

Src-dependent Phosphorylation of Membrane Type I Matrix Metalloproteinase on Cytoplasmic Tyrosine 573

ROLE IN ENDOTHELIAL AND TUMOR CELL MIGRATION*

Received for publication, August 22, 2006, and in revised form, March 27, 2007. Published, JBC Papers in Press, March 27, 2007, DOI 10.1074/jbc.M608045200

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Membrane type 1 matrix metalloproteinase (MT1-MMP) is a transmembrane MMP that plays important roles in migratory processes underlying tumor invasion and angiogenesis. In addition to its matrix degrading activity, MT1-MMP also contains a short cytoplasmic domain whose involvement in cell locomotion seems important but remains poorly understood. In this study, we show that MT1-MMP is phosphorylated on the unique tyrosine residue located within this cytoplasmic sequence (Tyr⁵⁷³) and that this phosphorylation requires the kinase Src. Using phosphospecific antibodies recognizing MT1-MMP phosphorylated on Tyr⁵⁷³, we observed that tyrosine phosphorylation of the enzyme is rapidly induced upon stimulation of tumor and endothelial cells with the platelet-derived chemoattractant sphingosine-1-phosphate, suggesting a role in migration triggered by this lysophospholipid. Accordingly, overexpression of a nonphosphorylatable MT1-MMP mutant (Y573F) blocked sphingosine-1-phosphate-induced migration of Human umbilical vein endothelial cells and HT-1080 (human fibrosarcoma) cells and failed to stimulate migration of cells lacking the enzyme (bovine aortic endothelial cells). Altogether, these findings strongly suggest that the Src-dependent tyrosine phosphorylation of MT1-MMP plays a key role in cell migration and further emphasize the importance of the cytoplasmic domain of the enzyme in this process.

The degradation of extracellular matrix (ECM)³ proteins by members of the matrix metalloproteinases (MMPs) plays a crucial role in several biological processes, including cell attachment, cell migration, invasiveness, cell proliferation, apoptosis,

and angiogenesis (1–4). Among the various MMPs described to date, there is now considerable evidence that MMPs that are intrinsically associated with the plasma membrane because of the presence of a transmembrane domain within their sequence, the so-called membrane type MMPs, represent key components involved in pericellular proteolysis and subsequent cell locomotion and invasion (5, 6). The prototypical member of this family, MT1-MMP, actively participates in the remodeling of the pericellular ECM by acting as a cellular receptor and activator of proMMP-2 (7) and as a potent matrix-degrading protease that proteolyzes a broad spectrum of ECM proteins (8–10) as well as a number of cell surface-associated adhesion receptors (11, 12). These events are likely to be important *in vivo* because MT1-MMP null mice fail to thrive and have a markedly reduced lifespan (13, 14).

In addition to its role in normal physiology, MT1-MMP is also overexpressed in many types of tumors (15, 16), and this overexpression appears crucial for tumor cell migration and invasion. For example, MT1-MMP-mediated degradation of some ECM proteins, such as the laminin-5 γ 2 chain (17), stimulates migration, whereas proteolysis of the dense, cross-linked meshwork of type I collagen fibrils by the enzyme confers neoplastic cells with tissue-invasive activity (18) and sustains tumor cell growth in otherwise growth-restrictive three-dimensional matrices (19).

Despite its importance to normal physiology and in the development of malignancy, the mechanisms underlying MT1-MMP-mediated cell invasion remain incompletely understood. During cell migration, MT1-MMP localizes predominantly to the cell adherent edge at the migration front, an appropriate location for the degradation of the ECM barrier (20). In addition, we and others have shown that MT1-MMP is preferentially localized into caveolae, specialized domains of the plasma membrane (21, 22), and this localization may contribute to the spatiotemporal regulation of its proteolytic activity by controlling adequate endocytosis and recycling of the enzyme (23, 24).

In addition to the importance of MT1-MMP-mediated proteolytic breakdown of ECM proteins for the induction of cell migration, recent studies suggested that the cytoplasmic domain of the enzyme may also play a role in this process. For example, MT1-MMP mutants lacking the cytoplasmic domain remain localized at the cell surface and failed to induce migration, suggesting an important role for the enzyme cytoplasmic sequence in the regulation of its activity (25–27). In this respect,

* This work was supported by a grant from the Canadian Institutes for Health Research (to R. B. and D. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by Cancer Research UK.

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³ The abbreviations used are: ECM, extracellular matrix; MT1-MMP, membrane-type matrix metalloproteinase; S1P, sphingosine-1-phosphate; siRNA, small interfering RNA; BAEC, bovine aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; HT-1080, human fibrosarcoma cells; FBS, fetal bovine serum; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MES, 4-morpholineethanesulfonic acid.

the cytoplasmic domain of MT1-MMP has been shown to be involved in several aspects of enzyme activity, including the formation of oligomers (28, 29), the localization of the enzyme to invadopodia (30), the activation of the extracellular signal-regulated protein kinase signaling pathway (31), and its interaction with a number of intracellular proteins such as cupin (32), the $\mu 2$ subunit of adaptor protein-2 (27), and tyrosine-phosphorylated caveolin-1 (33). Although the mechanisms by which the MT1-MMP cytoplasmic sequence is implicated in these processes remain poorly understood, recent observations indicate that it could involve an important function of this domain in the cooperation of the enzyme with serum-derived chemoattractant molecules such as sphingosine-1-phosphate (S1P), a bioactive lipid secreted by activated platelets (34).

In this study, we report that MT1-MMP is tyrosine-phosphorylated within its cytoplasmic domain, in a Src-dependent manner. This tyrosine phosphorylation is observable upon stimulation of endothelial and tumor cells with S1P and seems important for both tumor and endothelial cell migration triggered by this lipid.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Cell culture media, antibiotics (penicillin and streptomycin) and glutamine were purchased from Invitrogen. Trypsin was obtained from Sigma. Basic fibroblast growth factor was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). PolyFect and HiPerFect transfection reagents, control, and caveolin-1 siRNAs were purchased from Qiagen. The TransPass D₂ transfection reagent was from New England BioLabs Inc. (Ipswich, MA). Antibodies against caveolin (610059 and 610406) and caveolin pY14 (611338) were obtained from BD Transduction Laboratories (Mississauga, Canada); anti-MT1-MMP (AB815 and MAB3328) were from Chemicon International (Temecula, CA); anti-Tyr(P) (pY99) and c-Myc (clone 9E10) were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Src (clone GD11) was from Upstate Biotechnology, Inc.. Mouse and rabbit horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (Mississauga, Canada). Mouse Alexa 488-conjugated (A21202) and rabbit rhodamine-conjugated (T2769) antibodies were from Molecular Probes (Invitrogen). Protein G- and A-coupled Sepharose, immobilized pH gradient strips, and apparatus for isoelectric focusing were from Amersham Biosciences. Electrophoresis apparatus and reagents were purchased from Bio-Rad. Polyvinylidene difluoride transfer membranes and Western Lightening Chemiluminescence Reagent Plus were obtained from PerkinElmer Life Sciences.

