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Cytoplasmic tail–dependent internalization of membrane-type 1 matrix metalloproteinase is important for its invasion-promoting activity

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Membrane-type 1 matrix metalloproteinase (MT1-MMP) is an integral membrane proteinase that degrades the pericellular extracellular matrix (ECM) and is expressed in many migratory cells, including invasive cancer cells. MT1-MMP has been shown to localize at the migration edge and to promote cell migration; however, it is not clear how the enzyme is regulated during the migration process. Here, we report that MT1-MMP is internalized from the surface and that this event depends on the sequence of its cytoplasmic tail. Di-leucine ($Leu^{571–572}$ and $Leu^{578–579}$) and tyrosine^{573} residues are important for the internalization, and the μ2 subunit of adaptor protein 2, a component of clathrin-coated pits for membrane protein internalization, was found to bind to the LLY^{573} sequence. MT1-MMP was internalized predominantly at the adherent edge and was found to colocalize with clathrin-coated vesicles. The mutations that disturb internalization caused accumulation of the enzyme at the adherent edge, though the net proteolytic activity was not affected much. Interestingly, whereas expression of MT1-MMP enhances cell migration and invasion, the internalization-defective mutants failed to promote either activity. These data indicate that dynamic turnover of MT1-MMP at the migration edge by internalization is important for proper enzyme function during cell migration and invasion.

Introduction

Cells in tissue have to degrade and reconstitute the extracellular matrix (ECM)* at the periphery to carry out various cell functions such as migration, proliferation, and cell shape change. Matrix metalloproteinases (MMPs) anchored to plasma membrane, called membrane-type MMPs (MT-MMPs), play pivotal roles for such limited ECM remodeling (Werb, 1997; Nagase and Woessner, 1999; Seiki, 1999). Of the six MT-MMPs that have been identified to date, four (MT1-, MT2-, MT3-, and MT5-MMP) are anchored to the plasma membrane via a transmembrane domain that is followed by a short cytoplasmic tail (Pei, 1999; Seiki, 1999). The remaining two MT-MMPs, MT4- and MT6-, are tethered to the cell surface via a GPI anchor (Itoh et al., 1999; Kojima et al., 2000).

Of all the MT-MMPs, MT1-MMP has been studied the most. It degrades various ECM components including collagens type I, II, and III, laminins 1 and 5, fibronectin, fibrin, and proteoglycans (Pei and Weiss, 1996; Ohuchi et al., 1997; Koshikawa et al., 2000). It also activates other MMPs on the cell surface including proMMP-2 (Sato et al., 1994) and proMMP-13 (Knauper et al., 1996). MT1-MMP is expressed mainly in the mesenchymal tissues of embryos such as bone, muscle, and fibroblastic tissues (Kinoh et al., 1996; Apte et al., 1997). In the MT1-MMP–null mouse, formation of skeletal tissues is disturbed, and extensive fibrosis occurs after birth (Holmbeck et al., 1999). Activation of proMMP-2 was severely suppressed in this mouse (Zhou et al., 2000) and isolated fibroblasts failed to digest type I collagen (Holmbeck et al., 1999).

Overexpression of MT1-MMP is associated with malignant tumors (Seiki, 1999). Because MT1-MMP expression has been shown to augment the invasive and metastatic po-

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*Abbreviations used in this paper: AP-2, adaptor protein 2; ECM, extracellular matrix; GFP, green fluorescent protein; hTfnR, human transferrin receptor; IL2R, interleukin 2 receptor α-chain; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; TIMP, tissue inhibitor of metalloproteinases.

Key words: MT-MMP; metalloproteinase; internalization; invasion; migration

tential of cancer cells in experimental systems (Sato et al., 1994; Tsunezuka et al., 1996), the enzyme is believed to play a key role in the invasion of malignant tumors. Invasion requires the dynamic and coordinated action of many cell surface molecules including proteinases and adhesion molecules (Horwitz and Parsons, 1999; Murphy and Gavrilovic, 1999). During this process, MT1-MMP is important not only for ECM degradation but also for stimulating cell locomotion (Koshikawa et al., 2000; Kajita et al., 2001). During cell migration, MT1-MMP localizes at the migration front (Sato et al., 1997), particularly near the layer to which the cells adhere (Kajita et al., 2001). However, it is still unclear how MT1-MMP activity is regulated at the migration front during cell migration.

In general, MT1-MMP activity on the cell surface is regulated by several different mechanisms. ProMT1-MMP has a recognition site for proprotein convertases at the end of the propeptide (Sato et al., 1994). Therefore, MT1-MMP appears on the cell surface in its active form after proprotein convertases cleave off the propeptide intracellularly (Yana and Weiss, 2000). The enzyme activity on the cell surface can be inhibited by the tissue inhibitors of metalloproteinases (TIMPs)-2, -3, and -4 but not by TIMP-1. Of the TIMPs, TIMP-2 is the major inhibitor of MT1-MMP (Strongin et al., 1995). TIMP-2 also participates in the activation of proMMP-2 by MT1-MMP mediating the binding of proMMP-2 to MT1-MMP through the formation of a tri-molecular complex (e.g., proMMP-2–TIMP-2–MT1-MMP) (Strongin et al., 1995; Butler et al., 1998; Kinoshita et al., 1998). ProMMP-2 in the complex can then be activated by the adjacent TIMP-2–free MT1-MMP. Homophilic oligomer formation through the PEX domain facilitates such bi-molecular events and stimulates the activation of proMMP-2 (Itoh et al., 2001). MT1-MMP is then downregulated through the processing by MMP-2 and MT1-MMP itself. The cleavage occurs within its catalytic domain and generates a 43-kD inactive fragment on the cell surface (Lehti et al., 1998; Stanton et al., 1998). Such an inactive fragment may compete with the formation of homo-oligomers of intact MT1-MMP molecules and eventually inhibit proMMP-2 activation (Itoh et al., 2001). Another proposed mechanism by which cell surface MT1-MMP activity can be regulated is through its removal from the cell surface by internalization and subsequent degradation. This is suggested by a recent experiment showing that MT1-MMP–bound TIMP-2 is internalized and degraded (Maquoi et al., 2000).

In this paper, we report that MT1-MMP is indeed internalized at the cell surface and this process depends on tyrosine and di-leucine motifs in its cytoplasmic tail. In particular, the LLY^{573} motif was found to be the binding site of a component of clathrin-coated pits, adaptor protein 2 (AP-2). Although internalization is expected to regulate the net amount of MT1-MMP present on the cell surface, we found that the level of MT1-MMP activity in cells expressing internalization-defective mutants did not change significantly in relation to those expressing the wild-type enzyme. Despite this, however, the MT1-MMP mutants, unlike wild-type MT1-MMP, failed to promote cell migration and invasion into reconstituted basement membrane. Because MT1-MMP localizes at the cell adherent edge and is actively internalized there, our results suggest that turnover of MT1-MMP at the adherent edge by internalization is an important step in regulating the enzyme during cell migration and invasion.

Biochemical methods have been used to study membrane proteinases. Although a biochemical approach is powerful, especially for soluble enzymes, it is not sufficient to characterize the cell surface events that are coupled with various cell functions, an equally important aspect of membrane protease regulation. From this point of view very little information about cell surface proteinases is available. Our study provides an interesting initial step to understanding the dynamic regulation of MT1-MMP coupled with cell locomotion.

Results

Internalization of MT1-MMP in CHO-K1 cells

To study the internalization of MT1-MMP, we expressed human MT1-MMP having a FLAG tag at the NH_2 terminus (MT1-F) in CHO-K1 cells, and monitored the fate of MT1-F on the cell surface. Human transferrin receptor (hTfnR), a well-characterized marker of endocytosis (Zuk and Elferink, 1999), served as a positive control. The expression of MT1-F or hTfnR by the transfected cells was confirmed by Western blotting (Fig. 1 A, inset). To monitor the fate of the cell surface molecules, either ^{125}I-labeled anti-FLAG M2 antibody (^{125}I-M2) or ^{125}I-labeled human transferrin (^{125}I-hTfn) was used as a tracer. These ligands bound specifically to the cells expressing MT1-F and hTfnR, respectively (unpublished data; Fig. 1 B). The cell surface ligands could be removed almost completely by washing the cells with acid solution (Fig. 1 A). Thus, the internalized tracer molecules could be measured as the radioactivity that was resistant to the acid wash.

