin C (100 nM). ProMMP-2 activation is expressed as the percentage of MMP-2 in the active form, as determined by laser densitometry of the zymograms. Results are the means ± S.D. of three experiments. For cell migration, transfected COS-7 cells were preincubated for 1 h with the indicated inhibitors, harvested and seeded in Boyden chambers. The extent of migration was assessed as described in Fig. 1, following a 2 h incubation. The means ± S.D. of three experiments performed in triplicate are presented. B: Effect of PD98059 on basal and MT1-MMP-dependent migration. Mock- (open circles) or MT1-MMP-transfected cells (filled circles) were preincubated with various concentrations of PD98059 and subjected to migration assays. The means of two experiments performed in triplicate are presented.

3. Results

3.1. Overexpression of MT1-MMP induces cell migration

MT1-MMP has recently been shown to play an important role in the migration of various cell types [12–17]. In order to determine the mechanisms involved in MT1-MMP-dependent migration of COS-7 cells, we first compared the ability of various overexpressed MT1-MMP constructs to induce proMMP-2 activation as well as cell migration. COS-7 cells were transiently transfected with wild-type MT1-MMP, the catalytically inactive mutant E240A, or with three distinct cytoplasmic domain-truncated versions of the enzyme: MT1-MMP(CΔ20), in which the entire cytoplasmic domain is deleted, MT1-MMP(CΔ16), containing the sequence RRHV and MT1-MMP(CΔ10), containing the sequence RRHGTPRRLV. The extent of proMMP-2 activation was monitored by gelatin zymography, whereas cell migration was assessed by a modified Boyden chamber assay. As shown in Fig. 1A, overexpression of native as well as of the truncated mutants of MT1-MMP all induced a marked activation of an exogenous source

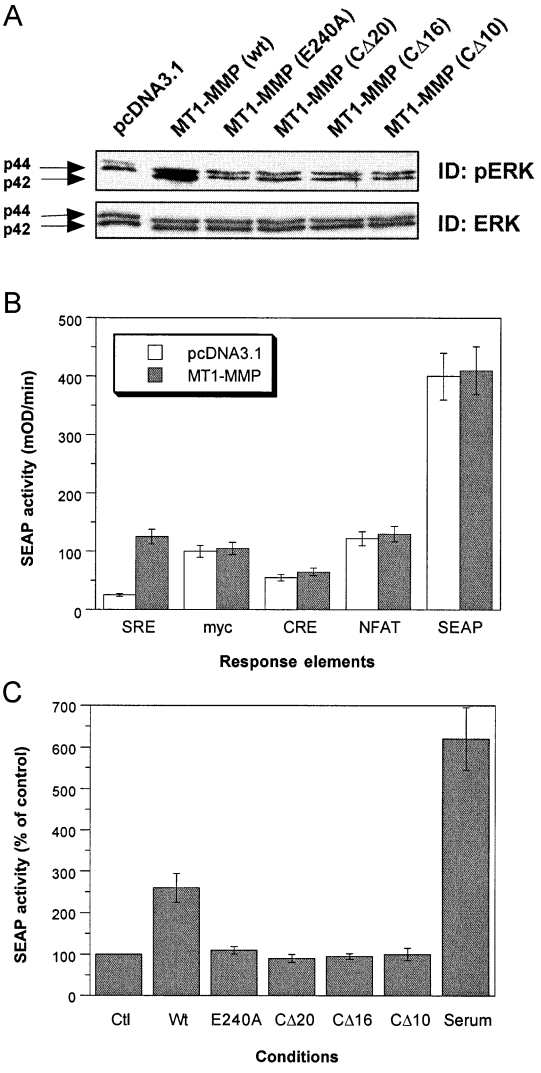

Fig. 3. MT1-MMP promote activation of the ERK cascade and of gene transcription. A: Cells were transfected with the various MT1-MMP constructs and allowed to recover for 48 h. Cells were harvested in lysis buffer and 20 μg of proteins were loaded on a 9% polyacrylamide gel. The activation of ERK was visualized using anti-phospho-specific-ERK antibodies (upper panel) whereas total ERK was detected using a monoclonal ERK antibody. A representative experiment is shown. B: Cells were co-transfected with plasmids containing the indicated response elements and with either empty vector (pcDNA3.1) or wild-type MT1-MMP and cells were allowed to recover for 48 h in serum-free medium. Aliquots of the resulting conditioned medium were used to measure alkaline phosphatase activity, using pNPP as the substrate. SEAP indicates a control vector in which SEAP is constitutively secreted by the cells. C: Cells were co-transfected with the indicated MT1-MMP constructs and with the SRE-SEAP plasmid and cells were allowed to recover for 48 h in serum-free medium. The extent of gene transcription driven by the SRE was monitored as described above. Transcription driven by the presence of 5% serum is presented to indicate the maximal transcription achieved under these conditions. Results are means ± S.D. of five distinct experiments.

