Distinct Roles for the Catalytic and Hemopexin Domains of Membrane Type 1-Matrix Metalloproteinase in Substrate Degradation and Cell Migration*

Jian Cao‡, Pallavi Kozarekar‡, Maria Pavlaki‡, Christian Chiarelli§, Wadie F. Bahou‡, and Stanley Zucker‡§¶

Received for publication, November 5, 2003, and in revised form, January 14, 2004
Published, JBC Papers in Press, January 15, 2004, DOI 10.1074/jbc.M312120200

Substrate degradation and cell migration are key steps in cancer metastasis. Membrane-type 1-matrix metalloproteinase (MT1-MMP) has been linked with these processes. Using the fluorescein isothiocyanate (FITC)-labeled fibronectin degradation assay combined with the phagokinetic cell migration assay, structure-function relationships of MT1-MMP were studied. Our data indicate that MT1-MMP initiates substrate degradation and enhances cell migration; cell migration occurs as a concurrent but independent event. Using recombinant DNA approaches, we demonstrated that the hemopexin-like domain and a nonenzymatic component of the catalytic domain of MT1-MMP are essential for MT1-MMP-mediated cell migration. Because the cytoplasmic domain of MT1-MMP was not required for MT1-MMP-mediated fibronectin degradation and cell migration, it is proposed that cross-talk between the hemopexin domain of MT1-MMP and adjacent cell surface molecules is responsible for outside-in signaling. Employing cDNAs encoding dominant negative mutations, we demonstrated that Rac1 participates in the MT1-MMP signal transduction pathway. These data demonstrated that each domain of MT1-MMP plays a distinct role in substrate degradation and cell migration.

Cell migration and invasion are critical coordinated events in the cancer dissemination process (1, 2). Cell migration involves the locomotion of a cell over an extracellular matrix (ECM) substrate (3). Extension of the leading edge is associated with adhesion, i.e. binding of integrins to their ECM ligands leading to subsequent migration and further invasion (4). Cancer cell invasion requires degradation of surrounding ECM and basement membrane by proteinases located at the leading edge of migrating cells. Extracellular proteolytic enzymes, i.e. matrix metalloproteinases (MMP’s), serine and cysteine proteinases have long been implicated in cancer metastasis (1, 5).

MMPs have been linked to the metastatic phenotype of tumor cells through both correlative and functional studies. Production and activation of MMPs in tumors are required for degradation of the ECM and dissemination of cancer cells to distant organs (2). MMPs also play an important role in tumor angiogenesis (6). The mechanism of activation of latent MMP-2 (pMMP-2) in tumors has been the focus of considerable recent interest based on the identification of a new category of intrinsic membrane-type MMPs (MT1, 2, 3, 4, 5, and 6-MMPs) (7).

MT1-MMP is able to activate pMMP-2 on the surface of tumor cells by assembling a unique triplex with tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) and pMMP-2; a second MT1-MMP molecule then cleaves the propeptide of pMMP-2, thereby activating the enzyme at the cell surface (8–10). Integrin receptors, α5β1 and αvβ3, participate in this response (11). Recombinant MT1-MMP hydrolyze collagen types I, II, and III and digests cartilage proteoglycan, fibronectin, fibrinogen, vitronectin, and laminin (12).

It is now recognized that the actions of MMPs are not restricted to the simple breakdown of ECM, but include revealing the cryptic site on ECM molecules hidden within folded or assembled protein structures (13, 14); an important aspect of this phenomenon is MMP-conducted cell migration. Recent studies have indicated that MMP-mediated cleavage of laminin 5 (15, 16) and type IV collagen (14) can expose a cryptic epitope that potentiates tumor cell migration.

All MMPs have an N-terminal signal sequence followed by a propeptide domain, a catalytic domain, a hinge region, and a hemopexin-like (PEX) domain (17). MT1-MMP is unique from secretory MMPs because of addition of transmembrane (TM) and cytoplasmic domains (8). This feature helps explain how MT1-MMP behavior differs from soluble MMPs (18). An important function of the PEX domain of MT1-MMP involves the formation of a homophilic complex that associates two MT1-MMPs together for the activation of pMMP-2 (19). Other structure-function relationships of MT1-MMP involved in cell migration and invasion remain to be defined.

In the current study, we examined the domain functions of MT1-MMP in substrate degradation and cell migration by employing mutations and exploring signal pathways in MT1-MMP-induced cell migration. Our data demonstrated that the catalytic domain of MT1-MMP is primarily required for enzymatic activity toward ECM substrates, whereas the PEX do-

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* This work was supported by a Scientist Development Grant from the American Heart Association and a New Investigator grant from the United States Army Medical Research and Materiel Command (to J. C.), a Merit Review grant and a Research Enhancement Award Program grant from the Department of Veterans Affairs (to S. Z.), and research grants (to W. F. B.) from the American Heart Association and National Institutes of Health Grant HL49141. The costs of publication are therefore to be hereby marked “advertisement.”

† To whom correspondence should be addressed: Mail Code 151, Veterans Affairs Medical Center, Northport, NY 11768. Tel.: 631-261-4400 (ext. 2861); E-mail: s_zucker@yahoo.com.

