The Second Dimer Interface of MT1-MMP, the Transmembrane Domain, Is Essential for ProMMP-2 Activation on the Cell Surface*

Yoshifumi Itoh†1, Noriko Ito‡, Hideaki Nagase§, and Motoharu Seiki§

From the ‡Department of Matrix Biology, Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College London, 65 Aspenlea Road, Hammersmith, London W6 8LH, United Kingdom and the §Division of Cancer Cell Research, Institute of Medical Science, The University of Tokyo, 4-6-1 Minatoku, Tokyo 108-8639, Japan

Activation of proMMP-2 and cell surface collagenolysis are important activities of membrane-type 1 matrix metalloproteinase (MT1-MMP) to promote cell migration in tissue, and these activities are regulated by homodimerization of MT1-MMP on the cell surface. In this study, we have identified the transmembrane domain as a second dimer interface of MT1-MMP in addition to the previously identified hemopexin domain. Our analyses indicate that these two modes of dimerization have different roles; transmembrane-dependent dimerization is critical for proMMP-2 activation, whereas hemopexin-dependent dimerization is important for degradation of collagen on the cell surface. Our finding provides new insight into the potential molecular arrangement of MT1-MMP contributing to its function on the cell surface.

Membrane-type 1 matrix metalloproteinase (MT1-MMP)2 is a type I transmembrane proteinase that promotes cell migration in tissue (1). MT1-MMP is implicated in many physiological and pathological conditions including wound healing (2), bone development (3, 4), lung development (5, 6), angiogenesis (3, 7, 8), cancer invasion (9) and growth (10), rheumatoid arthritis (11), and atherosclerosis (12–14). MT1-MMP promotes cellular invasion by degrading barrier extracellular matrix components including collagens I, II, III, fibronectin, laminins, vitronectin, and aggrecan (15–17); by activating other MMPs, namely proMMP-2 (9) and proMMP-13 (18); by shedding cell adhesion molecules such as CD44 (19) and syndecan 1 (20); and by activating extracellular signal-regulated kinase (ERK) through as yet undefined mechanisms (21, 22).

Having such diverse functions, MT1-MMP is regulated by different mechanisms including gene expression, activation of the zymogen (23, 24), inhibition by endogenous inhibitors, including tissue inhibitor of metalloproteinases (TIMPs) (25), RECK (26), and Testicans (27, 28), localization to the leading edge of migrating cells, including lamellipodia (29–31) and invadopodia (32), autolytic degradation and processing (33–35), endocytosis through clathrin- and caveolea-dependent mechanisms (36–38), palmitoylation at its cytoplasmic domain (39), recycling (40), and lysosomal degradation (41). Such regulation is thought to be essential to coordinate MT1-MMP activity with cellular events, enabling it to promote cell invasiveness (42).

ProMMP-2 activation is one of the MT1-MMP functions thought to be important in cancer invasion (9, 43) and growth (44), where its significance lies particularly on basement membrane degradation as MT1-MMP itself cannot degrade collagen IV, a major component of the matrix but activated MMP-2 does. In this activation process, MT1-MMP forms a complex with its endogenous inhibitor, TIMP-2 (45–47). TIMP-2 binds to the catalytic site of MT1-MMP through its inhibitory site in the N-terminal domain, leaving the exposed C-terminal domain of TIMP-2 to interact with the hemopexin (Hpx) domain of proMMP-2 (45–47). Thus the MT1-MMP-TIMP-2 complex acts as a receptor for proMMP-2. To activate proMMP-2 in this complex, a second MT1-MMP, which is free of TIMP-2, needs to be positioned in close proximity to the trimolecular complex. This is achieved by the formation of an MT1-MMP homodimer complex (31, 48).

