

Src-mediated Tyrosine Phosphorylation of Caveolin-1 Induces Its Association with Membrane Type 1 Matrix Metalloproteinase*

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We have recently shown that stimulation of endothelial cells with vascular endothelial growth factor (VEGF) induces dissociation of caveolin-1 from the VEGFR-2 receptor, followed by Src family kinase-dependent tyrosine phosphorylation of the protein (Labrecque, L., Royal, I., Surprenant, D. S., Patterson, C., Gingras, D., and Béliveau, R. (2003) *Mol. Biol. Cell* 14, 334–347). In this study, we provide evidence that the VEGF-dependent tyrosine phosphorylation of caveolin-1 induces interaction of the protein with the membrane-type 1 matrix metalloproteinase (MT1-MMP). This interaction requires the phosphorylation of caveolin-1 on tyrosine 14 by members of the Src family of protein kinases, such as Src and Fyn, because it is completely abolished by expression of a catalytically inactive Src mutant or by site-directed mutagenesis of tyrosine 14 of caveolin-1. Most interestingly, the association of MT1-MMP with phosphorylated caveolin-1 induced the recruitment of Src and a concomitant inhibition of the kinase activity of the enzyme, suggesting that this complex may be involved in the negative regulation of Src activity. The association of MT1-MMP with phosphorylated caveolin-1 occurs in caveolae membranes and involves the cytoplasmic domain of MT1-MMP because it was markedly reduced by mutation of Cys⁵⁷⁴ and Val⁵⁸² residues of the cytoplasmic tail of the enzyme. Most interestingly, the reduction of the interaction between MT1-MMP and caveolin-1 by using these mutants also decreases MT1-MMP-dependent cell locomotion. Overall these results indicate that MT1-MMP associates with tyrosine-phosphorylated caveolin-1 and that this complex may play an important role in MT1-MMP regulation and function.

There is considerable evidence that angiogenesis, *i.e.* the growth of novel capillaries from preexisting vessels, sustains the progression of many diseases including diabetic retinopa-

thy, psoriasis, rheumatoid arthritis, and tumor progression (1). Upon oxygen and nutrient deprivation, tumor cells promote neovascularization by inducing the expression of angiogenic cytokines, such as the vascular endothelial growth factor (VEGF).¹ VEGF is a potent and unique angiogenic protein that induces endothelial cell (EC) proliferation, migration, and vascular permeability and acts as a crucial survival factor for EC (2).

The mechanisms underlying the proangiogenic effects of VEGF on EC have been the subject of considerable interest in recent years, leading to the identification of a variety of signaling pathways that are involved in EC proliferation and migration (3). Among these, we have recently shown that the predominant VEGF receptor, VEGFR-2, is localized to caveolin-enriched membrane domains and that this localization is important for proper induction of downstream signaling events by VEGF (4). VEGFR-2 was shown to be associated with caveolin-1 in cells in “resting conditions,” whereas dissociation of this complex is observed following stimulation of EC with VEGF, leading to Src family kinase-dependent tyrosine phosphorylation of caveolin-1 (4). This suggests that caveolin-1 may play an important role in angiogenesis, an hypothesis strengthened by the observations that major changes in caveolin-1 levels are observed following treatment of EC with angiogenic cytokines (5) and during differentiation of these cells into capillary-like structures (6).

Caveolin-1 is the major structural protein of caveolae and also serves as a scaffold for a variety of signaling complexes because of the presence of a domain, corresponding to amino acids 82–101, that interacts with consensus motifs present in several signaling proteins, including VEGF, platelet-derived growth factor receptors, and the Src family kinases (SFK) (4, 7, 8). Caveolin-1 was first identified as a major tyrosine-phosphorylated protein in v-Src-transformed embryo fibroblasts (9), but paradoxically, the role of tyrosine-phosphorylated caveolin-1 still remains largely unknown. Caveolin-1 is phosphorylated on tyrosine 14 in response to a number of growth factors such as VEGF, epidermal growth factor, and platelet-derived growth factor (4, 10, 11), and many lines of evidence indicate that SFK play an essential role in this process (12). Tyrosine-phosphorylated caveolin-1 specifically interacts with the C-terminal Src kinase (Csk), a negative-regulator of SFK, suggesting that it may be involved in the negative regulation of Src kinase activ-

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This paper is dedicated to the memory of Dr. Francine Denizeau, whose gentleness, determination, and extraordinary passion for all aspects of biological sciences will always be remembered.

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¹ The abbreviations used are: VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1-MMP; ECM, extracellular matrix; EC, endothelial cell; BAEC, bovine aortic endothelial cell; SFK, Src-family kinases; Csk, C-terminal Src kinase; RT, reverse transcription; mAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; MES, 4-morpholineethanesulfonic acid.

ity (13). Tyrosine-phosphorylated caveolin-1 is also specifically associated with Grb7 (14), a signaling protein involved in growth factor-induced cell migration, and this association may serve to link phosphocaveolin-1 to the focal adhesion machinery involved in cell locomotion (14).

Cell migration is a highly complex process that requires the coordinate interplay of several extracellular matrix receptors and matrix-degrading enzymes, including members of the matrix metalloproteinase (MMP) family (15). There is now considerable evidence that MMPs that are intrinsically associated with the plasma membrane due to 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 (16). The prototypical member of this family, MT1-MMP, plays a major role in the ECM remodeling both directly, by degrading several of its components (17), and indirectly, by activating MMP-2 (18). Several lines of evidence support an important role for MT1-MMP in both tumor invasion and angiogenesis. Overexpression of MT1-MMP promotes cell migration of a number of cancer (19–21) and endothelial (22–25) cells, a process that involves the short cytoplasmic domain of the protein (19, 20, 26). MT1-MMP overexpression also induces tumorigenicity, growth, and vascularization of cancer cells (27, 28); these effects were mediated, at least in part, by an Src-dependent up-regulation of VEGF at both the mRNA and protein levels (27, 29). At the endothelial cell level, MT1-MMP expression is increased by angiogenic factors such as VEGF and fibroblast growth factor-2 (FGF-2), and the enzyme cooperates with both sphingosine 1-phosphate and VEGF to induce EC migration and morphogenic differentiation into capillary-like structures (24). Such an important role of MT1-MMP in angiogenesis was also supported by the observation that MT1-MMP-null mice failed to exhibit an angiogenic response to FGF-2 in an *in vivo* corneal angiogenesis assay (30).

Subcellular compartmentalization of ECM receptors as well as matrix-degrading enzymes into discrete areas of the plasma membrane is also important for the regulation of cell migration and invasion (15). Accordingly, MT1-MMP is concentrated at the leading edge of migrating cells, an appropriate location for the degradation of the ECM barrier to facilitate invasion of the ECM by tumor and endothelial cells. We (31) and others (32–34) have recently shown that MT1-MMP is also associated with caveolae membrane domains, suggesting that this localization may be important for the regulation of this activity. In this regard, caveolae were identified as a major endocytic pathway for MT1-MMP in both EC (32) and tumor (33) cells and are important for the constitutive endocytosis and recycling of the protein to the plasma membrane (33). In EC, this localization appears crucial for angiogenesis because chemical disruption of caveolae or selective interference with caveolin-1 expression disrupt MT1-MMP localization and activity, leading to diminished cell migration and morphogenic differentiation (32). This raises the interesting possibility that MT1-MMP may interact with a number of caveolae-associated signaling proteins to induce stimulation of EC function.