¶ The abbreviations used are: ECM, extracellular matrix; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; K44A, dominant negative dynamin mutation; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1-matrix metalloproteinase; PEX, hemopexin-like domain; pMMP-2, latent MMP-2; TIMP, tissue inhibitor of matrix metalloproteinase; TM, transmembrane domain; TRITC, tetramethylrhodamine isothiocyanate; IL2R, interleukin 2 receptor; PBS, phosphate-buffered saline.
Substrate Degradation/Cell Migration Utilize Different MT1-MMP Domains

main and a component of the catalytic domain of MT1-MMP are essential for MT1-MMP-induced cell migration.

EXPERIMENTAL PROCEDURES

Regents—Human fibroblast and oligo primers were purchased from Intronix (ITC) and was purchased from ID Biomedicals, Inc. (Costa Mesa, CA). The pcDNA3 and pSG5 expression vectors were described previously (20). Recombinant pMMP-2 was produced by COS-1 cells transfected with pMMP-2 cDNA as previously described (20). Anti-human MMP-2 (hemepoxin domain), TIMP-1, and MT1-MMP (catalytic domain, clone 141) monoclonal antibodies were purchased from Invitrogen Research Products (Carlsbad, MA). Anti-MMP-2 antibody (recognized N-terminal region of active form) was purchased from Chemicon International, Inc. (AB808, Temecula, CA). TET and e-Myo monoclonal antibodies were purchased from Novagen (Madison, WI) and Roche Diagnostics (Nutley, NY), respectively. Anti-MT1-MMP (hinge region) polyclonal antibody used for immunofluorescence staining was purchased from Triple Point Biologics (Portland, OR). Anti-Rac1 antibody (clone 102) was purchased from Transduction Laboratories (Lexington, KY). Rhodamine-conjugated phalloidin was purchased from Sigma.

Cell Culture and Transfection—CO3-1, NIH3T3, human prostate cancer cell line, LNCaP, and human breast cancer cell line MDA-MB-231 were purchased from ATCC (Manassas, VA) and were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen). Plasmids were transfected into cells using FuGENE 6 (Roche) according to the manufacturer’s instructions. Stably transfected LNCaP and MDA-MB-231 cell lines were generated using FuGENE 6 followed by isolation of single colonies after 4 weeks of G418 selection.

Construction of Plasmids—Expression vectors including MT1-MMP, TIMP-1, -2, pMMP-2, and soluble MT1-MMP (solMT1) without transmembrane and cytoplasmic domains have been described in detail previously (18, 21, 22). MT1 lacking the cytoplasmic domain of MT1-MMP was generated by introducing a stop codon after Phe562 based on the sequence of MT1-MMP frame with 11 additional amino acids (AAAGIHRPVAT) (QuikChange site-directed mutagenesis kit, Stratagene) to generate MT1E240A. A EcoRI-XhoI fragment converting Glu240 to alanine from MT1 E240A and a XhoI fragment from MT1-GFP chimera was then cloned into pSG5 vector. A PCR fragment was cloned into pcDNA3 at EcoRI and NotI sites. GFP was replaced by the hemagglutinin-tagged K44A mutant MT1-GFP chimera (monoclonal antibody against hemepoxin domain of MT1-MMP, 0.2 μg/ml in 0.1% bovine serum albumin/PBS) or TIMP-2 (0.2 μg/ml in 0.1% bovine serum albumin/PBS) at room temperature for 2 h followed by detecting with the secondary antibodies either fluorescein- or Texas Red-conjugated goat IgG (1:1500 dilution, Rockland, Gilbertsville, PA). After extensive washes, the coverslips were mounted on microscope slides with anti-mouse medium (Vectashield, Vector Laboratories, Burlingame, CA). The samples were examined and photographed with an Olympus fluorescence microscope.

Phagokinetic Migration Assay—The phagokinetic migration assay (25) has been widely used to evaluate cell migratory ability. Coverslips treated with 1-lysine (50 μg/ml) were coated with 0.5% gelatin for 1 h at room temperature followed by air drying. The coverslips placed in 12-well plates were incubated with freshly made colloidal gold particles for 40 min followed by washing with sterile PBS. Cells (1 × 10^6/well) were replated onto colloidal gold particle-coated coverslips for 18 h followed by fixation with 4% paraformaldehyde/PBS. Migratory cells were observed and photographed under light microscopy (Olympus). For a combination study between the phagokinetic assay and the FITC-substrate degradation assay (FITC-substrate degradation/phagokinetic migration assay), coverslips were sequentially coated with FITC-labeled fibronectin or gelatin and colloidal gold particles. Substrate degradation and cell migration were determined under fluorescent and light microscopy.