Cell Culture—Bovine aortic endothelial cells (BAEC), human umbilical vein endothelial cells (HUVEC), human fibrosarcoma cells (HT-1080), and monkey kidney cells (COS-7) were purchased from Clonetics and were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. BAEC were cultured in Dulbecco's modified Eagle's medium with low glucose supplemented with 10% bovine calf serum, 10 ng/ml basic fibroblast growth factor, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 4 mM glutamine. HUVEC were maintained in endothelial cell growth medium BulletKit (EGM-2) supplemented with 2% fetal bovine serum (FBS), human epidermal growth factor,

hydrocortisone, vascular endothelial growth factor, human basic fibroblast growth factor, insulin-like growth factor 1, ascorbic acid, heparin, gentamycin, and amphotericin-B. HT-1080 were grown in minimum essential medium supplemented with 1 mM pyruvate, 10% FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 4 mM glutamine. COS-7 were grown in Dulbecco's modified Eagle's medium with high glucose supplemented with 10% FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 4 mM glutamine.

Transfection—The cDNAs encoding the full-length human MT1-MMP, its cytoplasmic domain-deleted ($\Delta 20$), and catalytically inactive (E240A) mutants have been previously described (31, 34). The MT1-MMP cytoplasmic mutants L571A/L572A/Y573A and Y573F were produced by site-directed mutagenesis, as previously described (33). The Myc-tagged full-length MT1-MMP (MycMT1), in which the Myc epitope was inserted in the hinge region of the enzyme between proline 312 and threonine 313 (PEQKLISEEDLT), was synthesized using the overlap extension method. The wild-type and dominant-negative Src plasmids were kindly provided by Dr. Isabelle Royal (Université de Montréal, Montréal, Canada). Transient transfection of COS-7 cells was performed using the PolyFect transfection reagent (Qiagen). HT-1080 cells were transiently transfected using the FuGENE 6 transfection reagent (Roche Applied Science). HUVEC and BAEC were transiently transfected with TransPass D2 transfection reagent (BioLabs). All of the transfections were performed according to the manufacturer's instructions.

Production of Phospho-MT1-MMP(Y573) Antibodies—Polyclonal phosphospecific antibodies were produced by 21st Century Biochemicals (Marlboro, MA). Briefly, antigenic phosphopeptide (GTPRRLL[pY]CQRSL-amide) and nonphosphopeptide (GTPRRLLYCQRSL-amide) were synthesized based on the human MT1-MMP cytoplasmic sequence and purified by high pressure liquid chromatography. The sequences were verified by mass spectrometry. The rabbits were inoculated five times with the phosphopeptide conjugated with a immune carrier, and serum was collected and subjected to affinity depletion using the nonphosphopeptide, followed by affinity purification using a phosphopeptide affinity column. To verify the specificity of the antibodies against the phosphorylated peptide, pMT1-MMP(Y573) antibodies were preincubated for 45 min at 37 °C with a 5-fold molar excess of either the phosphopeptide or the nonphosphopeptide, and immunodetection was performed as described below.

Two-dimensional Gel Electrophoresis—For the first dimension, immunoprecipitates bound to protein A-coupled Sepharose beads were solubilized in rehydration buffer (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10, 0.3% dithiothreitol, bromophenol blue) containing ampholytes (pH 4–7). Solubilized proteins were incubated with 7-cm IPG strips containing a linear pH gradient (4–7) (Amersham Biosciences) at room temperature for 10 h, and isoelectric focusing was performed at 500 V for 30 min, 1000 V for 30 min, and then 5000 V for 13 h. For the second dimension, IPG strips were incubated for 15 min at room temperature in equilibration buffer (50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 2% SDS) containing 2% dithiothreitol and then for 15 min in equilibration buffer containing 5%

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iodoacetamide and bromphenol blue. IPG strips were loaded on 7.5% SDS-PAGE, and Western blotting was performed as described below.

Caveolae Isolation—Caveolae were purified using a hyperosmotic carbonate method, as described previously (33). Briefly, confluent COS-7 cells cultured in 100-mm² dishes were scraped into 3 ml of 0.5 M sodium carbonate (pH 11) and homogenized extensively using a Polytron (three pulses of 15 s, at speed 4) followed by sonication (five pulses of 15 s, at 70% of maximal power). 2.5 ml of the resulting homogenate was brought to 45% sucrose by the addition of 2.5 ml of 90% sucrose in MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl) and overlaid with two layers (6 ml each) of 35 and 5% sucrose in MES-buffered saline containing 0.25 M carbonate. The gradient was then centrifuged at 200,000 × *g* for 18 h using a Beckman SW41Ti rotor. For analysis of the resulting gradient, 1-ml fractions were collected from the top to the bottom of the gradient. Caveolae-enriched (5–8 fractions) or noncaveolae (12–15 fractions) fractions were pooled, diluted in 10 mM Tris-HCl, pH 7.5, and centrifuged at 100,000 × *g* for 1 h.

Stimulation of HT1080 and HUVEC Cells with S1P—HT1080 and HUVEC cells grown to 90% confluence were serum-starved for 18 h in medium containing 0.5% serum. The cells were then preincubated 2 h at 37 °C with 1 μM PP2 (or an equivalent amount of vehicle), followed by incubation for 2–30 min with 1 μM S1P. The cells were then solubilized in SDS lysis buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, 1 mM sodium orthovanadate), followed by boiling for 5 min at 100 °C and homogenization using a 26-gauge needle. Protein concentrations were determined by the bicinchoninic acid method (Pierce).

Immunoprecipitation and Western Blotting—The procedures have been described elsewhere (33). Briefly, equal amounts of proteins were incubated in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate) overnight at 4 °C in the presence of 1–2 μg/ml of specific antibodies, and the immune complexes were collected by incubating the mixtures with protein A- or G-coupled Sepharose beads. Bound material was solubilized in Laemmli sample buffer, boiled for 5 min, and separated by SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membranes, blocked overnight at 4 °C with Tris buffer saline with 0.1% Tween 20 (TBST) buffer containing 3% bovine serum albumin, and incubated for 1 h at room temperature with the desired primary antibodies. Immunoreactive bands were revealed following 1 h of incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies, and the signals were visualized by chemiluminescence. Densitometric analysis was performed using the IPLab Gel program.