After binding each ligand, the cells were incubated at 37°C to allow for internalization. The radioactivity in the acid-resistant fraction (referred to as "Internalize"), the culture medium (Medium), and the fraction released by acid wash (Cell surface) was measured after various incubation times (Fig. 1 A). After a 30-min incubation, almost 60% of the bound ^{125}I-hTfn became acid resistant, presumably reflecting its internalization, after which it decreased (Fig. 1 A). This is consistent with observations made previously by Zuk and Elferink (1999). The time-dependent transition of surface-bound ^{125}I-M2 to the acid-resistant fraction was similar to that of ^{125}I-hTfn, which showed 40% internalization after a 30-min incubation and a decrease thereafter. After a 60-min incubation, the majority of the radioactivity was found in the Medium fraction. Thus, the tracers that were once internalized were released again into the medium.

Almost 40% of the M2 antibody was released during the first 10-min incubation even in the cells fixed with 3% paraformaldehyde (unpublished data). Although we do not know how this weak binding occurs, a fraction of MT1-F may be processed partially from the NH_2-terminal FLAG sequence. Thus, there is a possibility that the efficiency of MT1-F internalization is underestimated. We also analyzed the internalization using a monovalent Fab fragment derived from the M2 antibody and obtained similar results, indicating that internalization is not an

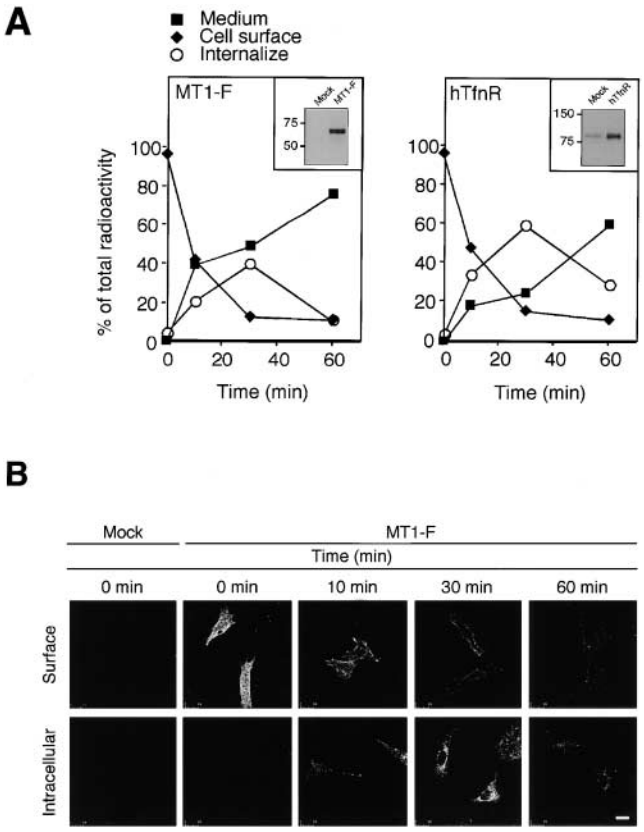


Figure 1. **Internalization of wild-type MT1-MMP in CHO-K1 cells.** (A) CHO-K1 cells were transfected with expression plasmids for MT1-F or hTfnR. 48 h later, the internalization of cell surface–expressed MT1-F or hTfnR was examined using ^{125}I-labeled anti-FLAG M2 antibody or ^{125}I-labeled hTfn as tracer ligands. The radioactivity relative to the amount of tracers bound to the surface at time 0 is plotted. ■, radioactivity spontaneously released into the culture medium (Medium); ◆, radioactivity released by acid wash (Cell surface); ○, acid wash–resistant radioactivity (Internalize). Mean values of three independent experiments are shown (mean ± SD). Transfected cells were also analyzed by Western blotting using anti-FLAG M2 or anti-hTfnR antibodies (inset). (B) CHO-K1 cells expressing MT1-F or mock-transfected cells were incubated with anti-FLAG M2 antibody, after which the cells were incubated at 37°C for the indicated periods of time. The cell surface and internalized MT1-F molecules were analyzed by immunolocalization using Alexa^{TM}488-conjugated anti–mouse IgG. Internalized MT1-F molecules were observed by washing the cells with acid solution before fixation and permeabilization. Signals were observed by confocal laser microscopy. Bar, 10 μm.

artificial event caused by the cross-linking of MT1-MMP molecules by the divalent antibody (unpublished data).

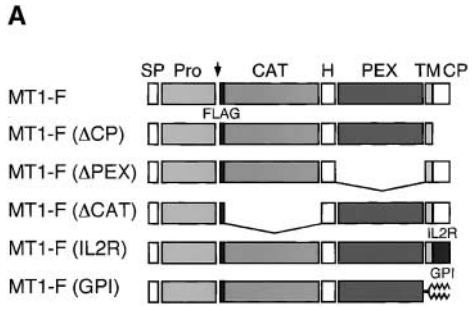
To ensure that the acid-resistant radioactivity of ^{125}I-M2 was a direct indication of the incorporation of molecules into the cells, the M2 antibody was visualized by immunostaining. The M2 antibody bound to cell surface MT1-F (Fig. 1 B, Surface fraction) was exclusively stained without permeation of the cells. The internalized antibody together with MT1-F was stained exclusively after removing the cell surface antibody by the acid wash and permeating the cell membrane. The M2 antibody bound to the cells expressing MT1-F, but not to the mock-transfected cells (Surface, 0 min). Internalized signals were not detected at time 0 (Intracellular, 0 min). The cell surface–bound M2 antibody decreased thereafter, suggesting that the M2 antibody was either being internalized together with the MT1-F molecules or was released from the surface. Intracellular signals of the M2 antibody increased according to the incubation time (Intracellular, 10 and 30 min) indicating that the M2 antibody bound to the cell surface MT1-F was incorporated into the cells. However both surface and intracellular signals became weak after a 60-min incubation (60 min). Thus, the internalization of the cell surface MT1-MMP molecules was visually confirmed again.

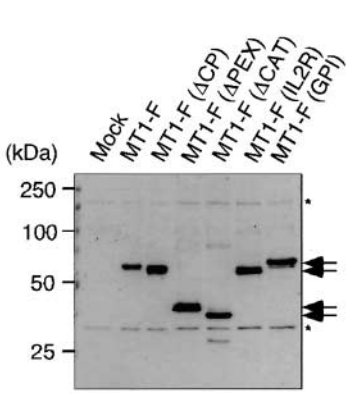
Internalization of MT1-MMP depends on sequences in its cytoplasmic tail

The internalization of surface proteins depends on specific sequences in the cytoplasmic tail that direct incorporation of the molecules into clathrin-coated vesicles (Heilker et al., 1999). MT1-MMP itself may bear such signals on its cytoplasmic tail. Alternatively, it may associate with other proteins having such internalization motifs. We first located the MT1-MMP domain that is essential for internalization by constructing various deletion mutants as shown in Fig. 2 A. Comparable levels of expression for each construct were confirmed by Western blotting (Fig. 2 B). The amount of each mutant on the cell surface was measured by the amount of cell surface–bound ^{125}I-M2 (Fig. 2 C). The internalization of each mutant was then analyzed after a 30-min incubation. Deletion of either the catalytic (ΔCAT) or the PEX domain (ΔPEX) did not affect internalization (Fig. 2 D). However, deletion of the cytoplasmic tail (ΔCP) or its substitution with the cytoplasmic tail derived from the interleukin 2 receptor α chain (IL2R) dramatically reduced internalization (Fig. 2 D). We also tested another chimeric MT1-F molecule, MT1-F(GPI), wherein the MT1-F transmembrane domain and the cytoplasmic tail had been substituted with the GPI-anchoring signal derived from MT4-MMP. The internalization of this mutant molecule was also reduced (Fig. 2 D). These observations indicate that the cytoplasmic tail of MT1-MMP contains the critical sequences required for internalization. These sequences are either internalization signals or the site through which MT1-MMP interacts with other molecules that contain internalization signal motifs.