Determination of Rac Activity—Rac activity was measured in a pull-down assay using the Rac-binding domain of p21-activated kinase fused to glutathione S-transferase (26). NIH3T3 cells transfected with both wild-type Rac1-GFP chimera and MT1-MMP or mock control cDNAs were incubated in serum-free conditioned medium for 18 h at 37 °C. Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM MgCl2, plus protease mixture inhibitors). Lysates were cleared by centrifugation at 13,000 rpm for 2 min, samples were withdrawn for analysis of total Rac, and the remaining lysate was incubated for 30 min at 4 °C with 20 μg of a 50% slurry of glutathione S-transferase-p21 activated kinase fusion protein on glutathione-coupled agarose beads (kindly provided by Prof. X. D. Ren, SUNY at Stony Brook). Beads were then washed.
washed and analyzed by SDS-PAGE and immunoblotting using mouse anti-Rac1 antibody.

Procedures for Gelatin Substrate Zymography, Immunoprecipitation, Preparation of Cell Lysates, and Western Blotting—Basic protocols for these techniques have been described in our previous paper (20).

RESULTS

Initiation of Substrate Degradation and Cell Migration by Cells Expressing MT1-MMP—To examine the contribution of MT1-MMP and MMP-2 to cellular invasion: inhibitory effects of TIMPs. COS-1 cells transfected with different combinations of cDNAs including MT1-MMP, MMP-2, TIMP-1, and TIMP-2 were plated onto FITC-fibronectin (Fn)-coated coverslips in serum-free conditioned medium for 18 h at 37 °C followed by immunofluorescent staining using anti-MT1-MMP (hinge region) antibodies and anti-TIMP-2 antibodies as indicated. The MT1-MMP-transfected cells, substrate degradation, and cell migration were determined under fluorescent microscopy with FITC and TRITC filters (A); the degradation products in spent conditioned medium were measured by FLEXStation using excitation wavelength at 488 nm and emission wavelength at 507 nm (B). MT1-MMP expressing cells, identified by surface staining with anti-MT1-MMP antibodies and anti-TIMP-2 antibodies, digested FITC-fibronectin readily and displayed cell migration. Arrow indicates cell migratory track. The combined effect of cell migration and fibronectin substrate degradation was inhibited by TIMP-2. The effects of MMP-2 and TIMP-1 were limited to enhancement and inhibition, respectively, of fibronectin degradation. C, initiation of substrate degradation and cell migration in human cancer cell lines stably expressing of MT1-MMP. Human prostate cancer cells, LNCaP stably transfected with MT1-GFP chimeric cDNA or human breast cancer cells, MDA-MB-231 stably transfected with MT1-MMP cDNA, were plated onto FITC-labeled fibronectin coverslips followed by immunocytochemistry of MDA-MB-231 cells using anti-MT1-MMP antibody. MT1-MMP expressing cancer cells were identified based on GFP expression in LNCaP cells and antibody staining employed for MDA-MB-231 cells. Both prostate cancer and breast cancer cells expressing MT1-MMP degraded FITC-fibronectin and migrated over digested substrate. Arrows identify MT1-GFP LNCaP cells and MT1-MMP MDA-MB-231 cells.

Substrate Degradation/Cell Migration Utilize Different MT1-MMP Domains 14131
effect of TIMP-2 on MT1-MMP-induced cell migration, we took advantage of a fluorescent marker gene encoding GFP that was mechanically fused to the 3'-end of MT1-MMP cDNA to generate MT1-GFP chimeric cDNA. The MT1-GFP fusion protein, expressed as the anticipated 90-kDa protein (Western blot analysis), was able to activate pMMP-2 and was visualized on the surface of the transfected COS-1 cells (data not shown). COS-1 cells transfected with MT1-GFP cDNA were plated onto colloidal gold-coated FITC-labeled fibronectin coverslips and cultured in serum-free conditioned medium with or without recombinant TIMP-2 (10 nM). In the absence of TIMP-2, MT1-GFP expressing cells were capable of degrading FITC-fibronectin (Fig. 3A, a). The migration of MT1-GFP expressing cells was confirmed in the phagokinetic migration assay based on the

FIG. 2. Enhanced cell migration in transfected cells by blocking MT1-MMP endocytosis. A, a schematic diagram of an engineered construct between MT1-MMP and the dominant negative dynamin mutation (K44A). To ensure transfected cell expression of both MT1-MMP and K44A, MT1-MMP and K44A cDNAs were inserted into the same vector controlled by separated expression elements: a cytomegalovirus promoter and a poly(A) tail. The construct was evaluated based on functional assays: pMMP-2 activation (gelatin zymography) and cell surface localization (immunocytochemistry) for the expression of MT1-MMP, and blockage of MT1-MMP endocytosis (endocytosis assay and immunocytochemistry) for the expression of K44A. B and C, accumulation of MT1-MMP at the cell surface by blocking endocytosis machinery resulted in enhanced cell migration and substrate degradation. COS-1 cells transfected with MT1-MMP or MT1/K44A were replated onto FITC-fibronectin-coated coverslips for 18 h in serum-free conditioned medium at 37 °C. MT1-MMP expression was determined by immunocytochemistry employing anti-MT1-MMP antibody. The cells were examined under fluorescent microscopy. With approximately the same number of plated cells between MT1 and MT1/K44A transfectants, the dramatic loss of FITC-fibronectin in the coverslips plated with MT1/K44A-transfected cells was noted as a result of MT1-MMP-induced cell migration.