In this study, we present evidence that MT1-MMP associates with phosphorylated caveolin-1 following stimulation of EC with VEGF. By using model cell systems, we show that this association occurs in caveolae membrane domains, is dependent on Src family kinase-dependent phosphorylation of tyrosine 14 of caveolin-1, and involves the cytoplasmic domain of MT1-MMP.

MATERIALS AND METHODS

Reagents and Antibodies—Cell culture media were obtained from Invitrogen, and serum was from HyClone Laboratories (Logan, UT).

Electrophoresis reagents were purchased from Bio-Rad. The anti-MT1-MMP polyclonal antibodies AB815 were from Chemicon (Temecula, CA). Anti-clathrin heavy chain mAb (clone 23), mAbs against caveolin-1 (C37120), phospho-caveolin (Tyr(P)-14, C91520), and polyclonal antibody against caveolin (C13630) were from Transduction Laboratories (Lexington, KY). mAb against Src (05-184) was from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal antibody against FAK (sc-557) and mAbs against Fyn (sc-434) and phosphotyrosine (PY99) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK polyclonal antibody was from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit IgG horseradish peroxidase-linked secondary antibodies were purchased from Jackson Immuno-Research (West Grove, PA), and enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences. FuGENE-6 Transfection Reagent was purchased from Roche Applied Science. TRIzol Reagent and the Superscript™ One-step RT-PCR with Platinum® Taq were from Invitrogen. Micro BCA protein assay reagents were from Pierce. All other reagents were from Sigma.

Plasmids—The cDNA encoding full-length human MT1-MMP was described previously (31, 35). Full-length wild-type human MT1-MMP cDNA without both 5'- and 3'-untranslated regions was subcloned between the HindIII and EcoRI sites of pCDNA3.1 Zeo⁺ mammalian expression vector (Invitrogen) and was used as template. All mutations were generated by site-directed mutagenesis using PCR. Plasmid template DNA (5–50 ng) was added to dNTP (10 μ M each), 1 \times Pfu buffer, Pfu/Pfu turbo DNA polymerase (2.5 units, Stratagene, Amsterdam, The Netherlands), and 125 ng of each mutagenic primers. Samples were then incubated at 95 °C for 30 s and then for 12–18 cycles of 95 °C for 30 s, 55 °C for 1 min, and 74 °C for 12 min. Methylated parental DNA template was next digested using DpnI (10 units) restriction enzyme for 1 h at 37 °C. An aliquot of the reaction was then directly transformed in XL1 Blue supercompetent *Escherichia coli* cells (Stratagene) and plated on LB agar plates containing 100 μ g/ml ampicillin. Clones were selected for each mutant and sequenced using Big Dye Terminator kit (Applied Biosystems, Warrington, UK) in order to verify the presence of the desired mutation.

A cDNA encoding caveolin-1 was kindly provided by Dr. S.-S. Yoon (Sung Kyon Kwan University, Korea). The Y14F caveolin-1 mutant was constructed by using PCR and using the full-length caveolin-1 cDNA as the template and a sense primer containing the desired mutation. The wild-type and dominant-negative Src plasmids were kindly provided by Dr. Isabelle Royal (Université de Montréal, Canada), and the plasmid encoding Fyn was from Dr. Marilyn Resh (Yale University).

VEGF Production—Vascular endothelial growth factor (isoform 165) was cloned into pTT vector (36) and produced following large scale transient transfection of human 293SFE cells in serum-free medium (37). The culture was harvested 5 days after transfection, and the medium was clarified by centrifugation at 3500 \times *g* for 10 min and filtered through 0.22- μ m membrane. Clarified culture medium was loaded on a heparin-Sepharose column, and bound VEGF was eluted by using a NaCl gradient in phosphate-buffered saline. A buffer exchange for phosphate-buffered saline was performed by gel filtration, and the final purified material was sterile-filtered, aliquoted, and stored at –80 °C.

Cell Culture—Bovine aortic endothelial cells (BAEC) were purchased from Clonetics (San Diego, CA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with low glucose, containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin, and grown in a humidified atmosphere containing 5% CO₂ at 37 °C. The monkey kidney cells COS-7 were purchased from Clonetics. The human embryonic kidney cell line 293T was kindly provided by Dr. M. Park (McGill University, Montreal, Quebec, Canada). COS-7 and 293T cells were maintained in DMEM high glucose containing 10% fetal bovine serum and antibiotics and grown as described above.

Transfection—The transient transfection of the plasmids (2 μ g) in subconfluent COS-7 cells (5×10^5) was performed using the nonliposomal formulation FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche Applied Science). All experiments using these cells were performed 40 h post-transfection. 293T cells (6×10^5) were transiently transfected with 2 μ g of the indicated cDNAs using the calcium phosphate precipitation method (38). 18 h post-transfection, the culture medium was replaced with fresh complete medium, and 60 h post-transfection, the 293T cells were harvested and used for immunoprecipitation assays.

Caveolae Isolation—Caveolae were purified using a hyperosmotic carbonate method described previously (39). Briefly, confluent COS-7 cells cultured in 100-mm² dishes were treated as described above and were scraped into 2 ml of 0.5 M sodium carbonate (pH 11) and homog-

enized extensively using a Polytron (three 15-s bursts at medium speed) followed by sonication (five 15-s bursts at 50% of maximal power). The resulting homogenate was brought to 45% sucrose by the addition of 2 ml of 90% sucrose in MES-buffered saline (MBS; 25 mM MES (pH 6.5), 150 mM NaCl) and overlaid with two layers of 35 and 5% sucrose in MBS containing 0.25 M carbonate (4 ml each). The gradient was then centrifuged at $200,000 \times g$ 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 (4–6) or non-caveolae (8–12) fractions were pooled, diluted in 10 mM Tris-HCl (pH 7.5), and centrifuged at $100,000 \times g$.

Stimulation of Endothelial Cells with VEGF—BAEC grown to confluence were serum-starved for 18 h in serum-free DMEM, followed by incubation for 2–30 min at 37 °C in 2 ml of serum-free DMEM containing 100 ng/ml human recombinant VEGF. In some experiments, cells were treated for 60 min with 10 mM β -cyclodextrin before stimulation with VEGF. After VEGF treatment, cells were washed once with phosphate-buffered saline containing 1 mM sodium orthovanadate and were incubated in the same medium for 1 h at 4 °C. Cells were then solubilized in SDS lysis buffer (10 mM Tris-HCl, 1% SDS, 1 mM sodium orthovanadate), followed by boiling for 5 min at 100 °C and homogenization using a 26-gauge needle. Cells were alternatively solubilized in lysis buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 1% Triton X-100, 60 mM *n*-octylglucoside, 1 mM sodium orthovanadate), and the resulting lysates were clarified by centrifugation at $10,000 \times g$ for 10 min. Protein concentrations were determined by the bicinchoninic acid method (Pierce).