Delineation of the internalization signals in the cytoplasmic tail

To delineate the sequences that are essential for MT1-MMP internalization, a series of deletions were introduced into the MT1-MMP cytoplasmic tail (Fig. 3 A). After confirming the expression levels and the cell surface amounts of each mutant (Fig. 3 B), which did not differ from the wild-type protein, the internalization of each mutant was measured (Fig. 3 C). Deletion of four (Δ579) and eight (Δ575) amino acids from the COOH terminus resulted in a slight decrease (20%) in internalization, whereas a significant reduction (60%) was observed with the Δ571 mutant that lacks 12 amino acids. Further deletion did not reduce the internalization significantly. Thus, it appears that the last four amino acids, LLYC^{574}, of the Δ575 mutant constitute an important internalization signal. Consistent with this observation, internal deletion of the LLYC^{574} amino acids (Δ571–574) reduced internalization by 50%.





C

MT1-MMP mutants	Cell-surface amount (fmol / 10^6 cells)
MT1-F	318.9 ± 18.4
MT1-F (ΔCP)	533.2 ± 30.6
MT1-F (ΔPEX)	300.4 ± 19.8
MT1-F (ΔCAT)	589.1 ± 15.1
MT1-F (IL2R)	166.8 ± 8.0
MT1-F (GPI)	417.0 ± 21.6

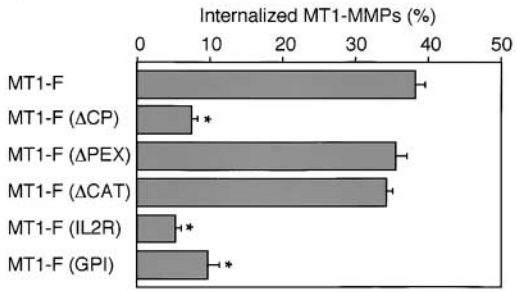


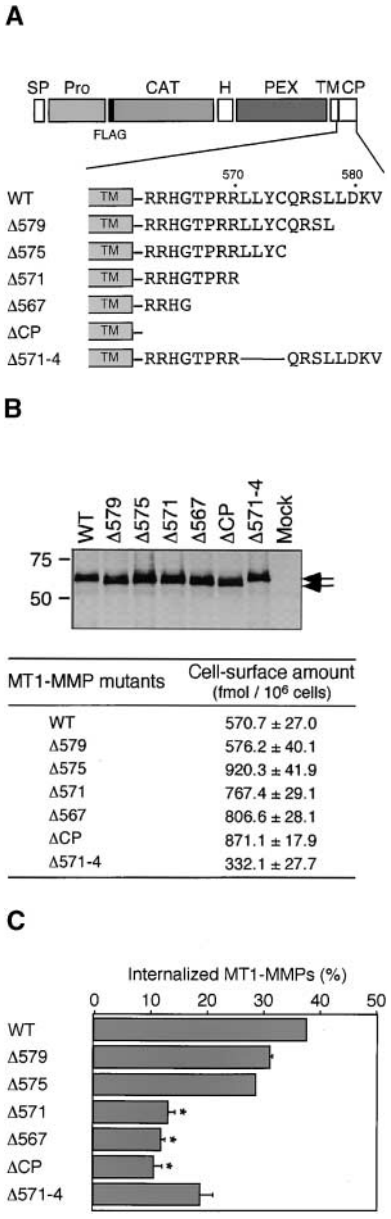
Figure 2. **Identification of the domain involved in MT1-MMP internalization in CHO-K1 cells.** (A) Schematic representation of MT1-MMP mutants used in this experiment. SP, signal peptide; Pro, propeptide; CAT, catalytic domain; H, hinge; PEX, hemopexin-like domain; TM, transmembrane domain; CP, cytoplasmic domain; FLAG, FLAG epitope; GPI, glycosylphosphatidylinositol anchor. (B) Western blot analysis of MT1-MMP mutants expressed in CHO-K1 cells. Molecules expressed in the transfected cells were detected by anti-FLAG M2 antibody. The asterisks (*) indicate the nonspecific band. (C) Amount of MT1-F and mutants expressed on the cell surface calculated from the bound ^{125}I-labeled anti-FLAG M2 antibody. Values are the mean ± SD of three experiments. (D) Internalization of MT1-MMP mutants after a 30-min incubation. Experiments were performed as described in Fig. 1 and the Materials and methods. Values are the mean ± SD of three experiments. The asterisks (*) indicate statistically significant differences ($P < 0.001$) between MT1-F and the mutant.

The tyrosine (Y^{573}) and di-leucine (LL^{572}, referred to as L1) residues in the deleted sequence are found in the internalization motifs that have been reported previously (Heilker et al., 1999). Another di-leucine sequence (LL^{579}, referred to as L2) was also found in the tail sequence. To determine which of these motifs participates in the MT1-MMP internalization, targeted mutations were introduced into one or more of the three motifs (Fig. 4 A). Mutation of either of the two di-leucine sequences to alanines (L1/A or L2/A) reduced internalization by 53 and 33%, respectively (Fig. 4 C). Mutation of both di-leucines showed a slightly additive effect (55% decrease). Mutation of tyrosine^{573} to alanine (Y/A) also reduced internalization by 40% and combining this mutation with L1/A (L1Y/A) inhibited it further (53%). Combined mutations in all three sites (L1YL2/A) reduced internalization to levels comparable with the cytoplasmic deletion mutant (ΔCP; 63%). In contrast, mutating either of the other two amino acids, i.e., serine^{577} (S/A) and valine^{582} (V/A), to alanine had no effect on internalization. The expression of the mutant MT1-MMPs and their cell surface amounts were confirmed and did not differ from the wild-type protein (Fig. 4 B).

Interaction of adaptor protein subunit μ2 with the LLY^{573} motif in the cytoplasmic tail suggests internalization of MT1-MMP via clathrin-coated vesicles

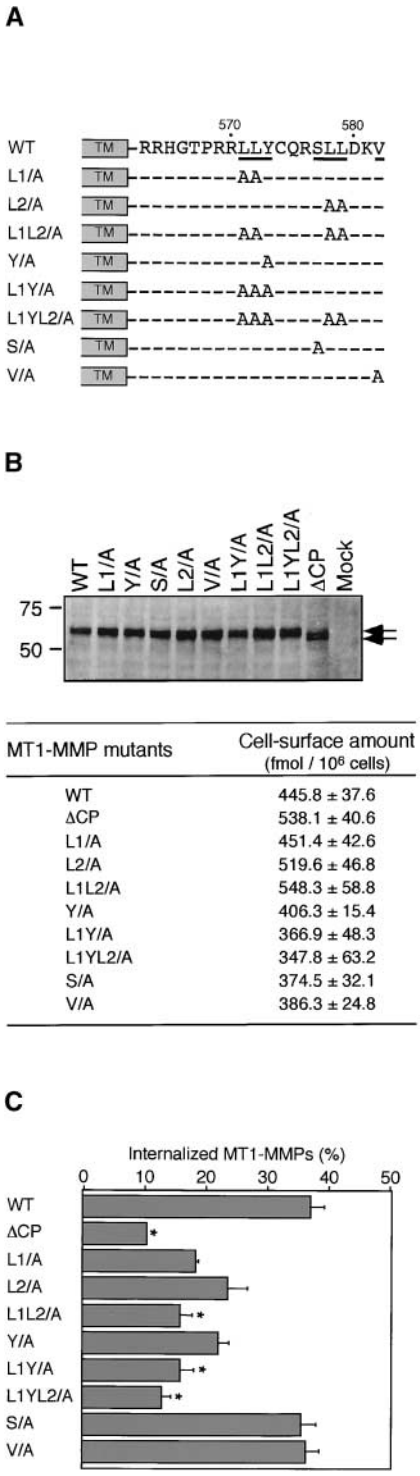
The binding of AP-2 to the cytoplasmic tail of membrane proteins is the first step in the process that causes internalization of the molecules via clathrin-coated pits. Tyrosine and di-leucine motifs are both potential binding sites for AP-2 (Hunziker and Fumey, 1994). Binding of AP-2 to the cytoplasmic tail of cell surface proteins is mediated by its subunit μ2, which recognizes tyrosine-containing motifs (Ohno et al., 1995). Such interactions can be detected by using the yeast two-hybrid system (Ohno et al., 1996). As we found that LLY^{573} serves as a cis-acting element in MT1-MMP internalization, we tested whether LLY^{573} is the binding site for the μ2 subunit. We used the 20–amino acid cytoplasmic tail of MT1-MMP as the "bait" by fusing it with the GAL4 DNA binding domain (GAL4DB), and the GAL4 activator domain fused with the μ2 fragment served as the "prey." The trans-Golgi network–specific integral membrane protein (TGN38) was used as a positive control for these experiments as its cytoplasmic tail is known to bind to μ2 (Ohno et al., 1996). GAL4DB alone (Mock) did not interact with μ2, whereas TGN38 did (Fig. 5 A, TGN38). When the cytoplasmic tail of MT1-MMP was tested, it gave a strong positive signal similar to TGN38 (Fig. 5 A, MT1-WT), suggesting that it interacts with the μ2 subunit. To confirm the importance of the LLY motif in this interaction, it was mutated to alanines (MT1-L1Y/A) and examined. MT1-L1Y/A did not interact with μ2. Thus, the LLY^{573} motif appears to be the site that interacts with clathrin-coated pits.