Immunofluorescence and Confocal Microscopy—Endothelial cells (HUVEC) were plated on cover glasses coated with 10 μg/ml fibronectin, serum-starved, and treated (or not) with 1 μM S1P for 15 min. After 5 min of incubation with Hoechst 20 μM for nuclei staining, the cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 5 min, blocked (1% bovine serum albumin in Tris-buffered saline containing 0.1% Tween) for 30 min, and stained with specific

primary antibodies against phospho-MT1-MMP (1/50 dilution), phospho-caveolin-1, and MT1-MMP (1/100 dilution). The cells were incubated with Alexa 488- or rhodamine-conjugated secondary antibodies. The slides were mounted with Immuno-Fluore mounting medium (MP Biomedicals). Immunostaining was visualized and photographed using a Zeiss LSM 510 Meta confocal microscope.

Cell Migration—Migration assays of BAEC, HUVEC, or HT1080 cells transfected with either pcDNA3.1, WT, or Y573F forms of MT1-MMP, were performed on transwells precoated with 10 μg/ml fibronectin. The transwells were assembled in 24-well plates, and the lower chambers were filled with serum-free media with or without 1 μM S1P or 10% FBS. Transfected cells were harvested, resuspended in 100 μl of fresh cell media at a density of 5 × 10⁵ cells/ml, and inoculated into the upper chamber of each transwell. The plates were then placed at 37 °C in 5% CO₂, 95% air for 3 h, and cells that had migrated were quantified using computer-assisted imaging (Northern Eclipse 6.0; Empix Imaging, Mississauga, Canada). The data are expressed as the average density of migrated cells/4 fields (original magnification, 50×) (34). Statistical analysis was performed by two-way analysis of variance followed by the Bonferroni post-tests. *p* < 0.05 was considered statistically significant. For the measurement of transfection efficiencies, the total membranes were isolated from HUVEC and BAEC as described (34), and MT1-MMP levels were monitored by immunoblotting. For HT-1080 cells, transfection efficiencies were monitored by zymographic analysis of MT1-MMP-dependent activation of proMMP-2 in as described (21).

RESULTS

MT1-MMP Is Phosphorylated on Cytoplasmic Tyrosine 573:

Involvement of Src Kinase—We previously showed that overexpression of Src induces the tyrosine phosphorylation of caveolin-1 and its subsequent association with MT1-MMP (33). Because this interaction required the cytoplasmic domain of MT1-MMP, we hypothesized that the stimulatory effect of Src could also involve the phosphorylation of the unique tyrosine residue located in the cytoplasmic sequence of MT1-MMP. As a first step to examine this possibility, COS-7 cells were transiently transfected with a Myc-tagged version of MT1-MMP along with Src or a kinase-inactive dominant-negative form of Src. MycMT1 was immunoprecipitated using an anti-Myc monoclonal antibody, and the extent of tyrosine phosphorylation was determined by immunoblotting using an anti-phosphotyrosine antibody. As shown in Fig. 1A, immunoprecipitation of MT1-MMP from cells overexpressing MT1-MMP and Src resulted in the appearance of two tyrosine-phosphorylated bands in the 63–66-kDa range, whereas co-expression of MT1-MMP with an inactive Src mutant failed to induce the tyrosine phosphorylation of these proteins. The upper phosphorylated band (66 kDa) shows an electrophoretic mobility similar to that of Myc-tagged MT1-MMP and was also observed upon reverse immunoprecipitation with anti-phosphotyrosine antibody, strongly suggesting it represents a tyrosine-phosphorylated form of MT1-MMP.