Immunoprecipitation and Western Blotting—For the immunoprecipitation studies, identical amounts of proteins from each sample were incubated in the SDS lysis buffer overnight at 4 °C in the presence of 1–4 μ g/ml of a specific antibody. The immune complexes were collected by incubating the mixtures with 25 μ l (50% suspension) of protein A (rabbit primary antibody) or protein G (mouse primary antibody)-Sephacrose beads. Nonspecifically bound proteins were removed by washing the beads three times in 1 ml of lysis buffer, and bound material was solubilized in 25 μ l of 2-fold concentrated Laemmli sample buffer, boiled for 5 min, and resolved by SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membranes, blocked 1 h at room temperature with TBST buffer (147 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.1% Tween 20) containing 2% bovine serum albumin, and incubated with primary antibody overnight at 4 °C. Immunoreactive bands were revealed following a 1-h incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies, and the signals were visualized with an ECL detection system.

In Vitro Src Kinase Assay—Src was immunoprecipitated from transfected COS-7 cells lysates as described above. The beads were washed three times with lysis buffer, followed by one additional wash with kinase buffer (20 mM Hepes, pH 7.4, 10 mM $MnCl_2$). The washed beads were then resuspended in 40 μ l of kinase buffer with 1 μ g of acid-denatured rabbit muscle enolase (Sigma) as an exogenous substrate. Enolase was denatured by incubation with an equal volume of 50 mM acetic acid for 15 min at 37 °C. Following a 2-min preincubation of the mixtures at 30 °C, the reactions were initiated by the addition of 10 μ Ci of [γ - 32 P]ATP (ICN Biochemicals), and reactions were stopped after 10 min by the addition of 5-fold concentrated Laemmli sample buffer. Samples were subjected to electrophoresis on 9% acrylamide/bisacrylamide gels and visualized by exposure to Fuji films.

RNA Isolation and Reverse Transcription-PCR—To monitor the effect of Src on MT1-MMP mRNA levels, total RNA was isolated from the transfected cells using the TRIzol Reagent, according to the instructions of the manufacturer. One μ g of total RNA isolated from cells was amplified with Superscript™ One-step RT-PCR with Platinum® Taq, using specific primers designed for human MT1-MMP: sense, 5'-CAACACTGCCTACGAGAGGA-3' and antisense, 5'-GTTCTACCTTCAGCTTCTGG-3'. RT-PCR was carried out under the following conditions: 1 cycle (50 °C, 30 min; 92 °C, 2 min) for the reverse transcription, 30 cycles (92 °C, 30 s; 50 °C, 30 s; 72 °C, 45 s) for the amplification, and 1 cycle (72 °C, 7 min) for the final extension. Products were analyzed using 1.0% agarose gels containing ethidium bromide.

Cell Migration—Cell migration assays were performed essentially as described (35). COS-7 cells were transfected with Src and either the wild-type or mutated versions of MT1-MMP and plated on gelatin-coated modified Boyden chambers. Migration was allowed to proceed for 3 h, and the amount of cells that had migrated to the lower face of the wells was quantitated by computer-assisted imaging (24).

RESULTS

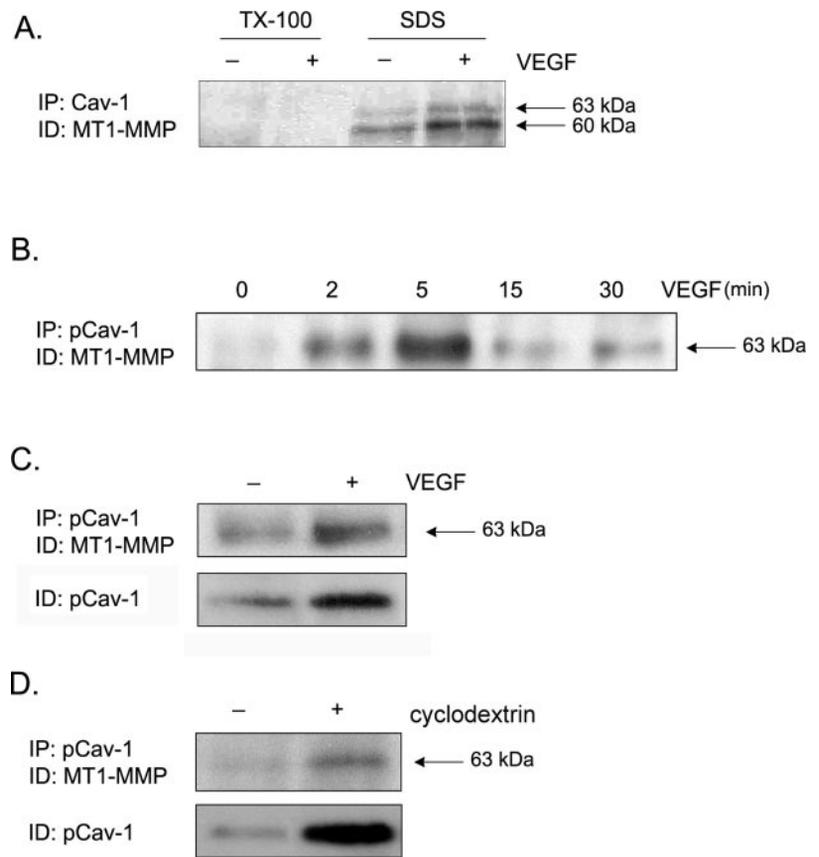
MT1-MMP Is Associated with Phosphorylated Caveolin-1 in Endothelial Cells—Previous work from our laboratory has shown that both caveolin-1 and MT1-MMP may play important roles in angiogenesis. On the one hand, caveolin-1 negatively regulates VEGFR-2 activity under resting conditions, and stimulation of EC by VEGF leads to dissociation of the complex and to the phosphorylation of the protein (4). On the other hand, MT1-MMP markedly increases EC migration and differentiation into capillary-like structures induced by VEGF and sphingosine 1-phosphate, suggesting that this protein is important for the angiogenic program triggered by these important stimuli (24). However, the potential relationship between caveolin-1 and MT1-MMP remains unknown.

We first attempted to determine whether these two proteins are physically associated following stimulation of endothelial cells with VEGF, a potent angiogenesis inducer. Because both proteins are localized in caveolae, which are characterized by their detergent-resistant properties (40), this possible interaction of MT1-MMP and caveolin-1 was evaluated by using two different lysis buffers, containing either nonionic detergents (1% Triton X-100 and 0.5% Nonidet P-40) or 1% SDS. Bovine aortic endothelial cells (BAEC) were serum-starved overnight and stimulated with VEGF for 5 min, and lysates from the treated cells were used to immunoprecipitate caveolin-1. As shown in Fig. 1A, there was no detectable association of caveolin-1 with MT1-MMP following lysis of VEGF-treated cells with nonionic detergents (Triton X-100). By contrast, lysis of the cells with SDS allowed the identification of the 60–63-kDa forms of MT1-MMP in the caveolin-1 immunoprecipitates (Fig. 1A). For unknown reasons, the presence of the 60-kDa form was highly variable and, in most experiments, immunoprecipitation of caveolin-1 resulted in the preferential recovery of the 63-kDa form of MT1-MMP. Under these conditions, however, the MT1-MMP polyclonal antibodies used in this study failed to precipitate significant amounts of the enzyme (data not shown), precluding analysis of the caveolin-1/MT1-MMP by reverse immunoprecipitation.