Next, we assessed whether the internalized MT1-F colocalizes with clathrin. Cells expressing MT1-F or its cytoplasmic mutants were reacted with Texas red–labeled anti-FLAG M2 antibody and incubated for 5 min to allow internalization. Then, cell surface antibodies were removed



MT1-MMP mutants	Cell-surface amount (fmol / 10^6 cells)
WT	570.7 ± 27.0
Δ579	576.2 ± 40.1
Δ575	920.3 ± 41.9
Δ571	767.4 ± 29.1
Δ567	806.6 ± 28.1
ΔCP	871.1 ± 17.9
Δ571-4	332.1 ± 27.7

Figure 3. **Effect of deleting the cytoplasmic domain on MT1-MMP internalization.** (A) Schematic representation of the MT1-MMP cytoplasmic domain deletion mutants. (B) Transiently expressed products in CHO-K1 cells were analyzed by Western blotting using anti-FLAG M2 antibody. Amount of cell surface MT1-F and mutant proteins was calculated from the bound ^{125}I-labeled anti-FLAG M2 antibody. (C) Internalization of MT1-F and its mutant proteins after a 30-min incubation was analyzed as in Fig. 1. Values in B and C are the mean ± SD of three experiments. The asterisks (*) indicate statistically significant differences ($P < 0.001$) between MT1-F and the mutant.



MT1-MMP mutants	Cell-surface amount (fmol / 10^6 cells)
WT	445.8 ± 37.6
ΔCP	538.1 ± 40.6
L1/A	451.4 ± 42.6
L2/A	519.6 ± 46.8
L1L2/A	548.3 ± 58.8
Y/A	406.3 ± 15.4
L1Y/A	366.9 ± 48.3
L1YL2/A	347.8 ± 63.2
S/A	374.5 ± 32.1
V/A	386.3 ± 24.8

Figure 4. **Effect of site-directed mutations on MT1-MMP internalization.** (A) Schematic representation of the MT1-MMP cytoplasmic sequence and mutations. (B) Transiently expressed products in CHO-K1 cells were analyzed by Western blotting using anti-FLAG M2 antibody. Amount of cell surface MT1-F and mutant proteins was calculated from the bound ^{125}I-labeled anti-FLAG M2 antibody. Values indicate the mean ± SD of three experiments. (C) Internalization of MT1-F and its mutant proteins after a 30-min incubation was analyzed as in Fig. 1. Values are the mean ± SD of three experiments. The asterisks (*) indicate statistically significant differences ($P < 0.001$) between MT1-F and the mutant.

completely by washing with acid solution, and the internalized molecules were selectively visualized. The internalized MT1-F molecules were detected in a dot-like pattern at the proximity of the adherent edge of the cell (Fig. 5 B). In the same cell, clathrin was immunostained as numerous dot-like aggregations scattering over the cell (green fluorescence). When these images were merged, the signals of internalized MT1-F overlapped closely with those of clathrin. When

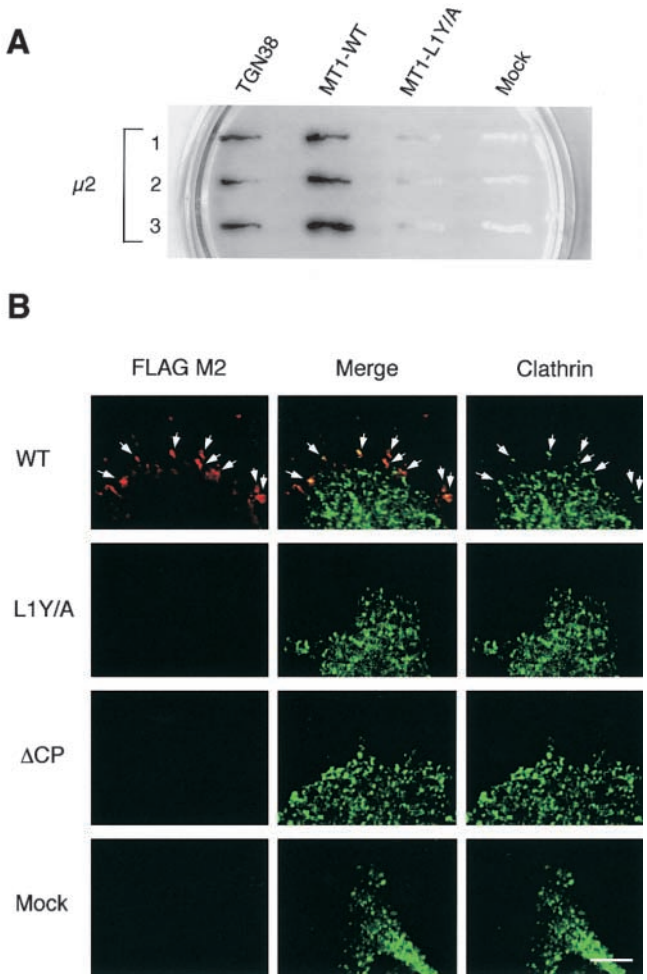


Figure 5. **Internalization of MT1-MMP through clathrin-coated vesicles.** (A) The interaction of the MT1-MMP cytoplasmic tail with the μ2 subunit of AP-2 was examined by yeast two-hybrid analysis. The cytoplasmic domain sequences derived from MT1-WT and MT1-LY/A were expressed as fusion proteins with the GAL4 DNA binding domain (bait), while the μ2 subunit was expressed as a protein fusion with the GAL4 transcription activation domain (prey). Protein–protein interaction was detected by the X-α-Gal assay. Three independent clones of transformed yeasts were analyzed for each plasmid combination. TGN38 was a positive control. (B) Colocalization of internalizing MT1-F and clathrin. Cells expressing MT1-F or its cytoplasmic mutants (L1Y/A and ΔCP) were incubated with Texas red–labeled anti-FLAG M2 antibody (red) on ice, washed, and then left at 37°C for 5 min to allow for internalization. Clathrin molecules were immunoreacted with goat anti-clathrin antibody and visualized with Alexa™488-conjugated anti–goat IgG (green). The colocalization of internalizing MT1-F and clathrin was observed by washing the cells with acid solution before their fixation and permeabilization. Signals were then examined by confocal laser microscopy. Bar, 5 μm.