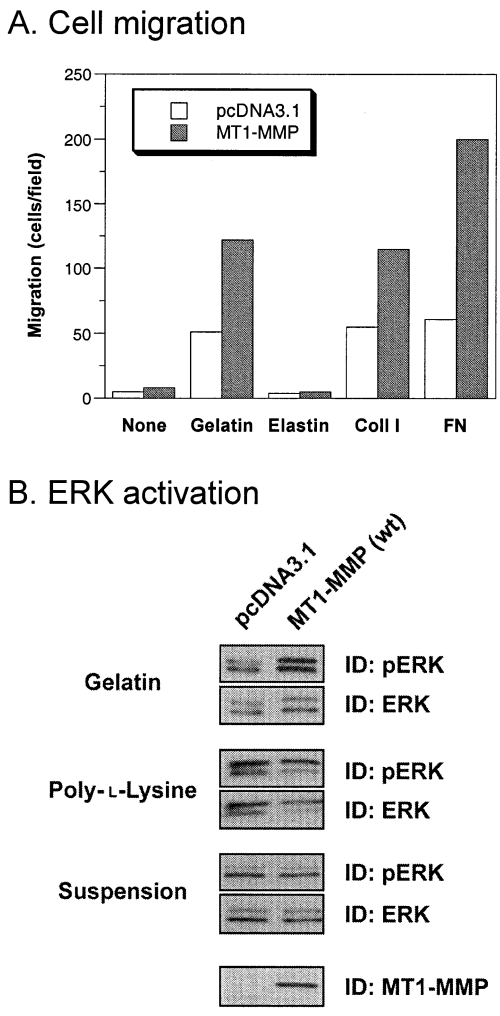

Fig. 4. MT1-MMP-induced activation of migration and of ERK requires cell adhesion. A: COS-7 cells were transfected with either pcDNA3.1 or wild-type MT1-MMP and cells were allowed to recover under serum-free conditions for 24 h. Cells were harvested and seeded into Boyden chambers that had been coated with the indicated matrix proteins. The extent of cell migration was assessed as described. A representative experiment is shown. B: Cells were transfected with either pcDNA3.1 or with the wild-type, E240A and CΔ20 versions of MT1-MMP. After recovery, cells were either kept in suspension by 1 h gentle rocking in serum-free medium containing 0.1% BSA or plated on dishes coated with either poly-L-lysine or gelatin. After a 2-h incubation, cells were collected, lysed and subjected to electrophoresis and immunoblotting with pERK and ERK antibodies. A representative experiment is shown.

of proMMP-2. As expected, this activation was however not observed in cells expressing the catalytically inactive E240A mutant. Truncation of the transmembrane domain of the protein resulted in the secretion of MT1-MMP in the CM with a concomitant loss of its ability to activate proMMP-2, as reported previously [21].

The effects of some of these mutants on cell migration were however very distinct. As reported in a number of recent reports [12,14,15,17], overexpression of full-length MT1-MMP promoted a marked increase in the migratory potential of COS-7 cells (Fig. 1B). However, MT1-MMP-dependent migration was significantly reduced in the CΔ16 mutant and was completely abolished in the CΔ20 mutant in which the cytoplasmic domain is completely removed (Fig. 1B). Similar results were obtained when these different constructs were transfected into bovine aortic endothelial cells (not shown). These results suggest that MT1-MMP-dependent activation of proMMP-2 is not sufficient to trigger cell migration, this process also requiring the presence of the cytoplasmic domain of the protein.

3.2. *Pharmacological inhibition of MT1-MMP-induced migration*

We next investigated whether inhibition of MT1-MMP-dependent proMMP-2 activation was correlated with the inhibition of MT1-MMP-dependent cell migration. Transfected COS-7 cells were preincubated with a number of pharmacological inhibitors specific to various signaling pathways and the extent of proMMP-2 activation or cell migration was monitored. As shown in Fig. 2A, addition of recombinant TIMP-2 to the transfected cells markedly reduced proMMP-2 activation, whereas TIMP-1 was ineffective to inhibit this process. TIMP-2 also strongly inhibited MT1-MMP-dependent migration (Fig. 2A). In addition, preincubation of the cells with PD98059, a well-characterized MEK1 inhibitor, strongly inhibited MT1-MMP-dependent migration, while this compound had no effect on proMMP-2 activation. Inhibition of protein kinase C by calphostin C, or of the stress-activated protein kinase p38 by SB203580 were without effect on both processes, as were inhibition of protein kinases A and G (results not shown).