Caveolin-1 has been shown to be tyrosine-phosphorylated in response to stimulation of endothelial cells with VEGF (4). To investigate the possibility that the association between MT1-MMP and caveolin-1 could be induced by phosphorylation of caveolin-1, serum-starved BAEC were stimulated with VEGF for 2–30 min, and the phosphorylated form of caveolin-1 was precipitated using an antibody specifically recognizing caveolin-1 phosphorylated on tyrosine 14. We observed that addition of VEGF to BAEC leads to a marked increase in the association of MT1-MMP with phosphocaveolin-1; this interaction was maximum at 5 min (Fig. 1B). The formation of the complex was correlated with VEGF-induced caveolin-1 phosphorylation (Fig. 1C). To investigate further the relationship between caveolin-1 phosphorylation and its association with MT1-MMP, BAEC were treated with β -cyclodextrin, a cholesterol-binding agent that extracts cholesterol from the plasma membrane and that leads to an increase in the phosphorylation of caveolin-1 (4). BAEC were incubated in the presence of 10 mM β -cyclodextrin and stimulated with VEGF, and phosphorylated caveolin-1 was immunoprecipitated from the resulting lysates. As observed following stimulation with VEGF, cholesterol depletion leads to an increase in caveolin-1 phosphorylation and its association with MT1-MMP (Fig. 1D). Overall, these results demonstrate that MT1-MMP associates with caveolin-1 in endothelial cells and suggest that phosphorylation of caveolin-1 is involved in this interaction.

Src Kinases Are Essential for the Association of MT1-MMP with Caveolin-1—VEGF-dependent tyrosine phosphorylation

FIG. 1. MT1-MMP associates with phosphorylated caveolin-1 in VEGF-stimulated endothelial cells. *A*, BAEC protein extracts prepared with a buffer consisting of 1% Triton X-100 and 0.5% Nonidet P-40 (TX-100) or using a boiling SDS lysis buffer, containing 1% SDS, were subjected to immunoprecipitation (IP) using a monoclonal antibody specific for caveolin-1, and associated MT1-MMP was monitored by Western blotting. *B* and *C*, BAEC were serum-starved for 18 h and incubated in the presence of 100 ng/ml VEGF for the indicated times (*B*) or for 5 min (*C*). Cells were lysed with SDS buffer, and phosphorylated caveolin-1 was specifically immunoprecipitated using an anti-phosphocaveolin-1 mAb. Associated MT1-MMP was monitored by Western blotting. *D*, cells were incubated for 1 h in the presence of 10 mM β -cyclodextrin prior to VEGF stimulation, and associated MT1-MMP was monitored by immunoprecipitation and Western blotting. *ID*, immunodetection.



of caveolin-1 likely involves SFK, based on the inhibitory effect of the Src inhibitor PP2 on this event (4). To determine the possible involvement of SFK in the association of MT1-MMP with caveolin-1, we used COS-7 cells, a monkey kidney cell line that expresses important levels of caveolin-1, but no detectable MT1-MMP. COS-7 cells were transiently cotransfected with wild-type MT1-MMP, in the absence or presence of Src or Fyn, and the extent of association of MT1-MMP to caveolin-1 was monitored by immunoprecipitation. As shown in Fig. 2*A*, we observed that the addition of Src or Fyn to MT1-MMP-transfected cells promoted a dramatic increase in the association of MT1-MMP to caveolin-1. A similar level of MT1-MMP was detected in immunoprecipitation performed using antibodies recognizing total caveolin-1 or with a mAb specific to the phosphorylated form of the protein, suggesting that the total fraction of caveolin associated with MT1-MMP is phosphorylated (Fig. 2*A*). Most interestingly, we routinely observed that Src or Fyn also induced a slight but significant increase in MT1-MMP expression (Fig. 2*A*), and this increase was correlated with an augmentation of MT1-MMP mRNA levels upon overexpression of Src (Fig. 2*B*). A similar finding was recently observed in Madin-Darby canine kidney cells transformed by v-Src, and in this latter case, MT1-MMP overexpression was associated with the invasive phenotype of the transformed cells (41).

Active Src Is Essential for the Association of MT1-MMP with Caveolin-1—To confirm further that the increase of caveolin-1/MT1-MMP association by Src overexpression is the result of caveolin-1 phosphorylation, COS-7 cells were transfected with a dominant-negative form of Src in which the ATP-binding site is inactivated and the intramolecular interaction of the SH2 domain is prevented (Y527F and K295R mutant) (42). As shown in Fig. 3*A*, we observed that the association of total or tyrosine-phosphorylated caveolin-1 with MT1-MMP is greatly reduced after overexpression of the dominant-negative Src mutant compared with wild-type Src. This effect of the mutant

was correlated with its lack of phosphorylation of caveolin-1 (Fig. 3*A*) and enolase (Fig. 3*B*), further suggesting that tyrosine phosphorylation of caveolin-1 by Src plays a central role in the interaction of the protein with MT1-MMP. Under these conditions, the Src mutant was expressed as two immunoreactive bands, suggesting that a subset of the enzyme was proteolytically processed in COS-7 cells (Fig. 3*A*). The presence of this proteolytic fragment is, however, not responsible for the lack of activity of the Src mutant because coexpression of the dominant-negative mutant along with MT1-MMP and caveolin-1 in 293T cells, a cell system in which the Src mutant was not proteolytically processed, also abolished phosphorylation of caveolin-1 and its interaction with MT1-MMP (not shown).

Unexpectedly, we observed that although Src activity was essential to induce caveolin-1 phosphorylation and its subsequent association with MT1-MMP, MT1-MMP expression significantly inhibited caveolin-1 phosphorylation (Fig. 3*A*), and this effect was correlated with reduced Src kinase activity toward enolase (Fig. 3*B*). This suggests that the formation of the caveolin-1-MT1-MMP complex may be involved in the negative regulation of Src kinase activity.