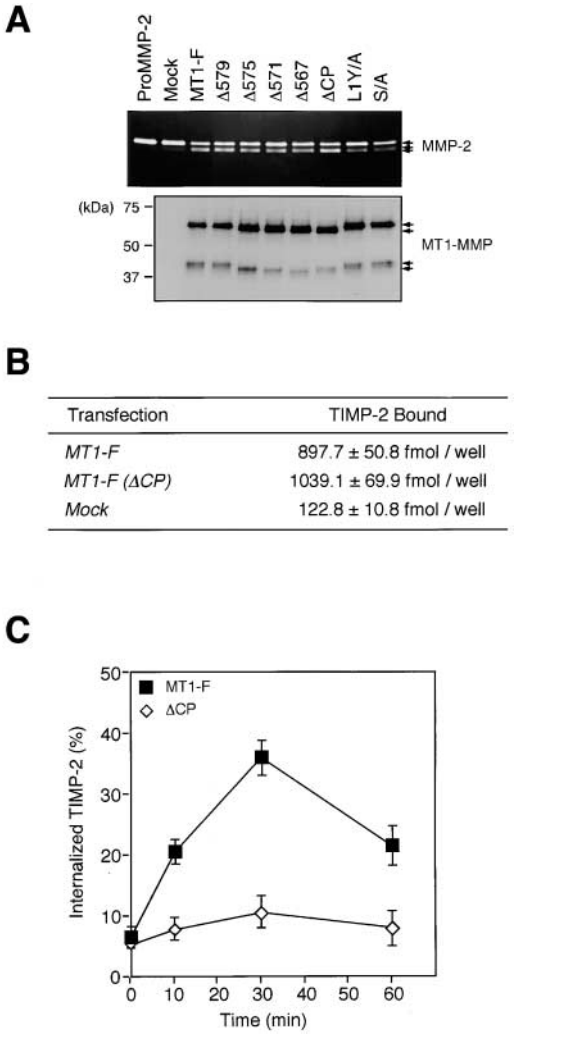


Figure 6. **Effect of cytoplasmic mutations on cell surface events mediated by MT1-MMP.** (A) CHO-K1 cells were transfected with the expression plasmids for various MT1-MMP mutants. The cells were incubated with purified proMMP-2 in serum-free culture medium. After 18 h, MMP-2 in the culture medium was analyzed by gelatin zymography (top). Cell lysates were subjected to Western blot analysis using the anti–MT1-MMP monoclonal antibody. (B) The cells expressing MT1-F or MT1-F(ΔCP) or mock-transfected cells were incubated with ^{125}I–TIMP-2, and the amount of TIMP-2 bound to the cells was calculated from the radioactivity. (C) Internalization of TIMP-2 bound to the cells was analyzed similarly as described in Fig. 1. Internalization of TIMP-2 bound to the cells expressing MT1-F (■) or MT1-F(ΔCP) (◇) is plotted. Values in B and C are the mean ± SD of three experiments.

MT1-F(L1Y/A)– or MT1-F(ΔCP)–expressing cells and mock-transfected cells were examined, such internalized M2 antibody signals were not observed at all.

A defect in MT1-MMP internalization does not affect its ability to activate proMMP-2

To examine whether MT1-MMP internalization affects MT1-MMP activity on the cell surface, we analyzed the ability of the MT1-MMP mutants to activate proMMP-2, which is one of the important functions of MT1-MMP. MT1-MMP constructs with various mutations in the cytoplasmic domain were expressed in CHO-K1 cells, and their ability to activate the proMMP-2 in the culture medium was examined (Fig. 6 A). None of the mutations altered proMMP-2 activation significantly. Thus, reflecting the expression levels of MT1-MMP mutants on the cell surface (Figs. 3 B and 4 B), their net cell surface proteolytic activity did not change significantly compared with that of the wild-type enzyme.

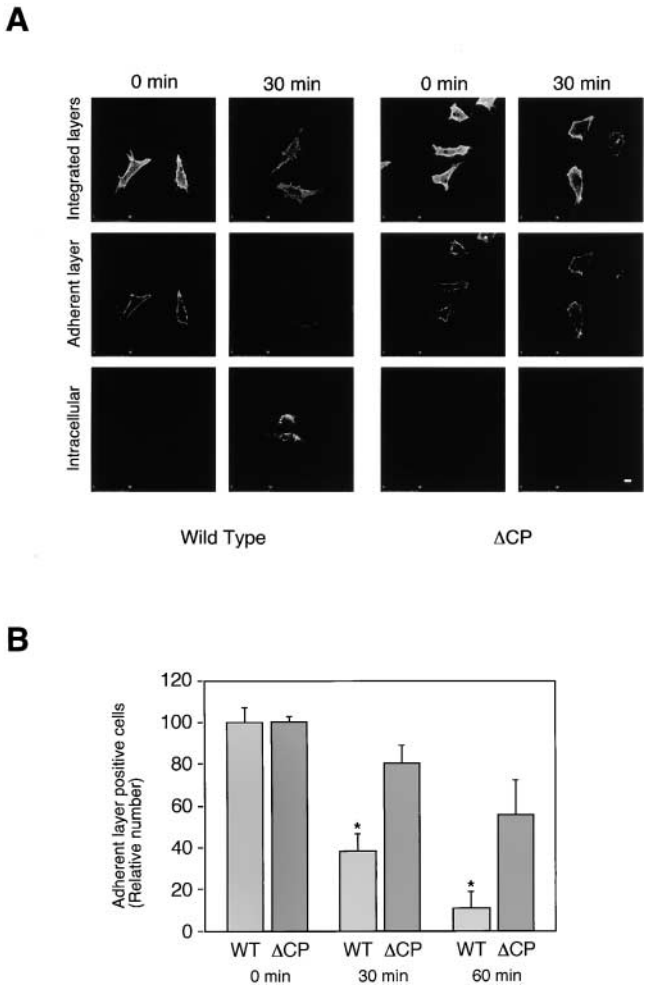


Figure 7. **Localization of MT1-F at the adherent edge and its internalization.** (A) CHO-K1 cells were cotransfected with the plasmids for either MT1-F or ΔCP mutant and that for GFP. The cells were incubated with anti-FLAG M2 antibody, washed, and then incubated at 37°C for the indicated periods of time. The M2 antibody was detected by immunostaining with Cy3-labeled anti–mouse IgG. Signals were observed by confocal laser microscopy. The internalized antibody was stained using the cells that had been acid washed. Adherent layer; confocal section at the adherent layer, Integrated layers; integrated confocal layers. Bar, 10 μm. (B) The number of cells that retained the signals at the adherent layer were counted. Transfected cells were identified by GFP expression. At time 0, almost 100% of GFP-positive cells expressed MT1-F or MT1-F(ΔCP) at the adherent layer. Cells that retained more than 30% of the original average fluorescence intensity at the adherent layer at each time point were counted as positive and presented. 100 cells were counted independently three times and the values presented are the mean ± SD.

Maquoi et al. (2000) reported recently that MT1-MMP–bound TIMP-2 is internalized into the cell. Thus, we examined whether TIMP-2 internalization is indeed mediated by MT1-MMP. The binding of ^{125}I–TIMP-2 to the cell surface was observed with cells that expressed either MT1-F or MT1-F(ΔCP) (Fig. 6 B). TIMP-2 was internalized if it was bound to MT1-F but not if it was bound to MT1-FΔCP (Fig. 6 C). Thus, the internalization of TIMP-2 was also dependent on the cytoplasmic sequence of MT1-MMP.

MT1-MMP is internalized at the adherent edge

MT1-MMP is mainly localized at the adherent edge and migration front of cells. This localization appears to be appropriate for promoting cell invasion and migration. The continuous internalization of MT1-MMP at the edge may serve to substitute inactivated molecules, including TIMP-2 inhibited molecules and processed fragments, with freshly synthesized ones during cell migration. To investigate this possibility further, we examined the internalization of MT1-F at the cell adherent edge using a confocal laser microscope. The localization of the enzyme at the adherent layer was not affected by the mutation that abolishes internalization (ΔCP) (Fig. 7 A, Adherent layer and Integrated layers, 0 min). When MT1-F–expressing cells were incubated for 30 min, the wild-type enzyme present at the cell adherent layer was drastically decreased and internalized molecules were detected (Wild Type, Integrated layers, Adherent layer, and Intracellular; 30 min). However, when MT1-F(ΔCP) was expressed, the signals at the cell adherent layer were not diminished even after a 30-min incubation (Adherent layer, ΔCP). Internalization at this point was also not observed (Intracellular, ΔCP). The number of cells that expressed MT1-F or MT1-F(ΔCP) at the adherent edge was also counted (Fig. 7 B). Thus, the mutation that disturbs the internalization of MT1-MMP clearly abolishes the active turnover of the molecule at the adherent edge. Similar results were obtained with other internalization-defective mutants (unpublished data).