Migration of Cells Expressing MT1-MMP Is Independent of Substrate Degradation—In the FITC-substrate degradation assay employed in this study, determination of cell migration is dependent on the loss of FITC-labeled fibronectin. This raises the question whether blockage of enzymatic activity of MT1-MMP in cells by TIMP-2 results in abrogation of cell migration (Fig. 1A, d). To clarify this issue, we examined cell migration on coverslips coated with both colloidal gold particles (25) and FITC-labeled fibronectin (FITC-substrate degradation/phagokinetic migration assay). To directly visualize the interfering

of substrate degradation (down to the basal digestion level resulting from MT1-MMP alone) induced by the cells producing both MT1-MMP and MMP-2, but did not interfere with cell migration (Fig. 1A, c). In contrast, overexpression of TIMP-2 in transfected cells expressing both MT1-MMP and pMMP-2 (as evidenced by immunofluorescent staining for both MT1-MMP and TIMP-2) (Fig. 1A, d and d1) totally abolished substrate degradation by transfected COS-1 cells. The independent effect of TIMP-2 on MT1-MMP-induced cell migration, however, could not be determined in this assay because of lack of indicator (substrate degradation, see Fig. 3A). Consistent with the loss of FITC-fibronectin, the release of degraded FITC-fibronectin in the conditioned medium from each group was matched to that of coverslips by analyzing solubilized fluorescent-labeled fibronectin (Fig. 1B).

Because transiently transfected COS-1 cells may result in high expression of foreign genes, it raised a question if the substrate degradation and the cell migration in COS-1 cells transfected with MT1-MMP cDNA were because of unusually high expression of the enzyme in the cells. To test this possibility, stable cell lines expressing MT1-GFP chimera in the human prostate cancer cell line LNCaP and MT1-MMP in the human breast cancer cell line MDA-MB-231 were examined using the FITC-substrate degradation assay. In agreement with the data obtained from COS-1 cells, LNCaP and MDA-MB-231 stable cells expressing MT1-MMP were able to digest FITC-labeled fibronectin and migrate over digested substrate (Fig. 1C). Interestingly, overexpression of MT1-GFP in COS-1 cells demonstrated that most of the MT1-GFP chimera was mainly distributed in the perinuclear region. The relative proportion of MT1-GFP expressed at the plasma membrane was considerably less than the perinuclear region; this observation was confirmed by cell surface biotinylation followed by Western blot analysis (data not shown).

The role of cell surface proteinases was further supported by interfering with MT1-MMP endocytosis in COS-1 cells transfected with a plasmid containing both MT1-MMP cDNA and a dominant negative dynamin mutation, K44A cDNA, controlled by a separate cytomegalovirus promoter (MT1/K44A, Fig. 2A). Increased MT1-MMP staining at the cell surface (employing an anti-MT1-MMP Ab) was found in cells expressing both MT1-MMP and K44A, indicating that MT1-MMP internalization occurs, in part, through clathrin-coated vesicles (Fig. 2B) (28, 29). Blocking endocytic machinery of MT1-MMP expressing COS-1 cells by overexpression of a dynamin mutation (K44A) resulted in ~70% of the FITC-labeled substrate degradation as compared with ~10% FITC-fibronectin digestion in COS-1 cells expressing MT1-MMP alone (Fig. 2C). This effect was not observed in K44A or dynamin cDNA-transfected control cells (lacking MT1-MMP cDNA) as examined by the FITC-substrate degradation/phagokinetic migration assay (data not shown). Because the number of transfected cells was the same (phase-contrast image, Fig. 2C, a1 and b1), it is concluded that the increased substrate degradation by MT1/K44A expressing cells was contributed by enhanced cell migration resulting from the accumulation of MT1-MMP at the plasma membrane.

Migration of Cells Expressing MT1-MMP Is Independent of Substrate Degradation—In the FITC-substrate degradation assay employed in this study, determination of cell migration is dependent on the loss of FITC-labeled fibronectin. This raises the question whether blockage of enzymatic activity of MT1-MMP in cells by TIMP-2 results in abrogation of cell migration (Fig. 1A, d). To clarify this issue, we examined cell migration on coverslips coated with both colloidal gold particles (25) and FITC-labeled fibronectin (FITC-substrate degradation/phagokinetic migration assay). To directly visualize the interfering
Substrate Degradation/Cell Migration Utilize Different MT1-MMP Domains

MT1E240A failed to digest fibronectin substrate (absence of fibronectin degradation as shown). Because this mutation disrupted catalytic activity, MT1E240A-GFP expressing cells were not able to degrade the fibronectin substrate (Fig. 3C, b). However, MT1E240A-GFP expressing cells exhibited unimpaired enhanced cell migration as examined by the phagokinetic migration assay (Fig. 3B, b). Taken together, these data demonstrated for the first time that the enzymatic function of MT1-MMP is not required for MT1-MMP-induced enhancement of cell migration but is required for substrate degradation.