Although caveolin-1 possesses several tyrosine residues that are known to be phosphorylated (43), tyrosine 14 represents the main site of phosphorylation by SFK (12). To determine the role of this residue in the formation of the caveolin-1-MT1-MMP complex, a caveolin-1 mutant in which tyrosine 14 was replaced with a phenylalanine residue was overexpressed in 293T cells, a human embryonic kidney cell line that expresses extremely low levels of endogenous caveolin-1 (44). As shown in Fig. 3*C*, coexpression of wild-type caveolin-1 with Src induces a strong phosphorylation of caveolin-1, and this effect was completely abolished upon mutation of tyrosine 14 of the protein. To test the effect of this residue on MT1-MMP/caveolin-1 association, both forms of caveolin-1 were transfected in the presence of MT1-MMP and Src, and caveolin-1 was immunoprecipi-

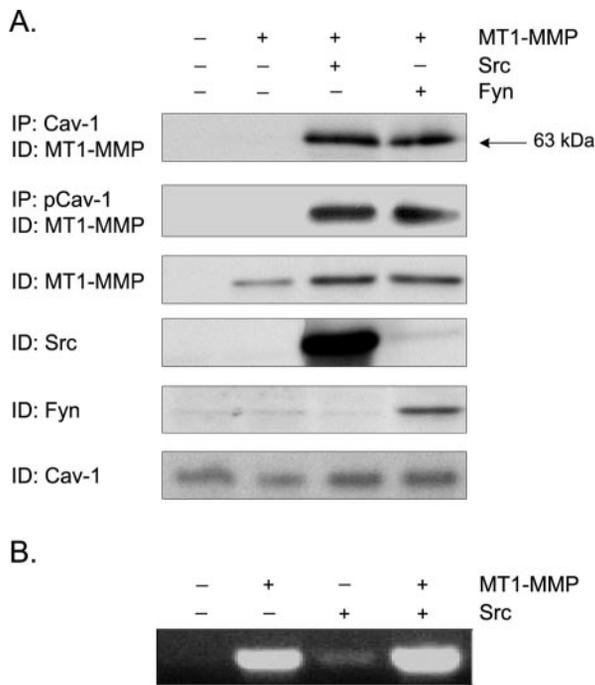


FIG. 2. Src family kinases induced association of MT1-MMP with caveolin-1. *A*, COS-7 cells were transfected with either pcDNA3.1 or wild-type MT1-MMP, Src, or Fyn, as indicated. 48 h post-transfection, cells were harvested and subjected to immunoprecipitation (IP) with a monoclonal antibody raised against caveolin-1 or against its phosphorylated form. Associated MT1-MMP was monitored by Western blotting. The expression levels of transfected proteins were monitored by Western blotting using specific antibodies. *B*, MT1-MMP mRNA levels in Src-transfected COS-7 cells were monitored by RT-PCR. *ID*, immunodetection.

tated. As shown in Fig. 3C, MT1-MMP is not detected in immunocomplexes when the Y14F caveolin-1 mutant is expressed along with MT1-MMP and active Src kinase. Overall, these results demonstrate that phosphorylation of caveolin-1 on tyrosine 14 by Src family kinases plays an essential role in promoting the association of MT1-MMP with caveolin-1.

The Association of MT1-MMP with Caveolin-1 Occurs in Caveolae Membranes—MT1-MMP has been reported previously (31, 33, 34) to be present in caveolin-1-enriched membrane domains. To investigate whether or not the interaction between MT1-MMP and caveolin-1 occurs in caveolae membranes, we purified caveolae and non-caveolae membrane fractions from COS-7 cells transfected with either MT1-MMP or Src, alone or in combination. We observed that under these experimental conditions, MT1-MMP is highly enriched in caveolae membranes and that interaction of the enzyme with caveolin-1 in these domains was markedly stimulated by the expression of Src (Fig. 4). Here again, tyrosine phosphorylation of caveolin-1 by Src was significantly reduced in the presence of MT1-MMP. These results thus suggest that the formation of the MT1-MMP-caveolin-1 complex occurs in caveolae and that these domains may be involved in the down-regulation of Src kinase activity.

MT1-MMP Potentiates Caveolin-1/Src Association—The association of caveolin-1 with Src is well documented and involves palmitoylation of cysteine 156 and the scaffolding domain of the protein (7, 45). Because this interaction has been shown to result in the inhibition of Src activity, we tested the possibility that the inhibition of Src activity observed upon expression of MT1-MMP could involve an increased association of Src with caveolin-1. COS-7 cells were transiently transfected with MT1-MMP or Src, alone or in combination. Caveolin-1 was then immunoprecipitated, and the presence of Src in the com-

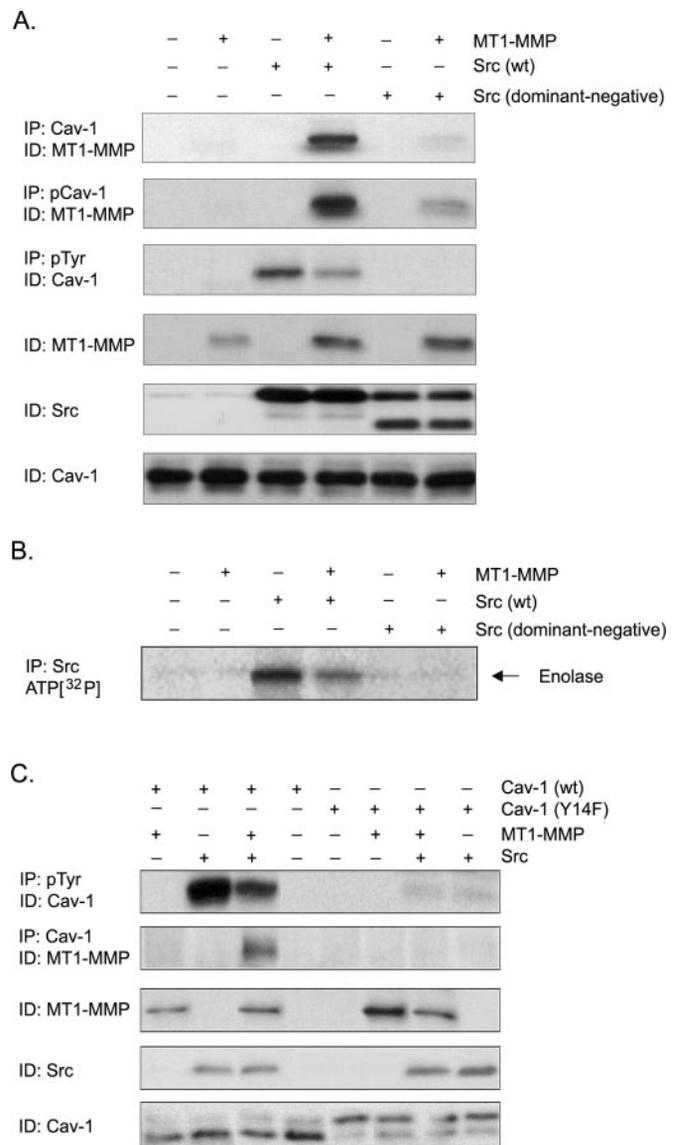


FIG. 3. Src kinase activity is essential for the association of MT1-MMP with caveolin-1. *A*, COS-7 cells were transfected with either pcDNA3.1 or wild-type MT1-MMP, Src, or a dominant-negative Src mutant, as indicated. 48 h post-transfection, cells were harvested and subjected to immunoprecipitation (IP) with monoclonal antibodies raised against caveolin-1, phosphorylated caveolin-1, or total phosphotyrosines (PY99). The presence of MT1-MMP or caveolin-1 in the immunocomplexes was monitored by Western blotting. The expression levels of transfected proteins were monitored by Western blotting using specific antibodies. *B*, Src was immunoprecipitated from whole cell lysates and incubated with acid-treated enolase as an exogenous substrate and 5 μ Ci of [γ -³²P]ATP. After electrophoresis on 9% acrylamide/bisacrylamide gels, the gels were stained with Coomassie Blue and exposed to Fuji films for 1 day. *C*, 293T cells were transfected with either pcDNA3.1 or wild-type MT1-MMP, wild-type, or mutant (Y14F) caveolin-1, and Src using the calcium phosphate method. 60 h post-transfection, cells were harvested and subjected to immunoprecipitation with specific antibodies, and associated proteins were monitored by Western blotting. The expression levels of transfected proteins were monitored by Western blotting using specific antibodies. *ID*, immunodetection.