Internalization-defective MT1-MMP cannot stimulate cell migration

We observed previously that expression of MT1-MMP promotes the migration of CHO-K1 cells (Kajita et al., 2001). We thus examined whether the internalization-defective mutations also affected the cell migration promoted by MT1-F (Fig. 8). Migration was analyzed by measuring phagokinetic tracks of cells on a coverslip coated with BSA and colloidal gold. Wild-type MT1-F promoted a threefold increase in CHO-K1 cell migration. This phenomenon requires the catalytic activity of MT1-F because the catalytically inactive E/A mutant did not promote the motility at all (Fig. 8, A and B). Interestingly, internalization-defective mutants (ΔCP and L1Y/A) did not stimulate motility, whereas the S/A mutation, which has no effect on internalization, did enhance motility. Other cytoplasmic tail mutants that did not affect internalization also stimulated motility (unpublished data). Thus, the internalization of MT1-F correlates with its ability to promote cell migration. We also confirmed that the internalization defect of MT1-F does not affect the internalization of other cell surface molecules, using hTfnR as a representative molecule (unpublished data).

The above-mentioned experiments were performed on a BSA-coated coverslip that is different from the gelatin-coated one used for the experiment in Fig. 7. To confirm that the coated material does not affect MT1-F internalization, we repeated the internalization experiments on a BSA-coated coverslip. Transfected cells were cultured in the same conditions that allow cell migration (but without colloidal gold) and were then analyzed. Although the cell morphology was different from that on the gelatin coat (Fig. 7), essen-

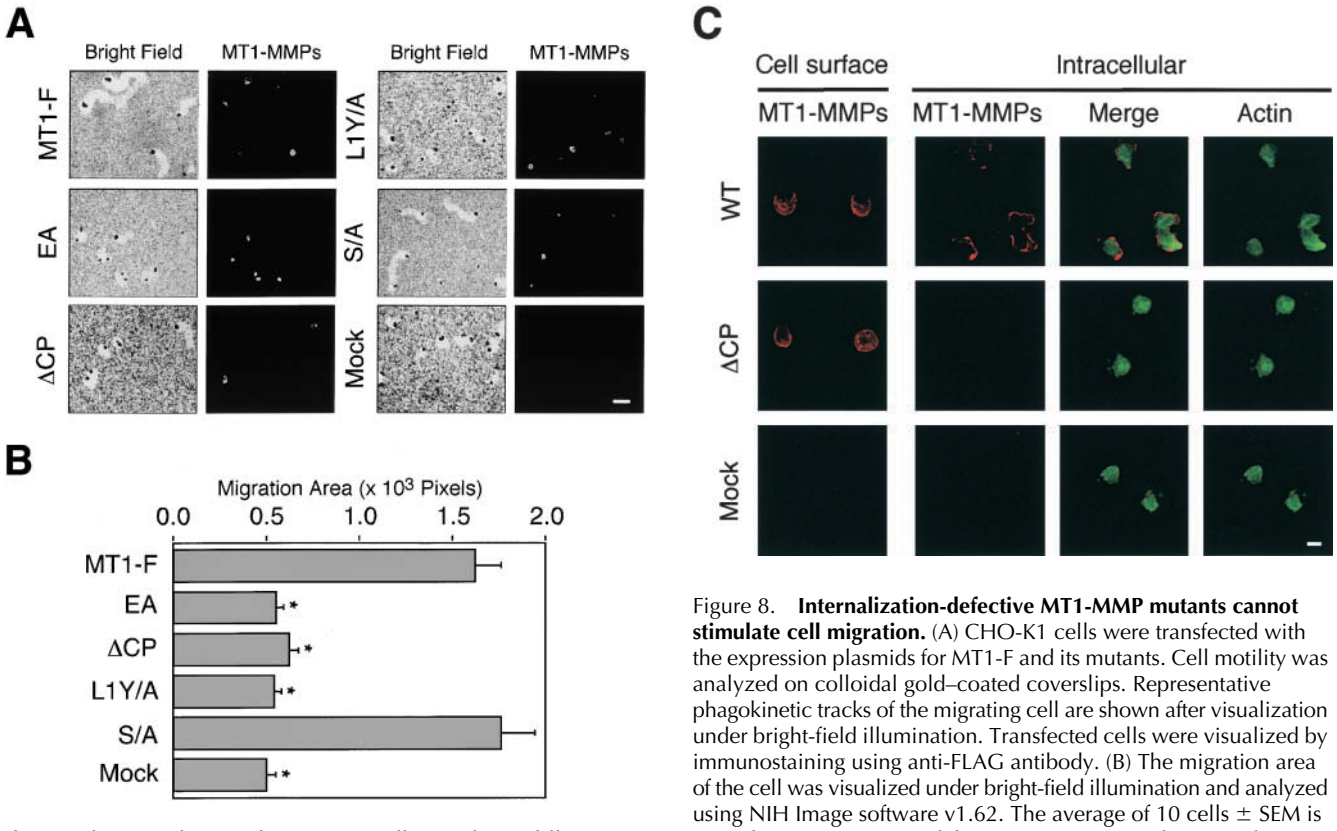


Figure 8. **Internalization-defective MT1-MMP mutants cannot stimulate cell migration.** (A) CHO-K1 cells were transfected with the expression plasmids for MT1-F and its mutants. Cell motility was analyzed on colloidal gold–coated coverslips. Representative phagokinetic tracks of the migrating cell are shown after visualization under bright-field illumination. Transfected cells were visualized by immunostaining using anti-FLAG antibody. (B) The migration area of the cell was visualized under bright-field illumination and analyzed using NIH Image software v1.62. The average of 10 cells ± SEM is shown. The asterisks (*) indicate statistically significant differences ($P < 0.001$) between MT1-F and the mutant. (C) Internalization of MT1-F during cell migration was monitored. Transfected cells were seeded on coverslips without colloidal gold and incubated. Cell surface MT1-F and its mutant were incubated with Texas red–labeled FLAG M2 antibody. After a 5-min incubation to allow for internalization, surface FLAG M2 antibody was removed by acid wash and internalized FLAG M2 antibody was observed by confocal laser microscopy. Actin was stained with Alexa™488-conjugated phalloidin. Bars, (A) 100 μm; (B) 10 μm.

tially the same results were obtained (Fig. 8 C). Thus, cell migration accompanies active internalization of MT1-MMP molecules at the migration front, and it appears that a defect in MT1-MMP internalization also disturbs the migration-promoting activity of the enzyme.

Correlation between internalization and invasion-promoting activity of MT1-MMP

It was reported previously that the MT1-MMP mutants with truncated cytoplasmic tails failed to promote invasion of the cells into Matrigel, a reconstituted basement membrane (Lehti et al., 2000). Invasion requires a combination of proteolytic and locomotive cell activities. As internalization-defective mutants also lost their ability to promote cell migration, there might be a possibility that the absence of invasion was caused by the impaired internalization of MT1-MMP. We expressed MT1-F and its mutants in CHO-K1 cells and cellular invasion of Matrigel was examined. The expression of MT1-F promoted a 6.5-fold increase in cell invasion (Fig. 9), whereas the expression of the catalytically inactive mutant (E/A) did not. Mutations that affect MT1-F internalization (Δ571, Δ567, ΔCP, and L1Y/A) all severely disturbed the invasion-promoting activity of the enzyme, whereas those that did not affect internalization (Δ579, Δ575, and S/A) promoted invasion as efficiently as the wild-type enzyme. The inability of the internalization-deficient mutants to promote invasion was not due to the differences in their net proteolytic activity or the amounts of MT1-F on the cell surface, as demonstrated in Fig. 6, A and B. Thus, internalization of MT1-MMP seems to be important in regulating the enzyme during cell migration and invasion.

Discussion

Internalization of MT1-MMP is regulated by signals in its cytoplasmic tail

In this study, we demonstrated that MT1-MMP can be removed from the cell surface by internalization, a process dependent on its cytoplasmic tail sequence. We initially thought that cell surface events such as processing, inhibition by TIMP-2, or oligomer formation might trigger or inhibit the internalization of MT1-MMP at the surface. To examine this, we studied MT1-MMP internalization using various deletion mutants that mimic processed products, or under conditions such as presence or absence of natural and synthetic inhibitors or monovalent and divalent antibodies (unpublished data). However, none of these affected the internalization efficiency; it was solely dependent on the cytoplasmic tail. Based on these observations, we prefer the idea that the internalization of MT1-MMP is independent of outside events such as TIMP-2 binding and autodegradation, and therefore this is presumably a system that constitutively cleans up all forms of MT1-MMP molecules at the surface.