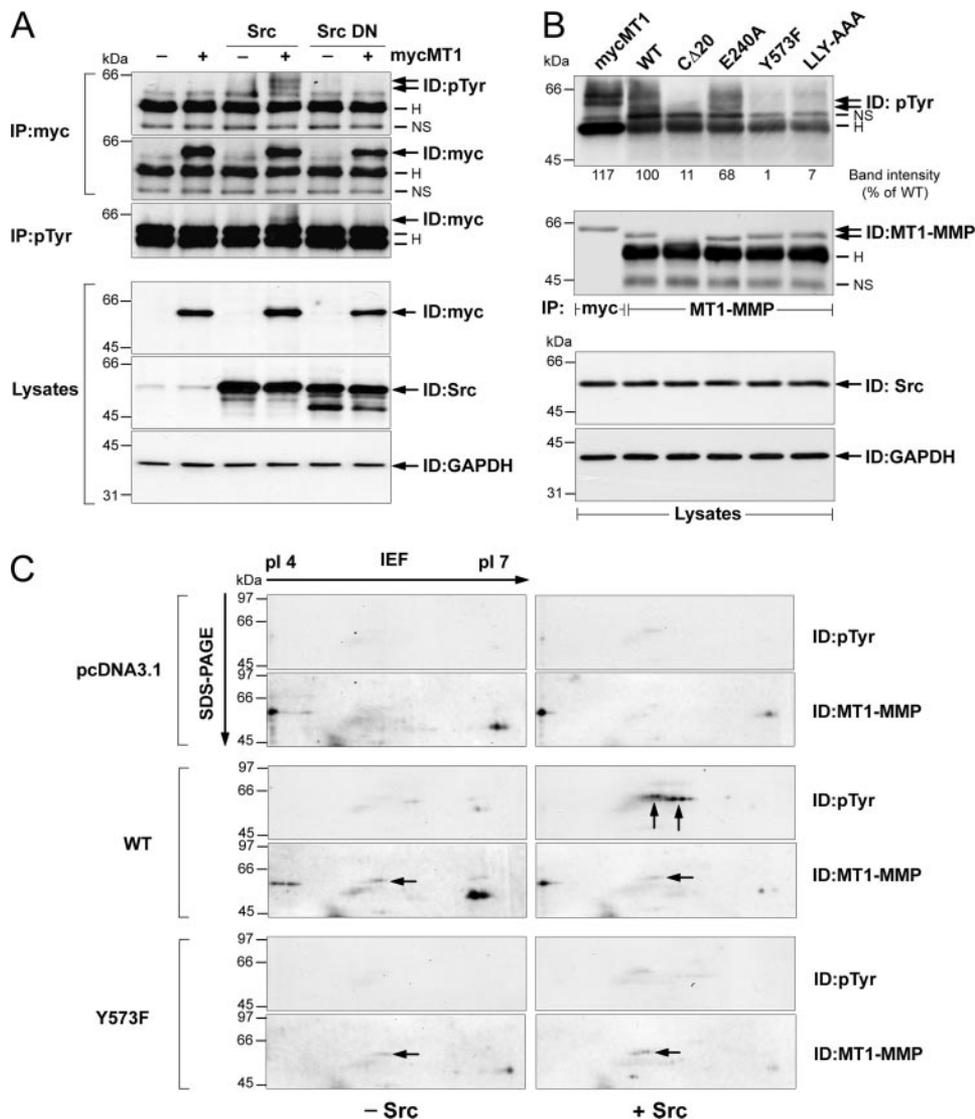


FIGURE 1. MT1-MMP is phosphorylated on its cytoplasmic tyrosine residue 573. *A*, COS-7 cells were transfected with empty vector (pcDNA3.1), wild-type Myc-tagged MT1-MMP (*mycMT1*), Src, or a dominant-negative form of Src. 48 h post-transfection, the cell lysates were subjected to immunoprecipitation (IP) with a monoclonal antibody raised against either Myc tag or total phosphotyrosines (*pTyr*), and phosphorylation was monitored by Western blotting. Levels of transfected proteins were monitored in cell lysates by Western blotting using specific antibodies. *B*, COS-7 cells were transfected with Src and different versions of MT1-MMP (Myc-tagged (*mycMT1*), WT, catalytically inactive mutant (E240A), cytoplasmic domain-deleted (Δ 20), or cytoplasmic mutants Y573F and L571A/L572A/Y573A), and the extent of MT1-MMP phosphorylation was monitored as in *A*. *C*, COS-7 cells were transfected with Src along with WT or Y573F forms of MT1-MMP. 48 h post-transfection, the cell lysates were immunoprecipitated with polyclonal antibodies raised against MT1-MMP. The phosphorylation of MT1-MMP was analyzed by two-dimensional gel electrophoresis: immunoprecipitated proteins were first subjected to isoelectric focusing (IEF) in a pI range of 4–7 and then applied to a 7.5% SDS-PAGE. Two-dimensional gels were then immunoblotted with antibodies against either pTyr or MT1-MMP. *ID*, immunodetection; *NS*, nonspecific; *H*, immunoglobulin heavy chain. These results are representative of three distinct experiments.

To further establish whether MT1-MMP is indeed tyrosine-phosphorylated in Src-expressing cells and whether this phosphorylation occurs at an intracellular location, we next examined the effect of various mutants of the enzyme on this event. As shown in Fig. 1*B*, immunoprecipitation of both Myc-tagged (*mycMT1*) or WT MT1-MMP resulted in the appearance of tyrosine-phosphorylated bands corresponding to the molecular masses of both forms of the enzyme, indicating that the observed phosphorylation was not restricted to the epitope-tagged version of MT1-MMP.

We next examined the requirement for the catalytic activity and cytoplasmic sequence of the enzyme using various mutants. The catalytically inactive mutant (E240A) was also phosphorylated but to a lower extent than the WT enzyme. Because this mutation impairs the refolding of a recombinant MT1-MMP polypeptide (35), it is possible that the E240A mutation could have induced a global conformational change in the enzyme structure, thus partially preventing MT1-MMP tyrosine phosphorylation. However, removal of the cytoplasmic domain (Δ 20) decreased almost completely tyrosine phosphorylation (Fig. 1*B*).

Because the MT1-MMP cytoplasmic sequence contains only one tyrosine residue at position 573 of the protein, we next examined the effect of mutated versions of MT1-MMP lacking this residue. Interestingly, overexpression of the Y573F and L571A/L572A/Y573A mutants abolished tyrosine phosphorylation, further suggesting that the intracellular tyrosine residue of MT1-MMP is the site of phosphorylation (Fig. 1*B*).

To unambiguously establish that MT1-MMP is phosphorylated on tyrosine 573, we next analyzed immunoprecipitates from cells expressing WT or Y573F forms of MT1-MMP along with Src, using two-dimensional gel electrophoresis. As shown in Fig. 1*C*, immunoprecipitation of MT1-MMP from cells co-expressing Src resulted in the appearance of at least four well defined tyrosine-phosphorylated spots (*vertical arrows*). We observed that the more acidic forms migrated at a position identical to immunoreactive MT1-MMP (63 kDa) (*horizontal arrow*), whereas the more basic forms, with slightly lower molecular masses (60 kDa), were not associated with detectable levels of the enzyme. These 60-kDa forms are, however, likely to represent low abundant tyrosine-phosphorylated MT1-MMP because overexpression of the Y573F mutant completely abolished the tyrosine phosphorylation of all MT1-MMP isoforms (Fig. 1*C*); these forms were also recognized by phosphospecific MT1-MMP antibodies (see Fig. 3*D*). Overall, these results indicate that MT1-MMP is tyrosine-phos-

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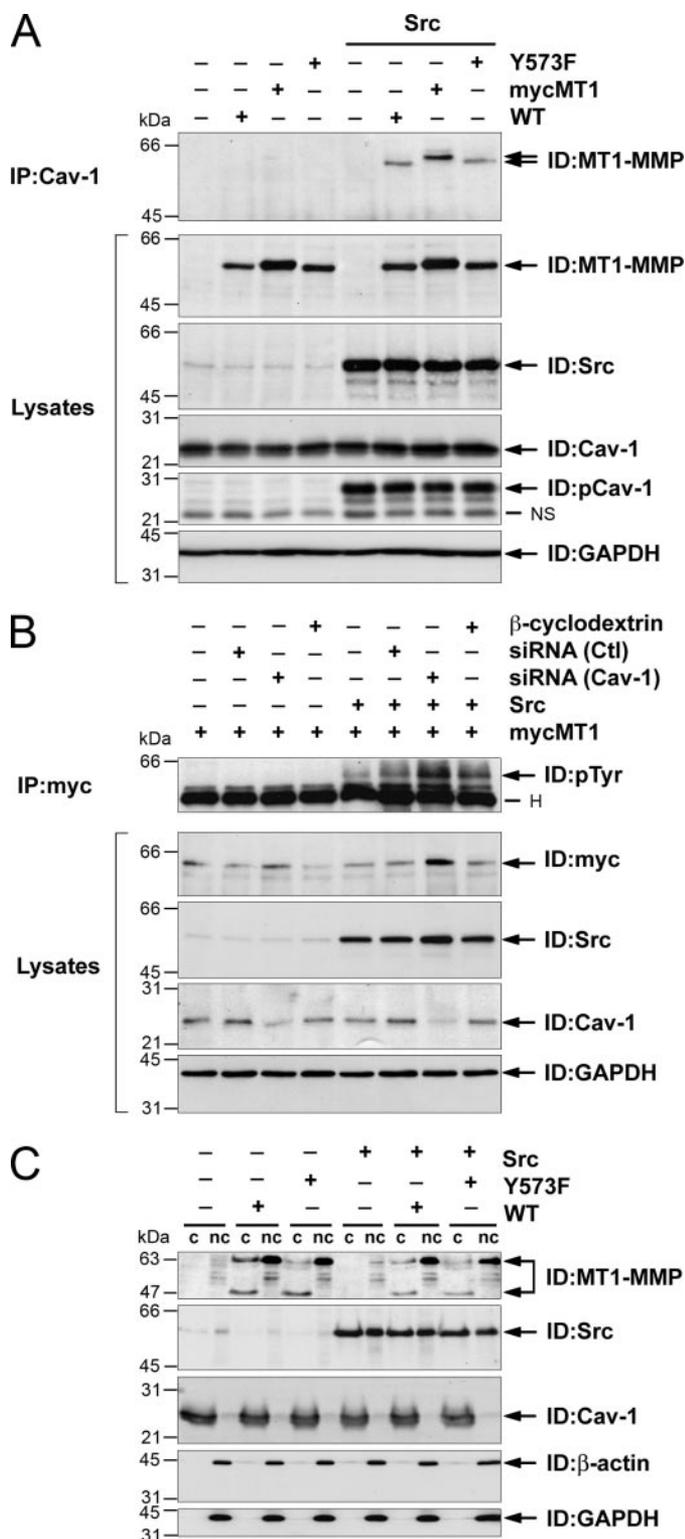