plex was detected. As shown in Fig. 5A, the formation of the caveolin-1-Src complex was markedly increased by the presence of MT1-MMP. Most interestingly, stimulation of the formation of the caveolin-1-Src complex was observed only in caveolae membrane domains (Fig. 5B). This result suggests the existence of a negative feedback loop in which the tyrosine phosphorylation of caveolin-1 by Src family kinases induces its

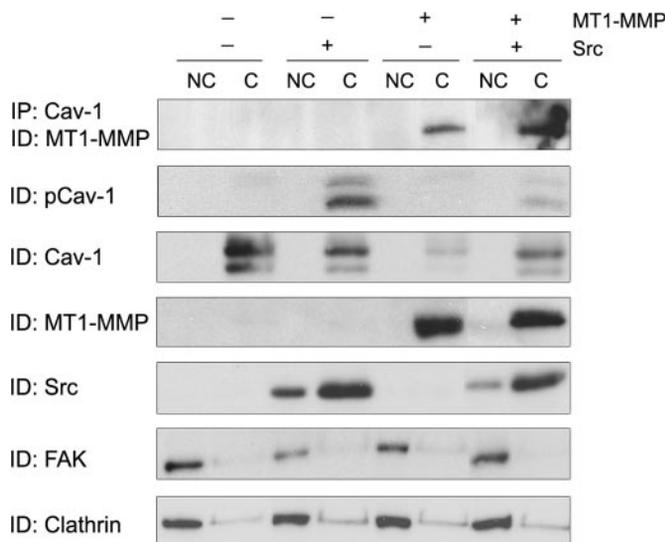


FIG. 4. **Association of MT1-MMP with caveolin-1 is preferentially observed in caveolae fractions.** COS-7 cells were transfected with wild-type MT1-MMP (MT) or Src, as indicated. 48 h post-transfection, cells were harvested and subjected to sucrose gradient sedimentation as described under "Materials and Methods." Equal amounts of proteins from caveolae (C) and non-caveolae (NC) membranes were separated by SDS-PAGE or subjected to immunoprecipitation (IP). ID, immunodetection.

association with MT1-MMP in caveolae, followed by recruitment and inactivation of the kinase activity.

Cysteine 574 and Valine 582 of MT1-MMP Are Involved in the Association with Caveolin-1—Caveolin-1 contains a membrane-spanning hairpin-like structure, with both N and C termini directed toward the cytoplasm. This suggests that the interaction between this protein and MT1-MMP likely involves the intracellular domain of the enzyme, an hypothesis strengthened by the observation that removal of the cytoplasmic region of MT1-MMP completely abolished Src-dependent association of caveolin-1 to MT1-MMP (data not shown). To locate more precisely the amino acid(s) responsible for the association of caveolin-1 with MT1-MMP, we generated a series of mutants of the cytoplasmic sequence of the protein. DNA for each mutant was transfected in COS-7 cells, and the formation of the caveolin-1-MT1-MMP and caveolin-1-Src complexes was monitored by immunoprecipitation. First, we used triple mutants, in which three adjacent amino acids were mutated to alanine residues (Fig. 6A) to identify the region of MT1-MMP cytoplasmic tail involved in the formation of the caveolin-1-MT1-MMP complex. Two mutants (C574A/Q575A/R576A and D580A/K581A/V582A) of the seven constructions used in this experiment inhibited significantly the formation of this complex (Fig. 6B). Most interestingly, these two constructs also decreased the association between caveolin-1 and Src, as well as the induction of phosphorylation of ERK, a key event involved in the stimulation of cell locomotion by MT1-MMP (35) (Fig. 6B). Next, in order to further identify the amino acids involved, single amino acid mutations were introduced in the MT1-MMP cytoplasmic tail sequence, and constructs were individually transfected in COS-7 cells to monitor the Src-dependent association between MT1-MMP and caveolin-1. All mutants were expressed at the cell surface, based on their capacity to trigger activation of an exogenous source of pro-MMP-2 (data not shown). By using these point mutants, we observed that formation of the MT1-MMP-caveolin-1 complex was markedly reduced in cells transfected with the C574S and V582A MT1-MMP cytoplasmic tail mutants (Fig. 6C). This suggests that the cytoplasmic domain of MT1-MMP plays an

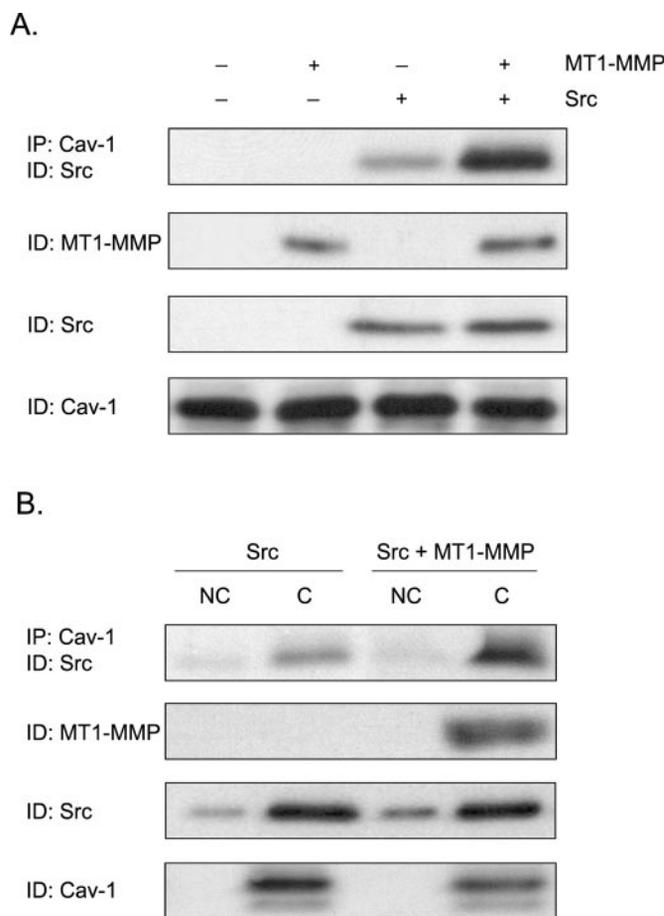


FIG. 5. **MT1-MMP increases association of caveolin-1 with Src.** A, COS-7 cells were transfected with wild-type MT1-MMP or Src, as indicated. 48 h post-transfection, cells were harvested and subjected to immunoprecipitation (IP) with polyclonal antibodies against caveolin-1, and associated Src was monitored by Western blotting. The expression levels of transfected proteins were monitored by Western blotting using specific antibodies. B, 48 h post-transfection, cells were harvested and subjected to sucrose gradient sedimentation as described under "Materials and Methods." Equal quantities of proteins from caveolae (C) and non-caveolae (NC) membranes were separated by SDS-PAGE or subjected to immunoprecipitation. ID, immunodetection.

essential role in the formation of a complex with phosphorylated caveolin-1 and that this process involves, at least in part, Cys⁵⁷⁴ and Val⁵⁸² of the protein.