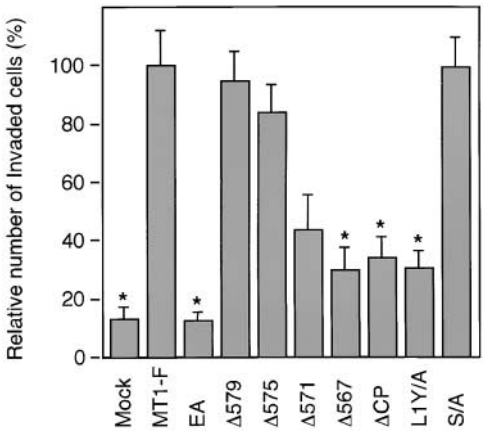


Figure 9. **Effect of the mutations on Matrigel invasion.** CHO-K1 cells were transfected with the expression plasmids for MT1-F, its mutants, or empty vector together with a GFP plasmid. Cells were then subjected to the Matrigel invasion assay. After 48 h of incubation, the invaded GFP-positive cells on the lower surface of the filters were counted under fluorescence microscopy. Each assay was performed in triplicate and numbers shown are the mean ± SD. The asterisks (*) indicate statistically significant differences ($P < 0.001$) between the cells expressing wild-type and mutant MT1-MMP molecules.

Unexpectedly, mutations that abrogated the internalization did not cause drastic accumulation of MT1-MMP molecules on the cell surface. They also did not increase the extent by which MT1-MMP activated proMMP-2 (Fig. 6 A). This may indicate that there are yet unknown regulations that adjust the level of MT1-MMP on the cell surface. However, it is also possible that the transient overexpression used for the experiments was not appropriate for reflecting the effect of internalization on the cell surface amount of the enzyme. Nevertheless, it is clear that the internalization is dependent on a specific sequence in the cytoplasmic domain, and mutating this sequence abrogates internalization at the adherent edge.

Fine mapping of the cytoplasmic domain sequence with mutants revealed the critical residues to be the LLY^{573} motif, which is a binding site for the μ2 subunit of AP-2, a component of clathrin-coated vesicles. Confocal laser microscopic analysis also revealed that the internalized MT1-MMP colocalized with clathrin-coated vesicles, indicating strongly that the MT1-MMP molecules were in the vesicles (Fig. 5 B).

Of the six MT-MMP proteins, four are integral membrane proteinases with a cytoplasmic tail, and two are tethered to the membrane by GPI anchors (Itoh et al., 1999; Kojima et al., 2000). The reported consensus sequence for μ2 binding is YXXØ (X, any amino acid; Y, tyrosine; Ø, bulky hydrophobic amino acids; Ohno et al., 1996). The cytoplasmic sequence in MT1-MMP (YCQR) is close to the consensus but does not match completely because Ø is substituted by the bulky but hydrophilic arginine. The cytoplasmic tails of the other three transmembrane MT-MMPs all contain similar YXXR sequences. The di-leucine motif preceding the tyrosine in the MT1-MMP cytoplasmic sequence is also found in MT2- and MT3-MMP, but not in MT5-MMP. Consistent with this sequence conservation, we found that MT2- and MT3-MMP are internalized as effectively as MT1-MMP, whereas MT5-MMP is internalized less efficiently (unpublished data). GPI-anchored type MT4- and MT6-MMP that lack a cytoplasmic tail are not internalized efficiently (unpublished data).

Internalization-defective MT1-MMP cannot promote cell migration and invasion

Although a defect in MT1-MMP internalization did not affect its net amount on the cell surface nor its ability to activate proMMP-2, this defect severely disturbed its ability to promote cell invasion in Matrigel. This observation is consistent with a previous study showing that deletion of the MT1-MMP cytoplasmic tail reduced its invasion-promoting activity (Lehti et al., 2000). We found that all the mutations resulting in poor internalization also abrogated the invasion-promoting activity of MT1-MMP. Similarly, poor internalization of MT1-MMP also affected its ability to promote cell motility. Cell surface MT1-MMP has to be regulated precisely when it functions as a part of invasion machinery. In addition to its proteolytic activity, the enzyme has to be at the right site at the right time on the cell surface together with the right partners, and then it is inhibited by TIMP-2 or partially degraded. These "old molecules" have to be cleared off to assure substitution with the newly synthesized molecules. Thus, it is very plausible that the proteolytic activity of MT1-MMP is critical but not sufficient for a biological outcome such as invasion.

During cell migration, MT1-MMP localizes predominantly to the cell adherent edge and the migration front. Because such localization is presumably important in regulating cell locomotion, we speculated first that the mutations in the cytoplasmic sequence affect such localization and eventually abrogate cell migration and invasion. However, this was not the case, as shown in Fig. 7. Consistent with the localization site of MT1-MMP, internalization of the enzyme was mostly observed there. Accordingly, whereas surface-labeled wild-type MT1-F disappeared over time at the adherent edge, the levels of the ΔCP mutant decreased very slowly (Fig. 8, A and B). Thus, the mutations that prevent internalization of MT1-MMP molecules disturb the turnover of the enzyme at the migration front. The adherent edge and migration front are where the proteases and cell adhesion molecules that regulate cell locomotion and invasion congregate. However, our knowledge of the molecules involved and the way they operate in these processes is at present not sufficient to completely understand the role of MT1-MMP internalization. One possible idea is that internalization may be a mechanism to prevent accumulation of inactivated MT1-MMP, such as TIMP-2–inhibited or partially degraded molecules, which may disturb their substitution with freshly synthesized enzymes. If this is the case, internalization-defective mutants are expected to disturb the function of the wild-type enzyme. Indeed, expression of such mutant MT-MMP molecules in the cells expressing the wild-type enzyme inhibited the migration and invasion-stimulating activity of the wild-type MT1-MMP (unpublished data). This dominant-negative effect of the mutant against the wild-type enzyme indicates again that proteolytic activity of

MT1-MMP is important but not sufficient to promote cell migration and invasion.

A recent study has described somewhat contrary results about the role of the MT1-MMP cytoplasmic tail on invasion-promoting activity. Namely, expression of the MT1-MMP mutant lacking a cytoplasmic tail still promoted invasion of COS-1 cells in a type I collagen gel, same as the wild-type enzyme did (Hotary et al., 2000). Because we used CHO-K1 cells and Matrigel for the assay, we tried to reproduce their experiments first. Expression of the MT1-F derivatives in COS-1 cells and analysis of the invasion in a type I collagen gel revealed that internalization-defective mutants also promoted invasion, confirming Hotary's results (unpublished data). However, when the same cells were analyzed in Matrigel, the results were similar to those obtained in this study using CHO-K1 cells. Thus, the requirement of the cytoplasmic tail for the invasion-promoting activity of MT1-MMP seems to differ depending on the matrix used for the assay. It should be noted that Matrigel contains complex ECM components, some of them cannot be degraded by MT1-MMP, whereas type I collagen gel contains a single component that is a good substrate for MT1-MMP (Ohuchi et al., 1997). Consequently, the collagen gel may be loosened more easily than Matrigel by MT1-MMP, and the cells may be able to move through the residual type I collagen matrix easily even when their locomotive force was reduced by the expression of internalization-defective mutants. Thus, the migration-promoting activity of MT1-MMP may play a more important role when the cells are moving through the residual Matrigel matrix rather than the loosened type I collagen gel.

Nakahara et al. (1997) have previously suggested that the cytoplasmic tail of MT1-MMP may play a role in localizing the enzyme to the protruding membrane structure known as the invadopodium, which is frequently associated with invasive cancer cells. In our experiments, CHO-K1 cells do not form obvious invadopodial structures and the localization of MT1-MMP at the cell adherent edge is not affected by mutations in the cytoplasmic tail. Similar observations were also made with COS-1 and HT1080 cells (unpublished data). Although the molecular characterization of invadopodia is not sufficient to understand how its formation is regulated (Chen and Wang, 1999), the cytoplasmic tail of MT1-MMP may interact with a certain molecule that exists in the invadopodia. It is also possible that the formation of the invadopodia itself might be disturbed by the internalization-defective MT1-MMP mutants because formation of these structures requires local invasion.