Interestingly, inhibition by PD98059 was specific to MT1-MMP-driven migration since this compound had negligible effect on basal, MT1-MMP-independent migration of COS-7 cells (Fig. 2B). This suggests that MT1-MMP-dependent migration involves a specific activation of MEK1.

3.3. *MT1-MMP induces activation of ERK and of gene transcription.*

The inhibitory effect of PD98059 on MT1-MMP-dependent cell migration led us to examine the potential activation of the ERK pathway by MT1-MMP, as well as the role of the cytoplasmic domain of the protein in this process. As shown in Fig. 3A, overexpression of full-length MT1-MMP in COS-7 cells induced a marked increase in the amounts of active, dually phosphorylated (Thr202/Tyr204) p44/p42 ERK in whole cell lysates, as monitored with phospho-specific ERK antibodies. This increase appears specific for ERK since MT1-MMP overexpression had no effect on p38 (data not shown). This increase in pERK levels by wild-type MT1-MMP was not observed upon overexpression of the catalytically inactive E240A mutant or of the cytoplasmic-deleted versions of MT1-MMP (Fig. 3A). This suggests that both the catalytic activity and the cytoplasmic domain of MT1-MMP are important for the induction of ERK.

In parallel experiments, we examined whether this increase in ERK activation could be correlated with the activation of gene transcription, a well-characterized intracellular consequence of ERK activation. Cells were co-transfected with wild-type MT1-MMP in the absence (pcDNA3.1) or in the presence of plasmids containing different response elements fused to SEAP. In this gene reporter assay, the activity of the response elements can be easily monitored by measuring SEAP activity in the culture medium. Under these conditions, we observed that co-transfection of full-length MT1-MMP with these constructs induced significant activation of the

SRE (Fig. 3B), whereas it had no detectable effects on the activity of other elements or on the control SEAP plasmid. This induction of SRE activity required the presence of the entire cytoplasmic domain of the protein since it was not observed in any of the three deletion mutants (Fig. 3C). In addition, the E240A mutant did not promote gene transcription, suggesting the requirement of the catalytic activity of the enzyme for the induction of these events by MT1-MMP.

The participation of serum- and matrix-derived factors to the induction of cell migration and ERK activation by MT1-MMP was also investigated. Under our experimental conditions, the induction of cell migration by MT1-MMP required the presence of serum (not shown). In addition, the stimulation of migration required the attachment of the cells to matrix proteins, MT1-MMP-dependent migration being maximal on fibronectin-, gelatin- and collagen-coated dishes (Fig. 4A). This behavior was not related to an increased adhesion of transfected cells to the matrix proteins, as determined in parallel cell attachment assays (results not shown). A similar requirement for matrix proteins was also observed for the induction of ERK. MT1-MMP-transfected cells kept in suspension were unable to activate ERK, whereas plating of the cells on gelatin-coated dishes induced a marked increase in ERK activation compared to the mock-transfected cells (Fig. 4B). Non-specific and integrin-independent attachment of the MT1-MMP-transfected cells to poly-L-lysine had no effect on ERK activation. These results imply that MT1-MMP co-operates with both serum- and matrix-derived signals to induce migration and activation of ERK in cells.

4. Discussion

Cell migration is an highly co-ordinated process involving the interplay of a variety of signaling pathways [1]. Among these, activation of the ERK cascade has been shown to play a critical role in the signaling events leading to cell migration [22–24]. These considerations prompted us to examine the involvement of this signaling pathway in cell migration induced by MT1-MMP.