Another important biological activity of MT1-MMP is collagen degradation (15, 49). Among MMP family members, at least six enzymes can degrade fibrillar type I collagen, namely MMP-1, MMP-2, MMP-8, MMP-13, MT1-MMP (50), and MT2-MMP (51). MT1-MMP and MT2-MMP are membrane-bound collagen-degrading enzymes, but MT2-MMP is the weakest of the collagenolytic MMPs, exhibiting 1⁄100 of the activity of MT1-MMP (51). Thus MT1-MMP is likely to be the major pericellular collagenase. MT1-MMP-null mice exhibit phenotypes thought to be due to a lack of cellular collagenolytic activity (3, 4). We have recently shown that MT1-MMP dimer formation is essential for collagen degradation on the cell surface (52), suggesting that dimerization of MT1-MMP may be an important regulatory mechanism to activate MT1-MMP on the cell surface for both collagenolysis and proMMP-2 activation (52).

The abbreviations used are: MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 matrix metalloproteinase; Hpx domain, hemopexin domain; TM domain, transmembrane domain; CP domain, cytoplasmic domain; NGFR, nerve growth factor receptor; TIMP, tissue inhibitor of metalloproteinases; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; PY, phospho-tyrosine.

* This work was supported, in whole or in part, by National Institutes of Health Grant AR40994. This work was also supported by Cancer Research UK Project Grant C1507/AS541 and the Welcome Trust equipment grant, and the Arthritis Research Campaign core grant to the Kennedy Institute of Rheumatology.

† To whom correspondence should be addressed. Fax: 44-20-8383-4760; E-mail: y.itoh@imperial.ac.uk.
§ The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
We have previously reported that dimerization of MT1-MMP is driven by homodimeric complex formation of the Hpx domains and that this interaction is crucial for proMMP-2 activation (31) and collagen degradation (52) on the cell surface. It was also reported that the Hpx and cytoplasmic domains can drive dimerization for proMMP-2 activation (48). On the other hand, it was recently reported that an Hpx domain-deleted MT1-MMP mutant retains the ability to activate proMMP-2 (53), suggesting that Hpx domain-dependent dimer formation may not play a role in this process. In this report, we have re-evaluated the role of the Hpx, linker-2, transmembrane (TM), and cytoplasmic domains in proMMP-2 activation and the collagenolytic activity of MT1-MMP and found that MT1-MMP has two modes of dimer formation: Hpx domain- and TM domain-dependent dimerization. For proMMP-2 activation, TM-dependent dimerization is essential, whereas Hpx-dependent dimerization is essential for collagenolytic activity. Inhibition of Hpx domain-dependent dimerization by co-expressing the Hpx domain resulted in inhibition of TM-dependent dimer formation, proMMP-2 activation, and collagenolytic activity. Our finding reveals an additional molecular arrangement contributing to MT1-MMP function on the cell surface.

MATERIALS AND METHODS

Cell Culture and Transfection—COS7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (BioWhittaker) supplemented with 10% fetal bovine serum and penicillin/streptomycin (BioWhittaker). TIMP-2-supplemented with 10% fetal bovine serum and penicillin/streptomycin (BioWhittaker). TIMP-2-null fibroblasts according to the manufacturer’s instructions. For transfection, cells were cultured in 6- or 12-well plates and were transfected with expression plasmids using FuGENE 6™ (Roche Applied Science, Basel, Switzerland) for COS7 cells and Lipofectamine 2000 (Invitrogen, Paisley, UK) for TIMP-2-null fibroblasts according to the manufacturer’s instructions.

Antibodies—Mouse anti-FLAG M1 and M2 monoclonal antibodies and alkaline phosphatase-conjugated goat anti–mouse IgG antibodies were purchased from Sigma-Aldrich (Dorset, UK). Mouse monoclonal anti-phospho-tyrosine antibody (PY-20) was purchased from ICN Biochemicals, and Alexa Fluor 488-conjugated and Alexa Fluor 568 conjugated goat anti–mouse IgG and anti–rabbit IgG antibodies were from Molecular Probes (Cambridge, UK).