In conclusion, our study indicates that the cytoplasmic tail of MT1-MMP is critical for the internalization of MT1-MMP molecules at the cell surface, presumably after their incorporation into clathrin-coated vesicles. During cell migration, MT1-MMP localizes predominantly to the migration front and is internalized there. Mutations that disturb the internalization caused accumulation of the molecules at the adherent edge and abrogated cell migration and invasion. Thus, dynamic turnover of MT1-MMP molecules at the migration front by internalization appears critical to the migration-stimulating activity of MT1-MMP. This is a newly discovered mechanism that regulates the action of pericellular proteases during cell migration and sheds light on the dynamic events at the migration front that control cell locomotion, a field that is still unclear. Further study is needed to precisely delineate the sequential events that regulate proteases and cell adhesion molecules in relation to actin dynamics.

Materials and methods

Cell culture and transfection

CHO-K1 cells were cultured at 37°C in Ham's F-12 medium (Sigma-Aldrich) supplemented with 10% FBS, 1% kanamycin, and MEM nonessential amino acid solution (Invitrogen). For transfection, cells were seeded in six-well plates at 1.5×10^5 cells/well and transfection was performed after 16 h. Expression vectors were transfected using FuGENE6™ (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Construction of FLAG-tagged human MT1-MMP mutants and hTfnR

FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys)–tagged MT1-MMP (denoted as MT1-F) was constructed as previously described (Itoh et al., 1999) and subcloned into the pSG5 expression vector (Stratagene). This plasmid was used as a template for PCR-based modification to generate mutant MT1-MMPs.

MT1-F(EA) is the catalytically inactive mutant (Glu^{240} to Ala). MT1-F(ΔPEX) is the hemopexin-like domain–deleted mutant (ΔIle^{318}–Gly^{535}), and MT1-F(ΔCAT) is the catalytic domain–deleted mutant (ΔTyr^{112}–Pro^{312}). MT1-F(IL2R) is the chimera of the ectodomain of MT1-F and the transmembrane/cytoplasmic domain of IL2R. MT1-F(GPI) is the chimeric mutant of MT1-F (Met^{1}–Cys^{508}) and the GPI-anchoring signal derived from MT4-MMP (Gly^{526}–Leu^{607}). Cytoplasmic deletion mutants of MT1-MMP (Δ579, Δ575, Δ571, Δ567, and ΔCP) were generated by introducing a stop codon in the plasmid, and site-directed mutations (Δ571–574, L1/A, Y/A, S/A, L2/A, V/A, L1Y/A, L1L2/A, and L1YL2/A) were generated by PCR-based mutation methods. All the PCR products were confirmed by DNA sequencing and the cDNAs subsequently generated were subcloned into a pSG5 mammalian expression vector (Stratagene).

Full-length hTfnR cDNA was amplified by reverse transcript PCR using total RNA obtained from HT1080 cells and subcloned into the pcDNA3.1(−) expression vector (Invitrogen).

Western blot analysis

Transfected cells were washed three times with PBS and lysed in SDS-PAGE loading buffer containing 2-mercaptoethanol. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech). After blocking the membrane with 10% fat-free dry milk in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl), the membrane was probed with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) (5 μg/ml) to detect FLAG-tagged MT1-MMP (MT1-F) and its mutants. An hTfnR-specific antibody was used to detect control cells transfected with hTfnR. The membrane was further probed with alkaline phosphatase–conjugated anti–mouse IgG (Sigma-Aldrich) to visualize the antibody.

Iodination of human holo-transferrin, anti-FLAG M2 antibody, and rTIMP-2

The proteins were labeled with ^{125}I using IODO-GEN (Pierce Chemical Co.) in TBS containing 0.05% Brij35. After the reaction, free ^{125}I was removed by gel filtration on Sephadex™ G-25 (Amersham Pharmacia Biotech).

Internalization assay

Transfected CHO-K1 cells were seeded onto gelatin-coated 12-well plates (10^6 cells/well). After 24 h, cells were incubated for 1 h on ice with 2 μg/ml ^{125}I-labeled anti-FLAG M2 IgG, holo transferrin, or TIMP-2 in internalization medium (Ham's F-12 medium containing 0.1% FBS, 1% kanamycin, and MEM supplemented with nonessential amino acids). 10 μM of peptidyl hydroxamate MMP inhibitor, BB94 (provided by Dr. Peter D. Brown, British Biotech Pharmaceuticals Ltd., Oxford, UK), was included during the incubation with the M2 antibody to avoid the autodegradation of MT1-MMP. Cells were washed with chilled medium three times and incubated further at 37°C for the indicated period of time in each experiment. At the end of the incubation, culture supernatants were collected to measure the ^{125}I-labeled proteins that had been released by the cells. ^{125}I-labeled proteins on the cell surface were collected by washing the cells

with acid solution (500 mM CH_3COOH, 150 mM NaCl) for 5 min at 4°C. This allows more than 98% of cell surface–bound ligand to be recovered. After the acid wash, cells were dissolved in lysis buffer (100 mM NaOH, 150 mM NaCl) to measure the proteins that had been internalized. Radioactivity in each fraction was measured by a gamma counter.

Indirect immunofluorescence staining

Transfected CHO-K1 cells were seeded on gelatin-coated coverslips. After 24 h, the cells were incubated for 1 h at 4°C with mouse anti-FLAG M2 (5 μg/ml) in internalization medium supplemented with 10 μM BB94. The cells were then washed with chilled medium three times, incubated at 37°C, and fixed with 3% paraformaldehyde in PBS. After blocking with 5% goat serum and 3% BSA in PBS for 1 h at room temperature, mouse anti-FLAG M2 antibody–treated cells were incubated with Alexa™488-conjugated goat anti–mouse IgG (Molecular Probes, Inc.) or Cy3-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories). For clathrin staining, fixed cells were incubated with goat anti-clathrin HC (C-20) antibody and visualized with Alexa™488-conjugated rabbit anti–goat IgG (Molecular Probes, Inc.). Actin was stained with Alexa™488-conjugated phalloidin. Fluorescence signals were detected using a Bio-Rad MRC-1024 confocal laser microscope.

Yeast two-hybrid analysis

Yeast two-hybrid assays were performed as described in the instructions for the MATCHMAKER two-hybrid kit using yeast strain Y190 (CLONTECH Laboratories, Inc.) with a modification described previously (Ohno et al., 1996). In brief, plasmids encoding the GAL4 binding domain fused with the MT1-MMP cytoplasmic domain (R^{563}–V^{582}) or the cytoplasmic domain mutant (LLY^{573}/AAA) were constructed using a PCR-based technique and subcloned into the pGBT9 vector. These constructs were tested for interaction with GAL4 fused to activation domain μ2 using X-α-Gal (CLONTECH Laboratories, Inc.).

Gelatin zymography

Gelatin zymography was performed as described previously (Sato et al., 1994). Samples were mixed with SDS sample buffer without a reducing agent and separated in 7.5% acrylamide gels containing 0.1% gelatin. The gels were incubated at 37°C after removing SDS by washing with 2.5% Triton X-100–containing buffer. The gels were stained with Coomassie brilliant blue R250, and the gelatinolytic activities were detected as clear bands against a blue background.

Matrigel invasion assay

The Matrigel invasion assay was performed as described previously (Itoh et al., 2001) according to manufacturer's instructions (Becton Dickinson). MT1-MMP (wild-type or mutant) expression plasmids were cotransfected into CHO-K1 cells with the green fluorescent protein (GFP) expression plasmid. The transfected cells were suspended in Ham's F-12 medium without serum and seeded onto Matrigel-coated transwell filters (8-μm pore) in invasion chambers. FBS (10%)-containing medium was added to the lower chambers and incubated for 48 h. Noninvading cells remaining on the upper surface of the filter were removed and fixed with 3% paraformaldehyde. The GFP-positive cells appearing on the lower surface of the filter were counted by fluorescence microscopy. Each assay was performed in triplicate.

Phagokinetic track motility assay

The phagokinetic track motility assay was performed as described previously (Kajita et al., 2001). Colloidal gold–coated coverslips were placed in a 12-well plate and transfected cells were seeded at 3×10^3 cells/well. After 12 h of incubation, the phagokinetic tracks were visualized under bright-field illumination using a CoolSNAP-fx monochrome CCD camera (Roper Scientific). The track area was measured using NIH Image software v1.62.

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