The Role of Transmembrane and Cytoplasmic Domains of MT1-MMP in Substrate Degradation and Cell Migration—MT1-MMPs are unique among other MMPs because of their plasma membrane anchor. MT1-MMP also possesses a short cytoplasmic tail that might play a critical role in outside-in signals for MT1-MMP-induced cell migration. To further define the role of the TM and cytoplasmic domain of MT1-MMP in substrate degradation and cell migration, we next examined the structure-function relationships of MT1-MMP. To this end, deletion and fusion mutants were generated by employing a two-step PCR (18). The TM and cytoplasmic domains of MT1-MMP were individually deleted to generate Sol.MT (22) and MTΔC, respectively (Fig. 4A). These mutants were expressed by transfection of COS-1 cells with corresponding cDNAs; wild-type and mutant MT1-MMP protein expressions were confirmed using anti-MT1-MMP catalytic domain antibodies (Fig. 4B). In agreement with previous reports (30), deletion of the cytoplasmic region of MT1-MMP did not interfere with pMMP-2 activation as compared with wild-type MT1-MMP transfected cells, whereas soluble MT1-MMP failed to result in pMMP-2 activation as examined by gelatin substrate zymogram (Fig. 4C) (18, 19, 22). Employing different experimental techniques, other investigators arrived at contradictory conclusions (29–32). The substrate degradation and cell migration of these deletion mutants were evaluated by plating transfected COS-1 cells onto FITC-fibronectin coverslips (Fig. 4D). MTΔC expressing cells displayed substrate degradation comparable with wild-type MT1-MMP-transfected cells as well as cell migration (Fig. 4D). The substrate degradation and cell migration induced by MTΔC expressing cells was neither cell type-dependent nor extracellular matrix component-dependent as examined with different cell lines, e.g. NIH3T3, breast cancer cell lines (MCF-7 and MDA-MB-436), prostate cancer cell line (LNCaP), and with different substrates, e.g. fibrinogen, fibrin, and gelatin (data not shown). This data suggest that the C-terminal cytoplasmic domain of MT1-MMP is not required for MT1-MMP-mediated substrate degradation and cell migration.

Despite Sol.MT1 displaying intrinsic gelatinolytic activity as demonstrated by gelatin zymography (Fig. 4C), no invasion over fibronectin was noted in cells expressing Sol.MT1 (Fig. 4D). The failure of cellular invasion is explained by the defect in plasma membrane anchoring (confirmed by lack of cell surface immunofluorescent staining using anti-MT1-MMP antibody (Fig. 4D, inset)). We previously demonstrated that a TM domain of MT1-MMP was essential for MT1-MMP-induced pMMP-2 activation in intact cells, but the unique sequence...
specificity was not essential (22). To test if this observation is also true for MT1-MMP-induced substrate degradation and cell migration, a non-related TM domain from the IL2R was amplified by PCR and fused to the 3'-end of Sol.MT cDNA to generate a chimeric cDNA between Sol.MT and the IL2R TM domain named MT/IL2RTM. As expected, MT/IL2RTM retained Sol.MT on the cell surface as examined by immunofluorescent staining using anti-MT1-MMP antibodies (Fig. 4D, inset) and retained the function of MT1-MMP in terms of activation of pMMP-2 as shown in Fig. 4C. Furthermore, fusion of the TM domain of IL2R to Sol.MT resulted in recovery of substrate degradation and cell migration (Fig. 4D). These data emphasize that the TM domain function of immobilizing MT1-MMP on the cell surface is necessary for MT1-MMP-mediated substrate degradation and cell migration. The specific peptide sequence of the MT1-MMP TM domain and the cytoplasmic
domain appear to be nonessential for substrate degradation and cell migration in fibronectin.

Distinct Roles of the Catalytic and PEX Domains of MT1-MMP in Substrate Degradation and Cell Migration—The role of the PEX domain of MT1-MMP has gained recent attention in terms of pMMP-2 activation and cell migration (7, 33). In this study, we found no effect on MT1-MMP-induced cell migration by effacing the enzymatic activity of MT1-MMP. These observations encouraged us to focus on the coordination role of the catalytic and PEX domains in MT1-MMP-induced substrate degradation and cell migration. The PEX domain of MT1-MMP shares low identity (from 20 to 35%) with secretory MMPs, but high identity with other MT-MMPs (55%, except MT4 and 6-MMP) (SeqWeb version 2, GCG Wisconsin package).