FIGURE 2. Caveolin-1 and caveolae are not necessary for MT1-MMP phosphorylation. A, COS-7 cells were transfected with Src and either MycMT1, WT, or Y573F forms of MT1-MMP. 48 h post-transfection, the cell lysates were immunoprecipitated (IP) with a monoclonal antibody raised against caveolin-1 (Cav-1), and associated MT1-MMP was detected with anti-MT1-MMP antibodies. Levels of transfected proteins were monitored in lysates by Western blotting using specific antibodies. B, COS-7 cells were transfected with control or caveolin-1 siRNA, followed, 24 h later, by transfection with MycMT1 and Src. The cells were treated for 2 h with β -cyclodextrin where indicated. The cell lysates were immunoprecipitated with a monoclonal antibody raised against Myc, and total phosphotyrosines were detected with an anti-Tyr(P). The levels of transfected proteins were monitored by Western blotting using

phosphorylated in Src-expressing cells and that this event involves its unique cytoplasmic tyrosine 573 residue.

Caveolin-1 and Caveolae Are Not Necessary for MT1-MMP Phosphorylation—Based on our observation that tyrosine-phosphorylated caveolin-1 interacts with MT1-MMP, we next examined whether the tyrosine phosphorylation of MT1-MMP is involved in its interaction with caveolin-1 (33). First, COS-7 cells were transfected with either wild-type MT1-MMP or the nonphosphorylatable MT1-MMP mutant (Y573F), and the presence of MT1-MMP in the caveolin-1 immunoprecipitates was determined. As shown in Fig. 2A, both the wild-type and the mutated version MT1-MMP associated with caveolin-1, ruling out phosphorylation of MT1-MMP as a necessary event for its interaction with caveolin-1.

Numerous proteins, such as endothelial nitric oxide synthase or epidermal growth factor receptor, are regulated by an interaction with caveolin-1 (36). Because MT1-MMP interacts with caveolin-1, we thus hypothesized that its phosphorylation status could be regulated by caveolin-1. First, to determine whether caveolin-1 is necessary for the tyrosine phosphorylation of MT1-MMP, cells overexpressing MT1-MMP and Src were incubated with a siRNA specifically targeting caveolin-1, and the resulting phosphorylation of MT1-MMP was monitored by immunoprecipitation. As shown in Fig. 2B, despite a dramatic reduction of the caveolin-1 levels by the siRNA, the tyrosine phosphorylation of MT1-MMP was not markedly affected by the treatment. However, we routinely observed that down-regulation of caveolin-1 significantly increases both MT1-MMP levels and its extent of phosphorylation, suggesting that caveolin-1 may play a role in the turnover rate of the enzyme. This effect is, however, unlikely to be related to the disruption of caveolae that are induced by the reduced levels of caveolin-1 because the addition of β -cyclodextrin, a well described caveolae-disrupting agent, had no significant effect on either MT1-MMP expression or phosphorylation (Fig. 2B). Finally, we tested whether phosphorylation of MT1-MMP alter its localization to caveolae. Although overexpression of MT1-MMP in COS-7 cells leads to a lower caveolar localization of the enzyme than in tumor (21) or endothelial (22) cells, we observed that the proportion of the WT form of the enzyme associated with these domains was slightly affected by either overexpression of Src or by mutation of the phosphorylation site within the cytoplasmic domain (Fig. 2C). The proportion of WT MT1-MMP associated with caveolae was higher than for the Y573F mutant. However, in the presence of Src, WT seems less abundant in caveolae, whereas the proportion the Y573F mutant associated with caveolae was not affected by the presence of Src. Thus, although these results indicate that caveolin-1 is not involved in the tyrosine phosphorylation of MT1-MMP, the phosphorylation state of the enzyme may alter its subcellular localization.

specific antibodies. C, COS-7 cells were transfected with Src and either pcDNA3.1, WT, or Y573F forms of MT1-MMP. 48 h post-transfection, the cells were harvested and subjected to sucrose gradient sedimentation. Equal amount of proteins from caveolae (c) and noncaveolae (nc) were subjected to immunoblotting with the indicated antibodies. ID, immunodetection; NS, nonspecific; H, immunoglobulin heavy chain. These results are representative of four distinct experiments.