Construction of MT1-MMP Mutants—FLAG (DYKDDDDK)-tagged MT1-MMP (MT1F) was constructed as described previously (54) and subcloned into pSG5 (Stratagene). A FLAG tag was inserted at the end of the propeptide (between Arg111 and Tyr112), and properly activated enzyme will have the FLAG tag at its N terminus and thus can be recognized by the anti-FLAG M1 antibody (54). MT1F ΔCat is a FLAG-tagged mutant MT1-MMP in which the region of Tyr112 to Pro112 was deleted. MT1F ΔHpx is a FLAG-tagged mutant MT1-MMP in which the region of Cys319–Cys508 was deleted. MT1F ΔHpxΔL2 is an Hpx domain and a linker-2 (L2) region deletion mutant of MT1-MMP where Phe336–Gly535 was deleted. MT1F ΔCatΔTM is a FLAG-tagged mutant MT1-MMP in which the regions of Tyr112 to Gly288 and also Ala336 to Val582 were deleted. MT1F ΔHpxΔL2A to MT1F ΔHpxΔL2J are MT1F ΔHpx derivatives in which the L2 region was further deleted as follows. In MT1F ΔHpxΔL2A, Pro509–Asp515 was further deleted; in MT1F ΔHpxΔL2B, Pro509–Glu523 was deleted; in MT1F ΔHpxΔL2C, Pro509–Val529 was deleted; in MT1F ΔHpxΔL2D, Val524–Ile527 was deleted; in MT1F ΔHpxΔL2E, Glu516–Glu523 and Asp530–Glu537 were deleted; in MT1F ΔHpxΔL2F, Pro509–Glu532 was deleted; in MT1F ΔHpxΔL2G, Asp530–Glu532 was deleted; in MT1F ΔHpxΔL2H, Val524–Glu532 was deleted; in MT1F ΔHpxΔL2I, Glu516–Glu532 was deleted; and in MT1F ΔHpxΔL2J, Glu516–Val529 was deleted. MT1F ΔCP and MT1F ΔHpxΔCP are cytoplasmic domain (Arg563–Val582) deletion mutants. MT1F/NGFRTM and MT1F ΔHpx/NGFRTM are chimera mutants in which corresponding regions of Ala536–Val582 in MT1-MMPs are replaced with Val412–Lys441 of nerve growth factor receptor (NGFR). MT1F/NGFR12TMCP, MT1F ΔCat/NGFR12TMCP, and MT1F ΔHpx/NGFR12TMCP are chimera mutants of MT1F and its mutants in which corresponding regions of Glu516–Val582 in MT1-MMPs were replaced with Glu384–Gly790 of NGFR. MT1F/NGFRCP, MT1F ΔCat/NGFRCP, and MT1F ΔHpx/NGFRCP are chimera mutants of MT1F and its mutants in which corresponding regions of Arg563–Val582 in MT1-MMPs were replaced with Asn434–Gly790 of NGFR. These mutants were generated by the PCR extension method (55). All the PCR-generated fragments were confirmed by DNA sequencing and subcloned into the pSG5 vector.

Western Blotting and Zymography—Western blotting was carried out as described previously (56). Total cell lysates were prepared by the addition of 1X SDS-PAGE loading buffer containing 2-mercaptoethanol to cells in the culture plate and subsequent boiling for 20 min. Zymography was carried out as described previously (54).

Surface Biotinylation and Subsequent Immuno-precipitation—Surface biotinylation and subsequent immuno-precipitation were carried out as described previously (31). Briefly, transfected COS7 cells were washed three times with chilled PBS containing 1 mM MgCl2, and 0.1 mM CaCl2. Cells were then incubated with sulfo-NHS-biotin (Pierce) in same buffer (2 mg/ml) at 4°C for 30 min. The reaction was terminated by further incubating the cells with 25 mM lysine in PBS. The cells were lysed in the buffer of 1% Nonidet P-40, 0.1% SDS, 1% deoxyacidic acid, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.02% NaN3, and the biotinylated proteins were precipitated with streptavidin-agarose beads (Sigma). The samples were analyzed by Western blotting using anti-FLAG M2 antibody.