Because the induction of cell migration by MT1-MMP was shown previously to involve the cytoplasmic domain of the protein, we next investigated the possibility that this effect could be correlated with an alteration of the ability of the enzyme to complex with caveolin-1. COS-7 cells were transfected with the wild-type or mutated versions of MT1-MMP, and the extent of cell migration on gelatin-coated filters was determined. Most importantly, we observed that migration was significantly impaired in cells overexpressing the C574S and V582A mutants (Fig. 7), whereas other mutants had no effect. Thus, reduced association of MT1-MMP with caveolin-1 is correlated with a significant reduction in MT1-MMP-induced cell migration, suggesting that the interaction of the enzyme with tyrosine-phosphorylated caveolin-1 likely plays a role in cell locomotion.

DISCUSSION

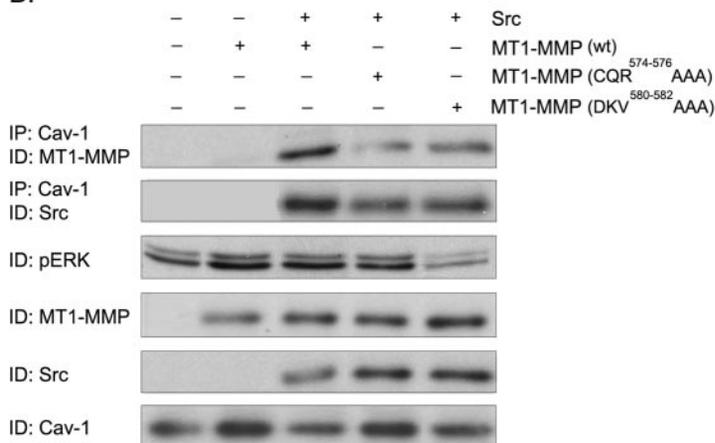
We have previously demonstrated that caveolin-1 plays an important role in VEGF-induced pathway by acting both as a negative regulator of VEGFR-2 activity under resting conditions and as a substrate that is tyrosine-phosphorylated under

A.

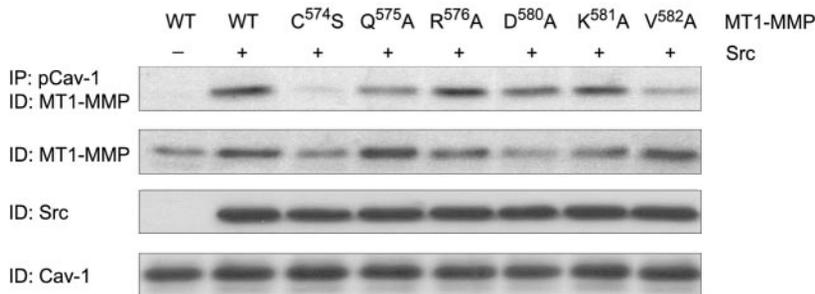
Wild type	FFFRRHGTPRRLLYCQRSLLDKV
FRR ⁵⁶²⁻⁵⁶⁴ AAA	FF AAA HGTPRRLLYCQRSLLDKV
HGT ⁵⁶⁵⁻⁵⁶⁷ AAA	FFFR RAA PRRLLYCQRSLLDKV
PRR ⁵⁶⁸⁻⁵⁷⁰ AAA	FFFRRHGT AAA LLLYCQRSLLDKV
LLY ⁵⁷¹⁻⁵⁷³ AAA	FFFRRHGTPRR AAA CQRSLLDKV
CQR ⁵⁷⁴⁻⁵⁷⁶ AAA	FFFRRHGTPRRLLY AAA SLLDKV
SLL ⁵⁷⁷⁻⁵⁷⁹ AAA	FFFRRHGTPRRLLYCQR AAA DKV
DKV ⁵⁸⁰⁻⁵⁸² AAA	FFFRRHGTPRRLLYCQRSL AAA

FIG. 6. Cysteine 574 and valine 582 from MT1-MMP are involved in its association with caveolin-1. *A*, schematic representation of the mutants used in this study. *B*, COS-7 cells were transfected with Src and either wild-type MT1-MMP or the indicated constructs containing triple mutations of adjacent residues. 48 h post-transfection, cells were harvested and subjected to immunoprecipitation with a monoclonal antibody specific for caveolin-1, and associated MT1-MMP was monitored by Western blotting. The expression levels of transfected proteins were monitored by immunoblotting using specific antibodies. *C*, COS-7 cells were transfected with Src and either wild-type MT1-MMP or the indicated point mutants, and the extent of association of MT1-MMP with caveolin-1 was determined as described above. *ID*, immunodetection.

B.



C.



activating conditions (4). In EC, phosphorylation of caveolin-1 on tyrosine 14 is required for caveolin-1 accumulation in the leading extension of transmigrating EC, a process essential for the initiation of angiogenesis (46). Extensions at the leading edge of EC are also enriched in matrix-degrading enzymes, such as MMP-2 and MT1-MMP, and cell adhesion receptors, such as the $\alpha_v\beta_3$ integrin, thereby restricting matrix proteolysis to a limited microenvironment at the cell surface (32, 34).

Although a number of reports have documented the cellular colocalization of MT1-MMP and caveolin-1 (31, 32, 34), our data show for the first time that this colocalization results from the physical association between the two proteins. We show that caveolin-1 associates with MT1-MMP and that this interaction is likely to play an important role in angiogenesis because it is modulated by stimulation of EC with VEGF and depends on the tyrosine phosphorylation status of caveolin-1. Most interestingly, the kinetics of MT1-MMP association with phosphorylated caveolin-1 follows that observed for VEGF-induced phosphorylation of caveolin-1 (4), suggesting that follow-

ing its dissociation from VEGFR-2 and subsequent tyrosine phosphorylation, caveolin-1 may interact with MT1-MMP.

This interaction was also observed in COS-7 cells overexpressing MT1-MMP, but in these cells, this process requires the simultaneous expression of SFK activity. This requirement for SFK is because of phosphorylation of caveolin-1 on tyrosine 14 because expression of a caveolin-1 mutant in which this phosphorylation site was abolished failed to associate with MT1-MMP. This suggests that MT1-MMP interacts only with tyrosine-phosphorylated caveolin-1, an hypothesis that is strengthened by the observation that in both EC and transfected COS-7 cells, equivalent quantities of MT1-MMP were immunoprecipitated using antibodies recognizing either total caveolin-1 or only the phosphorylated form of the protein.