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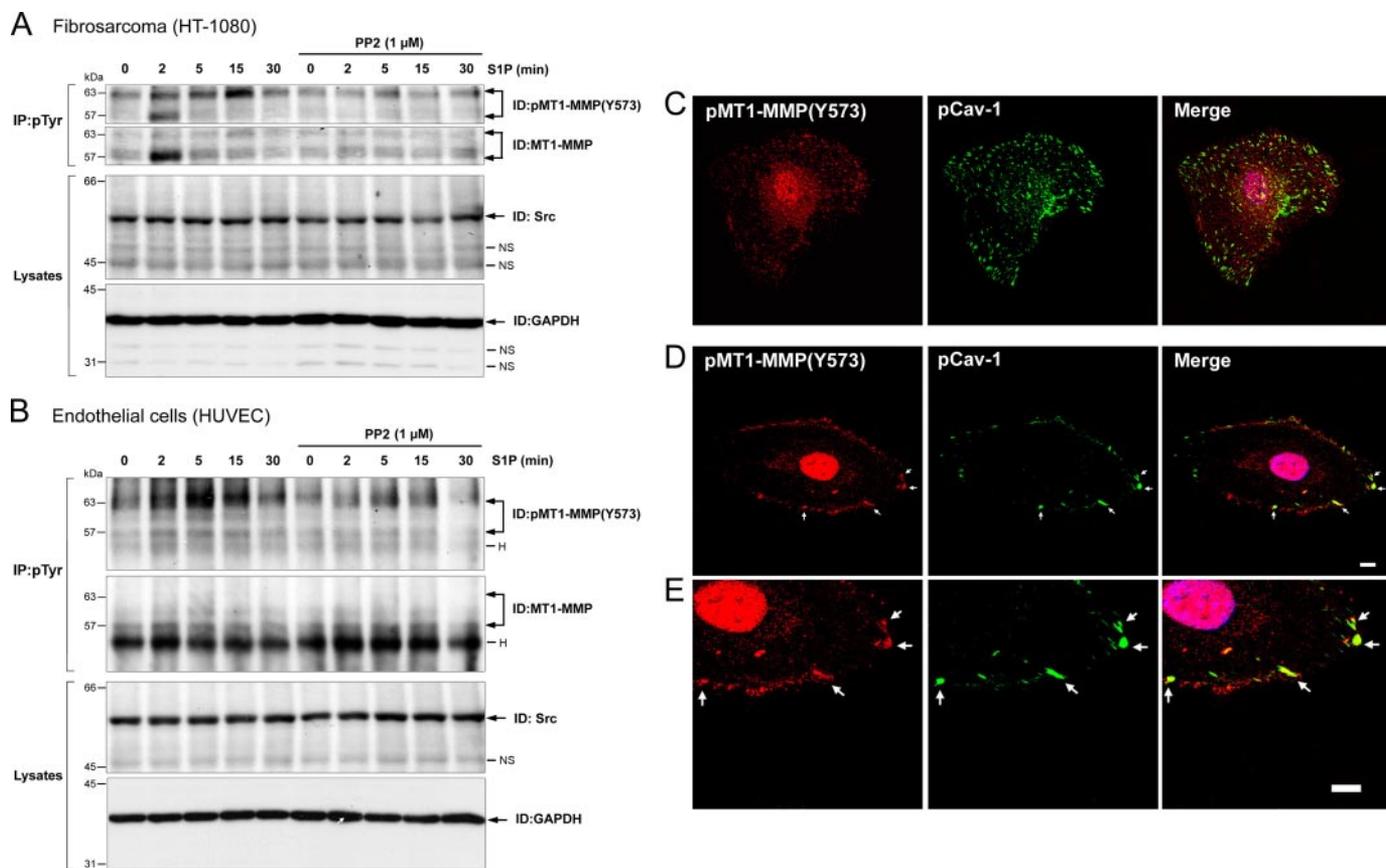


FIGURE 4. S1P-dependent phosphorylation of MT1-MMP in fibrosarcoma and endothelial cells. HT-1080 fibrosarcoma (A) or HUVEC endothelial (B) cells were serum-starved (1% serum) for 18 h. The cells were pretreated for 2 h with 1 μ M PP2 or equivalent amount of vehicle (Me₂SO) and stimulated with 1 μ M S1P for the indicated periods of time. The cells were harvested and immunoprecipitated (IP) with a monoclonal antibody raised against phosphotyrosine, and phosphorylated MT1-MMP was immunodetected with anti-pMT1-MMP(Y573) antibodies. ID, immunodetection; NS, nonspecific; H, immunoglobulin heavy chain. HUVECs were seeded on fibronectin-coated coverslips, serum-starved, and stimulated (D and E) or not (C) with S1P. The nuclei were then stained with Hoechst, and the cells were fixed and double stained with specific antibodies against phospho-MT1-MMP(Y573) and phosphocaveolin-1 (pCav-1), followed by incubation with rhodamine- and Alexa 488-conjugated secondary antibodies, respectively. E is an enlarged (2-fold) section of D. Representative cell images were collected by confocal microscopy as described under "Experimental Procedures." The white scale bar represents 10 μ m. These results are representative of three distinct experiments.

phosphopeptide had no effects. Furthermore, the anti-phospho-MT1-MMP(Y573) antibodies recognize MT1-MMP only in immunoprecipitates from COS-7 cells co-expressing MT1-MMP and Src, in agreement with previous results obtained with the anti-phosphotyrosine antibody. However, the antibodies did not recognize the MT1-MMP (Y573F) mutant, even in the presence of Src, further confirming their specificity toward MT1-MMP that is tyrosine-phosphorylated on its cytoplasmic tyrosine residue (Fig. 3C). The pattern of MT1-MMP phosphorylation detected by the phosphospecific antibodies following two-dimensional electrophoresis was also similar to that observed using the anti-phosphotyrosine antibody, again indicating that these antibodies indeed recognize phosphorylated MT1-MMP (Fig. 3D). Finally, antibodies against MT1-MMP and phospho-MT1-MMP show a similar immunostaining pattern in confocal microscopy (Fig. 3E), further indicating that phospho-MT1-MMP antibodies are specific to the tyrosine-phosphorylated forms of MT1-MMP.

S1P Stimulates Endogenous Endothelial and Tumor Cell MT1-MMP Phosphorylation—S1P is a platelet-derived lysophospholipid that acts as a powerful chemoattractant for both endothelial and tumor cells (38). Previous work from our labo-

ratory has shown that MT1-MMP cooperates with S1P to induce endothelial cell migration and their morphogenic differentiation into capillary-like structures (34). To examine whether MT1-MMP is tyrosine-phosphorylated in response to S1P stimulation, human fibrosarcoma (HT-1080) and human umbilical endothelial vein (HUVEC) cells were incubated for various periods of time with S1P, and the extent of MT1-MMP phosphorylation was monitored by immunoprecipitation. Because preliminary experiments indicated that the phosphospecific antibodies were ineffective in immunoprecipitation procedures (result not shown) and that anti-MT1-MMP antibodies failed to precipitate the enzyme from untransfected cells (33), total phosphotyrosines were immunoprecipitated, and the presence of phosphorylated MT1-MMP in the immune complexes was monitored using the anti-phospho-MT1-MMP(Y573) antibodies. As shown in Fig. 4A, stimulation of HT1080 cells with S1P resulted in a time-dependent increase of tyrosine-phosphorylated MT1-MMP, with a maximal phosphorylation occurring at 15 min. Tyrosine phosphorylation was predominantly observed for the 63-kDa form of the enzyme, but a 57-kDa form of MT1-MMP was also strongly phosphorylated after 2 min of stimulation with S1P. The extent of phos-