To explore secreted MMPs that would be appropriate for domain shuffling experiments, an active membrane-bound MMP-2 was engineered by deleting the propeptide domain of MMP-2 and by fusing the TM/cytoplasmic domains of MT1-MMP to the C terminus of MMP-2, designated as aMMP2-TM/Cyto. This chimera was detected at the plasma membrane of transfected COS-1 cells and bound TIMP-2 at the cell surface as examined by immunocytochemistry using anti-MMP-2 antibody against the hemopexin domain of MMP-2 and anti-TIMP-2 antibody, respectively. Cells expressing aMMP2-TM/Cyto digested FITC-gelatin, indicating trafficking of the chimeric protein to the cell surface (data not shown). Based on this information, the hemopexin domain of MMP-2 was chosen to examine the role of the PEX domain of MT1-MMP on cell migration. A domain shuffling experiment between MT1-MMP and pMMP-2 cDNAs was performed as follows: using a modified two-step PCR approach, MT1-MMP2CAT was engineered by substitution of the catalytic domain of MT1-MMP with that of pMMP-2 (Fig. 5A). Employing a similar approach, MT1-MMP2CAT-PEX and MT1-MMP2PEX were constructed by replacing the catalytic/PEX domains and PEX domain of MT1-MMP with those of pMMP-2, respectively. By gelatin zymography, MT1-MMP2CAT and MT1-MMP2CAT-PEX expressing cells failed to activate pMMP-2 (Fig. 5D). This failure of pMMP-2 activation was not because of a defect in protein synthesis as examined by Western blotting using anti-MMP-2 antibodies reacting with the catalytic domain of MMP-2 (Fig. 5B). Cells expressing MT1-MMP2PEX (confirmed by Western blotting using anti-MT1-MMP catalytic domain antibodies, Fig. 5C) displayed reduced activation of pMMP-2 (demonstrated by gelatin zymogram, Fig. 5D); fully active MMP-2 (62 kDa) was not noted within an 18-h incubation period. Although all of these chimeras were expressed on the plasma membrane as examined by immunocytochemistry, none of the MT-MMP2 chimera (MT-MMP2CAT, MT-MMP2PEX, and MT-MMP2CAT-PEX) transfected cells digested FITC-fibronectin (data not shown). These data support the concept that the unique sequence specificity within the PEX domain of MT1-MMP, which is essential for homodimer formation (19), is also required for cell migration.

Because the lysates of COS-1 cells transfected with the chimeric cDNAs (MT1-MMP2CAT, MT1-MMP2PEX, and MT1-MMP2CAT-PEX) were capable of directly degrading underlying gelatin as examined by gelatin zymography (data not shown), we sought to evaluate the migratory ability of these chimeric-expressing cells using FITC-labeled gelatin as an indicator (Fig. 5E). As expected, MT1-MMP expressing cells degraded FITC-gelatin and migrated over digested substrate. Surprisingly, the ability to digest FITC-gelatin by cells transfected with MT1-MMP2CAT, MT1-MMP2CAT-PEX, and MT1-MMP2PEX differed from gelatin zymograms of the cell lysate. Compared with MT1-MMP expressing cells, MT1-MMP2CAT- and MT1-MMP2CAT-PEX-transfected cells degraded reduced amounts of FITC-gelatin, whereas MT1-MMP2PEX expressing cells demonstrated full ability to degrade local gelatin. All three transfected cells, however, failed to initiate cell migration as examined in the FITC-substrate degradation assay (Fig. 5E, b-d). The role of the PEX domain of MT1-MMP on cell migration was further emphasized by employing the FITC-substrate degradation/phagokinetic migration assay. MT1-MMP2PEX expressing cells readily digested underlying FITC-labeled gelatin, but were not able to migrate (Fig. 5F, c and d). In agreement with the experiments depicted in Fig. 3, these data are consistent with the concept that the PEX domain of MT1-MMP is uniquely required for MT1-MMP-mediated cell migration.

Effect of Rac on MT1-MMP-mediated Cell Migration—Gingras et al. (34) previously implicated the extracellular signal-regulated kinase activation pathway in MT1-MMP-dependent cell migration (34). Hence, we tested the effect of wortmannin (a fungal metabolite that specifically inhibits phosphatidylinositol 3-kinase, mitogen-activated protein kinase, and myosin light chain kinase) and the extracellular-regulated kinase inhibitor PD98059 on the MT1-MMP-induced cell migration. None of these agents interfered with MT1-MMP-induced cell migration (data not shown).

Because Rho GTPases play an instrumental role in the actin cytoskeleton reorganization that is involved in cell migration, we analyzed their role in MT1-MMP-dependent cell migration using FITC-fibronectin as an indicator in the invasion assay. Coexpression of the dominant negative Rac1(N17) with MT1-MMP cDNAs led to complete inhibition of migration of transfected cells, but displayed no inhibitory effect on local fibronectin degradation (Fig. 6A, d). The expression of both MT1-MMP and N17Rac1 in the same cells was confirmed by substrate degradation for identification of MT1-MMP expression and immunostaining using anti-T7 antibody for the presence of N17Rac (Fig. 6A, d1), respectively. In contrast, coexpression of the dominant negative CDC42(N17) and RhoA(N19) had no effect on MT1-MMP-dependent cell migration (Fig. 6A, b and c), although these dominant negative inhibitors did change the pattern of actin reorganization as examined by rhodamine-conjugated phalloidin (data not shown). Thus, MT1-MMP-mediated cell migration is dependent on the activity of Rac but not CDC42 or RhoA.