Indirect Immuno-fluorescence Staining—To localize cell surface MT1-F and its mutants, transfected COS7 cells cultured on 4-well glass slide chambers (Nalge Nunc International) coated with F-gelatin were fixed with 3% paraformaldehyde in PBS. After blocking with 5% goat serum and 3% bovine serum albumin in Tris-buffered saline for 1 h at room temperature, cells were incubated with an anti-FLAG M1 antibody (5 µg/ml) at room temperature for 2 h without permeabilizing cells. 1 mM CaCl2 was included throughout the procedure of washing and incubation for the staining with the anti-FLAG M1 antibody. Alexa Fluor 488-conjugated goat anti-mouse IgG was used to visu-
alize the antigen signal. Note that anti-FLAG M1 antibody can only recognize FLAG tag at the N terminus of molecule (54); thus only active forms of the enzyme can be stained with this procedure. The signals were analyzed by CCD camera-equipped microscope (Nikon TE-2000) with \( \times 10 \) objective lens.

In Situ Gelatin Degradation Assay—4-well chamber slides (Nunc) were coated with Alexa Fluor 488-conjugated gelatin (F-gelatin) prepared with an Alexa Fluor 488 labeling kit (Molecular Probes) as described previously (31). Transfected COS7 cells were cultured in the chamber slides for 16 h. Cells were then fixed with 3% paraformaldehyde in PBS and analyzed using CCD camera-equipped microscope (Nikon TE-2000) with \( \times 10 \) (see Fig. 1) or \( \times 20 \) objective lens (the rest of the figures). The degraded area was visualized as a dark, non-fluorescent zone.

In Situ Collagen Degradation Assay—The experiments were done as described previously (52). 6-well culture plates were coated with a thin layer of chilled neutralized PureCol\textsuperscript{TM} collagen (Inamed Biomaterials, Fremont, CA) at 2.7 mg/ml in 1× RPMI medium (typically 100 \( \mu \)l/well) and incubated for 60 min at 37 °C for fibril formation, and COS7 cells (4 \( \times 10^5 \)/well) were then seeded on the film. 18 h later, cells were transfected with the expression plasmids in the growth medium (10% fetal bovine serum/DMEM) using FuGENE 6\textsuperscript{TM} according to the manufacturer’s instructions. The following day, culture medium was changed to serum-free DMEM, and cells were cultured for a further 3 days at 37 °C. The remaining collagen film was exposed by removing cells using repeated treatment with PBS containing 0.5 mg/ml trypsin and 1 mM EDTA. The collagen film was then fixed with 3% paraformaldehyde for 20 min at room temperature. Collagen was visualized by staining with Coomassie Brilliant Blue R250, and the images were captured by CCD camera-equipped microscope (Nikon TE-2000) with \( \times 20 \) objective lens. Degraded areas were visualized as a white, unstained, non-collagen-containing zone. In this assay, stained collagen was trypsin-resistant, suggesting that it was intact fibrillar collagen.

**RESULTS**

Hpx Domain Is Dispensable for proMMP-2 Activation by MT1-MMP—We have previously reported that MT1-MMP forms a homodimer through Hpx domain, and this interaction facilitates proMMP-2 activation on the cell surface (31). In contrast, Wang et al. (53) have reported that the Hpx domain is not required for proMMP-2 activation by showing that Hpx domain-deleted mutant activates proMMP-2. To address this discrepancy, we re-evaluated the roles of the Hpx domain by analyzing the following deletion mutants. MT1-\( \Delta \)Hpx is the mutant lacking Hpx domain only (Cys\textsuperscript{319}–Cys\textsuperscript{358}), and MT1-\( \Delta \)HpxL\( \Delta \)L2 further lacks the linker-2 region (L2 region, Pro\textsuperscript{509}–Gly\textsuperscript{537}) linking Asn\textsuperscript{317} and Ala\textsuperscript{536} (Fig. 1A). COS7 cells were transfected with expression plasmids for these mutants and tested for proMMP-2 activation ability. In our previous study, we used MT1F-\( \Delta \)HpxL\( \Delta \)L2 to examine the role of the Hpx domain in proMMP-2 activation (31), and here we confirm that MT1F-\( \Delta \)HpxL\( \Delta \)L2 is unable to activate proMMP-2 as shown in Fig. 1B. However, MT1F-\( \Delta \)Hpx activates proMMP-2 as efficiently as full-length MT1F, supporting the results of Wang et al. (53). To test whether these enzymes are expressed on the cell