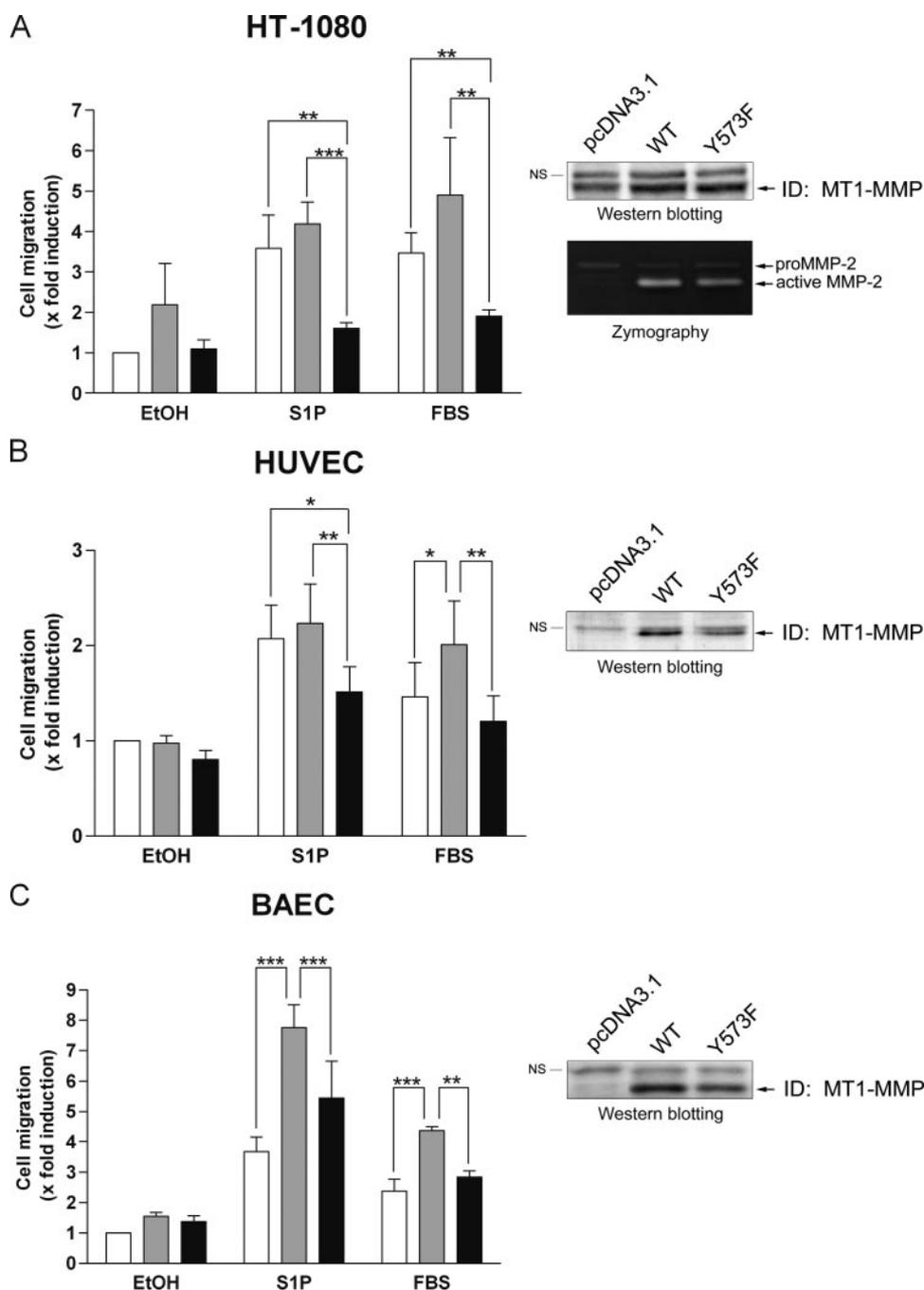


FIGURE 5. Mutation of cytoplasmic tyrosine 573 of MT1-MMP impairs tumor and endothelial cell migration. HT-1080 (A), HUVEC (B), and BAEC (C) were transfected with either pcDNA3.1 (white bars), WT (shaded bars), or Y573F (black bars) forms of MT1-MMP. 48 h post-transfection, the cells were harvested, and 5×10^4 cells were subjected to migration assays as described under "Experimental Procedures," using transwells pre-coated with 10 $\mu\text{g}/\text{ml}$ fibronectin and 1 μM S1P or 10% FBS as chemoattractants. The values (means \pm S.D.) are expressed relatively to control cells ($n = 3$ for HT-1080, $n = 4$ for HUVEC and BAEC). Statistical analyses were performed with analysis of variance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The remaining cells and conditioned media were used for the evaluation of transfection efficiencies (right panels). MT1-MMP expression in transfected cells was monitored by Western blotting of isolated membranes and by MT1-MMP-dependent activation of proMMP-2 (zymography). ID, immunodetection; NS, nonspecific.

phorylation of this form, however, rapidly declined, possibly reflecting the high turnover rate of MT1-MMP in these cells (39). The addition of PP2, a Src kinase inhibitor, markedly inhibited S1P-dependent phosphorylation of MT1-MMP, again suggesting the importance of Src in this process. In a similar manner, S1P stimulated MT1-MMP phosphorylation in

HUVEC, with a maximum at 5 min, and this phosphorylation was sensitive to PP2, indicating the involvement of Src kinase activity.

S1P Induces Subcellular Relocalization of Phospho-MT1-MMP—Because MT1-MMP is localized to caveolae and interacts with phosphorylated caveolin-1 (Fig. 2C and Refs. 33 and 21) and overexpression of Src appears to slightly modify the proportion of MT1-MMP in caveolae, we next examined the effect of S1P-dependent phosphorylation of MT1-MMP on its subcellular localization in endothelial cells (HUVEC). In unstimulated cells, a basal and diffuse staining of phospho-MT1-MMP was observed (Fig. 4C), in agreement with the results obtained by immunoprecipitation. Interestingly, stimulation of the cells with S1P induced a striking relocalization of the enzyme to the cell periphery, and this relocalization was correlated with a partial colocalization of phospho-MT1-MMP with phosphocaveolin-1 within these structures (Fig. 4, D and E, white arrows). The localization of phospho-MT1-MMP at the cell periphery in migrating cells thus suggests that tyrosine phosphorylation of the enzyme may play an important role in cell migration induced by S1P.