To confirm whether Rac1 is activated in MT1-MMP cDNA-transfected cells, the GTP-bound Rac was examined in cells transfected with a combination of cDNAs. NIH3T3 cells were transfected with wild-type Rac-GFP chimeric cDNA along with mock control or MT1-MMP cDNA and cultured in the absence of serum (serum starved) for 18 h. Activated Rac was precipitated from cell lysates by the Rac-binding domain of p21-activated kinase fused to glutathione S-transferase (26). As shown in Fig. 6B, a 4- and 2.8-fold increase in the activity of endogenous Rac and transfected wild-type Rac-GFP, respectively, were observed in cells co-expressing MT1-MMP. This data indicates that Rac1 is activated during MT1-MMP-induced cell migration.

**DISCUSSION**

Tumor invasion requires loss of stable cell-cell contacts, enzymatic degradation of ECM components and cellular locomotion (1, 2). Recently, this concept has been challenged based on in vitro experimental data showing that protease-independent migration can occur through pre-existing matrix gaps in the ECM (35). Coordination between substrate degradation and cell migration mechanisms has been emphasized based on in vitro observations that MT1-MMP cleavage of laminin 5, fibrin, and type 1 collagen promotes tumor cell motility in vitro (16, 30, 36, 37). MT1-MMP not only has broad ECM substrate degradation capacity (12), but is able to cleave a number of
FIG. 5. A unique role of hemopexin (PEX) domain of MT1-MMP in cell migration. A, schematic diagram of substitute mutations of MT1-MMP. By employing a PCR approach, the catalytic, catalytic-PEX, and PEX domains of MT1-MMP were replaced by the corresponding regions of MMP-2 to generate MT1-MMP and MMP-2 chimeras. B–D, evaluation of enzymatic activity of MT1-MMP-2 chimeras: COS-1 cells were transiently transfected with wild-type and substitute mutants of MT1-MMP cDNAs. The cells were incubated in serum-free conditioned medium containing recombinant pMMP-2 for 18 h at 37 °C. The conditioned medium and cell lysates were then examined by gelatin zymography for MMP-2 activation and Western blotting for protein expression. All the transfectants expressed the expected proteins using anti-MMP-2 catalytic domain antibodies for MMP-2, MT1-MMP2CAT, and MT1-MMP2CAT-PEX, and anti-MT1-MMP catalytic domain antibodies for MT1-MMP and MT1-MMP2PEX, respectively (B and C). Among mutants, only MT1-MMP2PEX resulted in the partial activation of pMMP-2 to the intermediate form (iMMP-2) (D). E, sequence specificity of MT1-MMP in cell migration/substrate degradation: COS-1 cells transfected with wild-type and substituted mutants of MT1-MMP were plated onto FITC-labeled gelatin in serum-free conditioned medium for 18 h at 37 °C. Cell migration/substrate degradation was determined under fluorescent microscopy. Replacement of the catalytic and PEX domains of MT1-MMP by those of MMP-2 in transfected cells resulted in loss of cell invasion. Arrows indicate transfected cells. F, requirement of MT1-MMP PEX domain in cell migration. COS-1 cells transfected with MT1-MMP, MT1-MMP2PEX, and mock control cDNA were replated onto colloidal gold-coated FITC-labeled fibronectin coverslips for 18 h. Substrate degradation and cell migration were determined by cleared colloidal gold particles and loss of FITC-labeled gelatin substrate. Arrows indicate a cell migratory track in MT1-MMP-transfected cells.
Fig. 6. Involvement of a small GTPase, Rac in MT1-MMP-induced cell migration. A, interference with MT1-MMP-induced cell migration by a constitutively inactive Rac1 (N17Rac1). COS-1 cells co-transfected with both MT1-MMP and dominant negative inhibitors of CDC42 (N17CDC42), RhoA (N19RhoA), and Rac1 (N17Rac1), respectively, were replated onto FITC-labeled fibronectin coverslips followed by immunofluorescent staining using corresponding antibodies as indicated. MT1-MMP expression was identified by substrate degradation and the presence of constitutively inactive small GTPase in cells was determined by antibody staining. Arrows indicate transfected cells. MT1-MMP-induced cell migration was inhibited by co-expression of constitutively inactive Rac, and the presence of constitutively inactive small GTPase in cells was determined by antibody staining. Arrows indicate endogenous and recombinant Rac, respectively. 

other protein substrates that may affect cell migration (16, 38, 39). We recently reported that non-invasive COS-1 cells can be induced to degrade fibronectin and migrate over digested substrate after transfection of MT1-MMP cDNA into the cells (27). In this report, we further characterized the function-structure relationship of MT1-MMP in substrate degradation and cell migration using FITC-labeled fibronectin as an indicator. 

We provided evidence for the first time that the catalytic and PEX domains of MT1-MMP play independent roles in substrate degradation and cell migration. MT1-MMP-induced substrate degradation and cell migration are dependent on coordinated interactions of unique subdomains of MT1-MMP and signaling through a small GTPase, Rac pathway.