The exact nature of the interaction between caveolin-1 and MT1-MMP remains to be determined. Most of the signaling proteins known to interact with caveolin-1 possess a consensus binding sequence allowing association with caveolin-1 scaffolding domain (7). Close examination of the MT1-MMP sequence

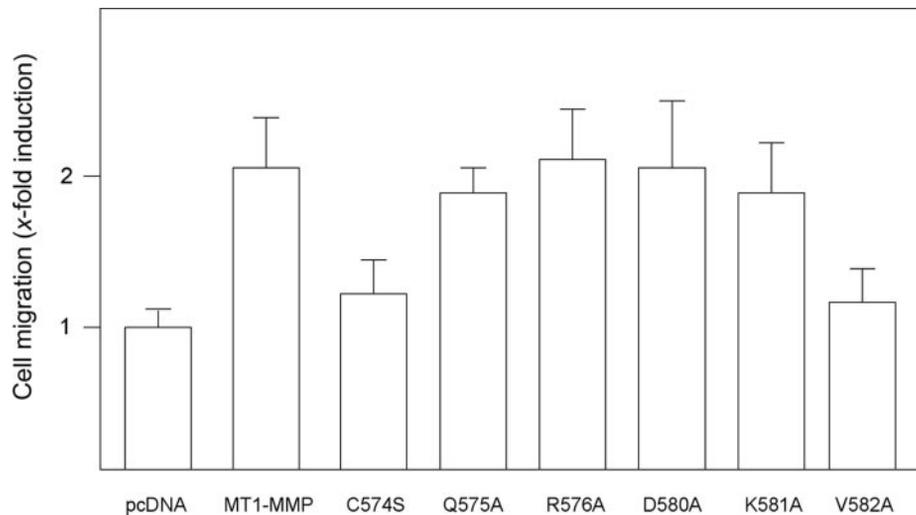


FIG. 7. **Inhibition of MT1-MMP interaction with caveolin-1 correlates with an inhibition of cell migration.** Cell migration assays were performed essentially as described, using transwells precoated with 0.15% gelatin (24). COS-7 cells transfected with Src and either the wild-type MT1-MMP or the C574S or V582A mutants were harvested by trypsinization, centrifuged, resuspended in 100 μ l of fresh DMEM at 2.5×10^5 cells/ml, and inoculated into the upper chamber of each transwell. The transwells were assembled in 24-well plates, and the lower chambers were filled with 600 μ l of media containing 10% serum. The plates were then placed at 37 $^{\circ}$ C in 5% CO₂, 95% air for 3 h. Cells that had migrated to the lower surface of the filters were fixed and stained with 0.1% crystal violet, 20% MeOH. The migration was quantified using computer-assisted imaging as the average density of migrated cells per four fields (magnification $\times 50$). Data are means \pm S.D. of three different experiments.

indicates that it contains several caveolin-1 binding consensus sequences, but these sequences are located within the extracellular hemopexin domains and are thus very unlikely to interact with intracellular caveolin-1. Our results rather suggest that the formation of the phosphocaveolin-MT1-MMP complex involves the cytoplasmic domain of the enzyme because point mutation of two residues, Cys⁵⁷⁴ and Val⁵⁸², markedly reduce the formation of the complex. However, because these residues do not represent known caveolin-1- or phosphotyrosine-binding sites, it is likely that additional proteins are involved in the phosphocaveolin-1/MT1-MMP association. In this respect, it is noteworthy that juxtamembrane cysteine residues are often post-translationally modified by palmitoylation, and this lipid modification has been proposed to provide a means to trigger the specific association of transmembrane proteins lacking signaling motifs with proteins involved in signal transduction (47). Whether such a post-translational modification of the cysteine residue in the cytoplasmic region of MT1-MMP is involved in its association with caveolin-1 or with caveolin-1-binding proteins is currently under investigation. However, because the formation of the MT1-MMP-caveolin-1 complex requires phosphorylation of caveolin-1, it is tempting to speculate that phosphocaveolin-1-binding proteins, such as Grb7 or Csk, could play a role in this process.

The cytoplasmic domain of MT1-MMP plays an important role in the endocytosis of the protein (48, 49), in the activation of ERK signaling cascade (26), the induction of the VEGF gene (29), and in cell locomotion (20, 26, 48, 49). It is noteworthy that the two residues we have identified as important structural determinants for the caveolin-1/MT1-MMP association were previously found to play important roles in MT1-MMP function. Cys⁵⁷⁴ was suggested to be involved in the formation of a disulfide bridge linking two MT1-MMP molecules, and mutation of this residue impairs cell migration (20). Based on these results and on those reported here that a decrease in MT1-MMP interaction with caveolin-1 by mutation of Cys⁵⁷⁴ correlates with a decrease in the MT1-MMP-dependent induction of cell migration, it is tempting to speculate that the association of MT1-MMP with caveolin-1 plays a role in cell locomotion. It is also interesting to note that mutation of Cys⁵⁷⁴ completely abolished the up-regulation of the VEGF gene expression by

MT1-MMP in tumor cells (29). Because this process involves SFK activity (29), this raises the interesting possibility that the association of MT1-MMP with caveolin-1 following tyrosine phosphorylation of the protein by SFK could play an important role in triggering the intracellular pathways leading to the VEGF gene transcription.

Mutation of the C-terminal valine residue (Val⁵⁸²) also markedly inhibited MT1-MMP association with caveolin-1 as well as MT1-MMP cell migration. This residue has been suggested to be important for efficient maturation and trafficking of the protein (50). Recently, the C-terminal sequence DKV of MT1-MMP was also shown to be required for its recycling (51). It is thus likely that inappropriate localization of this mutant at the plasma membrane may be responsible for its reduced capacity to interact efficiently with caveolin-1.

We also observed that the interaction of MT1-MMP with phosphorylated caveolin-1 promotes a significant reduction of Src kinase activity. This effect was correlated with increased association of Src with caveolin-1, a complex that has been shown to result in the inhibition of Src activity (7), suggesting that interaction of caveolin-1 with MT1-MMP could induce a negative feedback loop leading to inactivation of Src activity. Alternatively, the endogenous Src inhibitor, C-terminal Src kinase (Csk), which has been shown to interact specifically with caveolin-1 phosphorylated on tyrosine 14 (13), could be recruited to the complex and induce inactivation of SFK that are highly enriched in caveolae. Although further work is needed to precisely identify the mechanisms involved in the inhibition of Src activity, there is compelling evidence that this process may play an essential role in cell migration during angiogenesis because in Csk^{-/-} embryos vessels failed to engage into normal sprout formation (52). Cell spreading and migration is a temporally and spatially regulated event, with an initial rapid membrane extension followed by assembly of focal adhesions behind the leading edge, and both events involve activation and inactivation of Src activity (53). The formation of the phosphocaveolin-MT1-MMP complex, leading to subsequent inactivation of Src, could thus represent a mechanism by which this fine-tuning of Src activity is achieved.

In summary, our results provide evidence that MT1-MMP interacts with phosphocaveolin-1 and that this process is de-

pendent on Src family kinase activity. Given the importance of both MT1-MMP (24, 25) and phosphorylated caveolin (46) in endothelial cell migration, the identification of MT1-MMP as a binding partner for phosphocaveolin-1 could be of considerable importance for the elucidation of the role of this enzyme in angiogenesis.

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