Under our experimental conditions, activation of ERK is apparently essential for MT1-MMP-promoted cell migration since the mitogen-activated protein (MAP) kinase kinase (MEK) inhibitor, PD98059, completely blocked the effect of overexpressed MT1-MMP without affecting the basal level of migration. The catalytic activity of MT1-MMP appears important for the ability of the protein to induce both cell migration and activation of ERK since these processes were markedly reduced by addition of exogenous TIMP-2, but not TIMP-1, as well as by a point mutation within the catalytic domain of the enzyme. However, our results also show that the catalytic activity of MT1-MMP is not sufficient per se for the effects reported here since cytoplasmic domain-deleted mutants still retained the ability to activate proMMP-2 but failed to induce cell migration and ERK activation. These results thus suggest that the cytoplasmic domain of MT1-MMP represents a crucial region of the protein involved in both cell migration and activation of the ERK cascade. A similar requirement for the cytoplasmic region was recently described for the MT1-MMP-dependent invasion by melanoma and breast carcinoma cells [15]. In this latter case, a single point mutation of a cysteine residue located in the cytoplasmic segment of the protein (C574) was sufficient to inhibit cell migration and invasion, possibly by abolishing the formation of MT1-MMP dimers [15]. Under our experimental conditions, however, removal of this cysteine residue (MT1-MMP(CΔ10)) did not significantly affect migration but completely abolished activation of ERK. The reason for this discrepancy is unclear but may suggest that additional signals originating from the cytoplasmic domain of MT1-MMP may contribute to the biological effects triggered by this enzyme. Close examination of this sequence, however, indicates that it is devoid of known catalytic activities, suggesting that it may provide signals by recruiting and/or binding other proteins or messenger molecules, leading to cell migration and activation of the ERK cascade. In this respect, it is noteworthy that the sequence **R**H**G**TP**RR** present in the MT1-MMP cytoplasmic domain shows significant homology with the **KXG**FF**KR** sequence of integrin α subunits [25]. This sequence has been shown to be involved in the interaction of α integrin with proteins such as calreticulin [26] and the guanine nucleotide exchange protein Mss4 [27]. It is thus tempting to speculate that the binding of specific proteins to this region of MT1-MMP could trigger the changes observed in ERK activation and transcription. This interaction would however require the catalytic activity of the enzyme since these processes are abolished by either endogenous inhibitors of MT1-MMP (e.g. TIMP-2) or by a catalytically mutated form of the enzyme. The binding of the enzyme to matrix proteins may thus induce conformational changes within its structure that may lead to its interaction with these proteins, triggering activation of the ERK cascade. The observation that MT1-MMP-dependent activation of migration and of ERK requires adhesion of cells to matrix-coated dishes strongly support this hypothesis.

The matrix requirement for the MT1-MMP-dependent effects also suggest a potential role of integrins in the regulation of MT1-MMP activity. Activation of the ERK cascade in response to integrin-mediated cell adhesion has been widely described (for a review see [28]). In this context, it is interesting to note that MT1-MMP and αvβ3 are co-localized on the cell membrane of tumor cells [29,30] and that αvβ3 co-operate with MT1-MMP for efficient activation of MMP-2 [31]. It is thus tempting to speculate that MT1-MMP may co-operate with integrins in order to mediate its effects on migration and ERK activation. Alternatively, MT1-MMP-dependent cleavage of cell surface proteins, such as the hyaluronan receptor CD44 and tissue transglutaminase was recently suggested to play an important role in the induction of cell migration by MT1-MMP [32,33] and it will be interesting to determine whether the cleavage of such proteins is required for the induction of the ERK cascade reported here.

The activation of ERK by MT1-MMP also seems to have important effects on gene transcription since transient transfection of a reporter vector containing SRE, a *cis*-acting DNA response element that can be activated through the MAPK/JNK pathway [34], induced a marked increase in the activity of this element. This effect of MT1-MMP appears specific, as no activation of other response elements was detected under our experimental conditions, and also requires the catalytic activity and cytoplasmic domain of the protein. While the effects of MT1-MMP on gene transcription remain to be further characterized, it is interesting to note that transgenic mice overexpressing the MT1-MMP gene show increased mammary gland abnormalities and adenocarcinoma [35] and that

overexpression of MT1-MMP in a gastric cancer cell line enhances lymph node metastasis of these cells [36]. Further studies aimed at the identification of genes whose expression is altered upon overexpression of MT1-MMP should help to determine the importance of MT1-MMP-driven gene transcription in tumor cell growth and metastasis.

In summary, our results suggest that MT1-MMP-dependent cell migration is correlated with the activation of the ERK cascade, these processes being highly dependent on the catalytic activity of the enzyme as well as on the cytoplasmic domain of the protein. Further studies aimed at the identification of the cellular proteins that interact with the cytoplasmic domain of MT1-MMP should provide important information on the mechanisms involved in these novel signal-transducing properties of MT1-MMP.

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