There are several technical approaches to study the invasive ability of cells, e.g., Boyden chamber coated with extracellular matrices, chick chorioallantoic membranes, and chicken heart invasion assay (40). The approach used in this study was based on direct visualization of substrate degradation (loss of fluorescence) and cell migration (track lacking fluorescence). In the absence of substrate degradation, however, cell migration cannot be observed using the latter technique. To overcome this obstacle, we modified an assay involving coating colloidal gold particles on FITC-labeled fibronectin coverslips. This improved technique provided a tool to evaluate structure-function relationships of MT1-MMP required for cell migration and substrate degradation. In preliminary experiments, we compared our assay to the Boyden chamber assay containing a 8-μm pore size membrane coated with or without fibronectin. Cells transfected with MT1-MMP mutants lacking protease activity did not invade through the coated membrane in the Boyden chamber assay regardless of whether the mutants have the capability to migrate. In contrast, our modified FITC-substrate degradation/phagokinetic migration assay permitted the simultaneous assessment of migration and substrate degradation by individual cells in situ. This approach facilitated the evaluation of the role of distinct domains of MT1-MMP in either substrate degradation or cell migration.

A previous study reported that migration of tumor cells with low invasive ability on ECM components was accompanied by re-arrangement of the cellular substrate without proteolytic process (41). Mechanical pushing of FITC-labeled substrate, rather than degradation is considered unlikely in our experiments because there was no accumulation of FITC-fibronectin at the edge of the cell path and there was gradual release of the degraded fluorescent substrate into conditioned medium (Fig. 1B).

Employing the fluorescent substrate degradation assay, we found that TIMP-2 inhibited MT1-MMP-induced substrate degradation and “cell migration” (Fig. 1A, d). This could be explained by TIMP-2 interfering with the proteolytic activity of MT1-MMP (42) and hence cryptic sites required for cell migration were not available (16). To our surprise, blockage of enzymatic activity of MT1-MMP by TIMP-2 was not accompanied by the loss of motility of cells expressing MT1-MMP examined in a FITC-substrate degradation/phagokinetic migration assay. This observation was further confirmed by demonstrating unperturbed migration of cells transfected with a constitutively inactive mutant MT1-MMP cDNA (MT1E240A-GFP) (Fig. 3). These data imply that migration of cells expressing MT1-MMP was independent of proteolytic cleavage of surrounding ECM components. In contrast, studies employing other experimental approaches have identified cryptic site triggers that elicit cell migration (14, 16). In agreement with our finding, Hotary et al. (37) reported that TIMP-2 did not affect the migratory response of fibroblasts in two-dimensional fibrin gels, but had an inhibitory effect in three-dimensional invasion. Because the PEX domain of MT1-MMP has been suggested to play a critical role in cell migration by forming homodimers at the cell surface (19, 43) or interacting with CD44 (44), this domain was re-evaluated in terms of cell migratory ability. Substitution of the PEX domain of MT1-MMP with that of MMP-2 failed to initiate cell migration, but did not interfere with cell-mediated substrate degradation. On the other hand, the PEX domain of MT1-MMP appears to coordinate with other components of the catalytic domain, not involving the HEXGH active-site cleft to facilitate cell migration (Fig. 5E, b). This result emphasizes that both the nonenzymatic component of the catalytic domain and PEX domain of MT1-MMP contribute to cell migration.

Although the overall structure of all MMP catalytic domains are similar (17), the MT1-MMP catalytic domain deviates the most with the insertion of an elongated and more exposed MT loop (45). It has been demonstrated that the insertion of eight amino acids (Pro163-Gly170) between strands βII and III in the catalytic domain is required for efficient activation of pMMP-2 (46). Although MMP-2 has strong gelatinolytic activity, substi-
Substrate Degradation/Cell Migration Utilize Different MT1-MMP Domains

tution of the catalytic domain of MT1-MMP with that of MMP-2 (MT1-MMP2CAT) reduced gelatinolytic activity of the chimera (Fig. 5E).

The role of the cytoplasmic domain of MT1-MMP in cell invasion is controversial (29–32). The cytoplasmic tail of MT1-MMP has been reported to play an important role in cellular invasion in Matrigel (29), but not in a type I collagen gel (30). A role for the cytoplasmic domain of MT1-MMP in the turnover of cell surface MT1-MMP has also been demonstrated. MT1AC does not undergo endocytosis (28). Fusion of a nonrelated cytoplasmic domain from the IL2R to the C terminus of MT1AC rescued the internalization of MT1AC. Our data demonstrated that MT1AC expressing cells were able to digest fibronectin substrate and migrate over the digested substrate. Similar data (not shown) were obtained by employing different substrates and cell lines. It is possible that the ECM degradation ability of MT1-MMP differs depending upon the matrix used for the assay (29). This possibility is currently being evaluated by employing the FITC-substrate degradation assay using different FITC-labeled matrices. Because MT1AC does not undergo internalization, it is reasonable to speculate that accumulation of MT1AC at the transfected cells surface led to more aggressive cellular behavior. Indeed, enhanced type I collagen degradation was seen in Madin-Darby canine kidney cells transfected with MT1AC (30). In support of this hypothesis, 4- and 2.8-fold increase in the activity of endogenous Rac and trans- 

2 J. Cao, P. Kozarekar, M. Pavlaki, C. Chiarelli, W. F. Bahou, and S. Zucker, manuscript in preparation.

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