**FIGURE 1.** Hpx domain is dispensable for proMMP-2 activation but important for collagenolytic activity. A, schematic representation of mutant constructs used in the experiments. S, signal peptide; Pro, propeptide; FLAG, FLAG tag (DYKDDDDK); Cat, catalytic domain; L1, linker 1 (hinge); Hpx, hemopexin domain; L2, linker 2; TM, transmembrane domain; Zn, catalytic zinc ion. B, COS7 cells were transfected with empty vector (Mock), MT1F, MT1F-\( \Delta \)Hpx, and MT1F-\( \Delta \)HpxL\( \Delta \)L2 as indicated. Cells were then incubated with purified proMMP-2 in serum-free culture medium for 18 h. ProMMP-2 activation in the media was analyzed by zymography (upper panel), and cell lysates were analyzed for expression of the proteins by Western blotting using anti-FLAG M2 antibody (lower panel). The arrows indicate MT1-MMP mutants expressed. P, proMMP-2; A, active MMP-2. C, transfected cells were subjected to surface biotinylation as described under “Materials and Methods” and analyzed by Western blotting using anti-FLAG M2 antibody. The upper panel is biotinylated samples, and the bottom panel is whole cell lysates. D, in situ gelatin degradation assay was carried out as described under “Materials and Methods.” Transfected COS7 cells were seeded on Alexa Fluor 488-labeled gelatin-coated 4-well chamber slides and cultured for 18 h. The cell surface-localized active form of FLAG-tagged MT1-MMP mutants was visualized by staining with anti-FLAG M1 antibody in the presence of 1 mM CaCl\textsubscript{2} without permeabilization. Green channel (F-gelatin) and red channel (FLAG M1) fluorescent images were captured in each field using \( \times 10 \) objective lens. Merged images are shown in the bottom. The bar indicates 200 \( \mu \)m. E, in situ solid-phase collagen degradation assays were carried out as described under “Materials and Methods.” The bar indicates 100 \( \mu \)m.
surface, the transfected cells were subjected to surface biotinylation experiments. As shown in Fig. 1C, all of the MT1-MMPs were biotinylated in a similar manner, suggesting that they are all expressed on the cell surface. To further confirm this result, these cells were cultured on a fluorescent-labeled gelatin (F-gelatin) film and stained with anti-FLAG M1 antibody without permeabilizing the cells. As in all enzymes, the FLAG tag is inserted immediately downstream of the $^{108}$RRKR$^{111}$ sequence, where proprotein convertases recognize and process (23, 57), only correctly processed enzyme can be recognized by the M1 antibody (54). In Fig. 1D, the wideview of a representative area of FLAG M1 staining/F-gelatin film degradation is shown. Anti-FLAG M1 antibody staining indicates that all the enzymes were expressed as correctly processed active forms on the cell surface and that the absence of the L2 region has no effect on this. Although active MT1F-DHpxΔL2 was present on the cell surface, it was unable to degrade F-gelatin film, whereas both MT1F and MT1F-DHpx did degrade F-gelatin effectively as evident by the numerous dark non-fluorescent patches. However, deletion of the Hpx domain did abrogate collagenolytic activity on the cell surface as reported previously (Fig. 1E) (53). These results suggest that the Hpx domain is not required for proMMP-2 activation, and the L2 region may play an important role in proMMP-2 activation and F-gelatin degradation, whereas the Hpx domain is critical for collagenolysis.

L2 Domain Is Not Responsible for proMMP-2 Activation—

Based on the results above, we postulated that the L2 region may play an important role in proMMP-2 activation by MT1F-DHpx. We therefore made a series of deletions or mutations in the L2 region of MT1F-DHpx to test this possibility (Fig. 2A).