MT1-MMP Phosphorylation Is Important for Tumor and Endothelial Cell Migration—It is well established that MT1-MMP plays a key role in cell migration (reviewed in Ref. 5). To assess the role of phosphorylated MT1-MMP in tumor and endothelial cell migration, fibrosarcoma (HT-1080) or endothelial (HUVEC and BAEC) cells were transfected with either WT or Y573F forms of MT1-MMP. In cells that express relatively high levels of endogenous MT1-MMP (HUVEC and HT-1080), overexpression of

WT MT1-MMP had a modest stimulatory effect on migration, but the Y573F mutant strongly diminished migration induced by either S1P or serum, suggesting that the mutant acted in a dominant-negative manner (Fig. 5, A and B). As reported previously (34), overexpression of MT1-MMP in BAEC, which are largely devoid of the enzyme, induced a marked increase in

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migration induced by either S1P or serum (Fig. 5C). However, this stimulatory effect was markedly reduced upon expression of the nonphosphorylatable MT1-MMP mutant. These results thus indicate that the tyrosine residue located within the MT1-MMP cytoplasmic domain plays an important role in cell migration, possibly by acting as a tyrosine phosphorylation site within the enzyme.

DISCUSSION

Although the involvement of MT1-MMP in cell migration has been mostly studied in terms of its proteolytic activity toward a wide variety of ECM (10, 17–19) or cell surface-associated (11, 12) proteins, there is also increasing evidence supporting an important role of the short intracellular cytoplasmic domain of the enzyme in this event. For example, the migration of a number of tumor (25, 27, 29, 31) or endothelial (34) cell lines is impaired following deletion of the intracellular sequence of MT1-MMP, an inhibitory effect that may be related to defective endocytosis of the protein (27), reduced activation of the extracellular signal-regulated protein kinase signaling cascade (31), as well as impaired interaction of the enzyme with tyrosine-phosphorylated caveolin-1 (33).

The results reported in the current work suggest that the importance of the cytoplasmic domain in MT1-MMP-dependent cell migration may also be related to the phosphorylation of the unique tyrosine residue located within this intracellular domain. First, we observed that tyrosine-phosphorylated proteins associated with the MT1-MMP immunoprecipitates had molecular masses and isoelectric points very similar to the enzyme, strongly suggesting that they indeed represent phosphorylated forms of MT1-MMP. This was supported by the inhibition of phosphorylation by site-directed mutagenesis of this tyrosine residue as well as by the specific recognition of the phosphorylated forms of the enzyme by phosphospecific antibodies raised against the phosphorylated sequence. Because these antibodies did not recognize MT1-MMP lacking a single tyrosine residue located within its intracellular domain, these observations clearly indicate that, at least under the experimental conditions used in our study, MT1-MMP was phosphorylated on its unique cytoplasmic tyrosine residue.

The tyrosine phosphorylation of MT1-MMP required concomitant expression of Src and was completely abolished by overexpression of a kinase-defective Src mutant, indicating that Src and/or a kinase activated by Src plays an essential role in this event. Although under these conditions, Src also induced phosphorylation of caveolin-1, leading to the interaction of MT1-MMP with phosphocaveolin-1 (Fig. 2 and Ref. 33), our results indicate that this interaction was neither necessary for nor a consequence of the phosphorylation of MT1-MMP. However, we observed that reduction of caveolin-1 levels increased the expression and the extent of phosphorylation of MT1-MMP, suggesting that caveolin-1 may regulate the turnover of the enzyme.

Interestingly, tyrosine phosphorylation of MT1-MMP was not restricted to cells overexpressing Src but could also be detected in untransfected cells stimulated with the chemoattractant S1P. This tyrosine phosphorylation of MT1-MMP was correlated with the relocalization of the enzyme at the cell

periphery following stimulation with S1P, raising the interesting possibility that this phosphorylation could participate in the migratory processes induced by this lipid. Moreover, S1P-mediated phosphorylation of MT1-MMP induced a partial colocalization of the enzyme with phosphocaveolin-1, a phosphoprotein that was previously shown to be important for endothelial cell movement (40). The importance of MT1-MMP phosphorylation is also suggested by the ability of a nonphosphorylatable MT1-MMP mutant to inhibit migration of cells endogenously expressing MT1-MMP, such as HT1080 and HUVEC, as well as by its failure to stimulate migration in BAEC, a cell line that expresses much lower levels of the enzyme. This S1P-dependent tyrosine phosphorylation of MT1-MMP could thus explain the preferential cooperation of the enzyme with this lipid for the induction of migration and morphogenic differentiation of endothelial cells (34) and the absence of promigratory effects of S1P in cells lacking MT1-MMP (41).

Phosphorylation of tyrosine 573 within the MT1-MMP intracellular domain was unexpected because this region lacks known recognition motifs for tyrosine kinases (R/K/Q) X_{2-4} (D/E) X_{2-3} (pY) (42) or binding motifs for either Src homology 2 (pYXX(I/L)) or 3 (PXXP) domains that would facilitate recruitment of tyrosine kinases. Despite the lack of these consensus sequences known to be involved in signal transduction, previous results have nevertheless suggested that the sequence encompassing this tyrosine residue plays an important role in MT1-MMP function. The LLY sequence was found to be essential for internalization of the enzyme at the migration front through its involvement in the interaction of MT1-MMP with the adaptor 2 complex (27), suggesting that reversible phosphorylation of this tyrosine residue may play a role in this interaction. In this respect, it is noteworthy that the internalization of the T cell surface receptor CTLA-4 from the plasma membrane involves the interaction of the receptor cytoplasmic sequence 165 YVKM 168 with the adaptor complex μ 2 and that phosphorylation of the tyrosine residue abolishes this binding, possibly by disrupting the hydrophobic pocket necessary for the interaction (43, 44). Such a negative regulatory role of phosphorylation on the endocytosis of MT1-MMP is also suggested by the recent observation that v-Src-transformed cells activate a FAK-dependent mechanism that reduces endocytosis of MT1-MMP, increasing cell surface expression of the enzyme and the resulting degradation of the ECM (45). Based on our observation that MT1-MMP is tyrosine-phosphorylated and that a nonphosphorylatable mutant of the enzyme inhibited cell migration, it is thus tempting to speculate that the induction of MT1-MMP-dependent migration by chemoattractants such as S1P could involve tyrosine phosphorylation of the enzyme, leading to alteration in its rate of internalization and to a resulting increase in ECM degradation and migration. Further studies on the impact of tyrosine phosphorylation of MT1-MMP on its rate of internalization are currently underway and should provide interesting insights on the role of this event in the regulation of the function of the enzyme.

Although the important role of MT1-MMP in tumor invasion and angiogenesis makes it an attractive drug target, the participation of the enzyme in normal skeletal development suggest that the inhibition of its catalytic activity may lead to

severe side effects (46). In this context, the observation that tyrosine phosphorylation of the MT1-MMP cytoplasmic domain plays an important role in both tumor and endothelial cell migration suggests that interfering with the intracellular pathways involved in this event may represent an alternative strategy to inhibit tumor progression triggered by this crucial enzyme.

Acknowledgments—We thank Marguerite Buchanan for helpful advice on two-dimensional gel electrophoresis and Marcos Di Falco for critical analysis of mass spectrometry studies.

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