According to its sequence, L2 can be divided into five parts: 509–514, which is a non-polar region, 515–523, which is an acidic region, 524–529, which is a hydrophobic region, 530–532, which is a short acidic region, and 533–535, which is a flexible region consisting of three glycines. Therefore, deletions or mutations were made to modify these different regions. As shown in Fig. 2B, among these mutants, MT1F-DHpxΔL2B and MT1F-DHpxΔL2E showed inefficient proMMP-2 activation, whereas all other variants were as good as MT1F-DHpx. MT1F-DHpxΔL2B and MT1F-DHpxΔL2E are expressed on the cell surface as active forms as they degraded F-gelatin, although the level of degradation seems to be lower. The level of F-gelatin degradation may not completely reflect the amount of cell surface enzyme as cells expressing MT1F-DHpxΔL2A, which activates proMMP-2 efficiently, also showed weaker F-gelatin degradation. The common feature of MT1F-DHpxΔL2B and MT1F-DHpxΔL2E is a lack of Glu$^{516}$–Glu$^{523}$, which is an acidic region immediately upstream of the hydrophobic region. However, this is not a region responsible for proMMP-2 activation as MT1F-DHpxΔL2I and MT1F-DHpxΔL2J, which lack this region, activate proMMP-2. Furthermore, MT1F-DHpxΔL2F,

FIGURE 2. Effect of L2 region mutations on proMMP-2 activation. A, a schematic representation of mutant MT1-MMPs used in the experiments. Different deletions were made in the L2 region of MT1F-DHpx. S, signal peptide; Pro, propeptide; FLAG, FLAG tag (DYKDDDDK); Cat, catalytic domain; L1, linker 1 (hinge); Hpx, hemopexin domain; L2, linker 2; TM, TM domain; CP, cytoplasmic domain; Zn, catalytic zinc ion. B, proMMP-2 activation ability of these mutant MT1-MMPs were analyzed as in Fig. 1B. ProMMP-2 activation in the media was analyzed by zymography (upper panel), and cell lysates were analyzed for expression of the proteins by Western blot (lower panel). The arrows in the Western blot (lower panel) indicate MT1-MMP mutants expressed. P, proMMP-2; A, active MMP-2. Mock, cells transfected with empty vector. C, in situ gelatin degradation assay was carried out as described under “Materials and Methods.”
which lacks most of the L2 except the three glycines, activates proMMP-2 very well. Only further deletion of these glycines (MT1F-ΔHpxΔL2) made the enzyme inactive for proMMP-2 activation (Fig. 1). This suggests that L2 may not play a direct role in proMMP-2 activation but may be important for providing flexibility and/or correct arrangement to the ecto-domains and thereby orientation of the catalytic domain for proMMP-2 activation to take place.

TM Domain Plays an Important Role in proMMP-2 Activation—We next examined the role of the cytoplasmic (CP) and TM domains in proMMP-2 activation. To test this, we deleted CP domain and exchanged the TM region with that of NGFR. As NGFR dimerization requires binding to the ligand in its ecto-domain, we did not expect the TM of NGFR to dimerize efficiently by itself. Indeed, our previous results support this notion (31). These mutations were incorporated into both MT1F and MT1F-ΔHpx as shown in Fig. 3A. The CP domain deletion did not affect the ability to activate proMMP-2 on the cell surface as reported previously (Fig. 3B) (37). This suggests that the CP domain is dispensable for proMMP-2 activation. On the other hand, replacing the TM domain with that of NGFR caused significant reduction in proMMP-2 activation by both full-length mutant (MT1F/NGFR<sub>TM</sub>) and the Hpx-deleted mutant (MT1F-ΔHpx/NGFR<sub>TM</sub>) (Fig. 3B). The reduction in proMMP-2 activation was due not to a reduction in cell surface localization as indicated by surface biotinylation study (Fig. 3C). Also, it is not due to a lack of activation as F-gelatin degradation was similar for all mutants (Fig. 3D).

The TM Domain Acts as Dimer Interface—One of the possible roles of TM domain in proMMP-2 activation could be that it acts as a dimer interface. To address this question, we utilized chimera mutants of MT1F and NGFR. We have previously shown that such chimera mutants can be used to test the ability of the MT1-MMP to form a dimer by monitoring tyrosine phosphorylation at their CP domain (31, 52). We created two sets of chimera mutants. The first group of the chimeras has L2, TM, and CP regions derived from NGFR with other ecto-domains from MT1-MMP, and in the second group of chimeras, the portion derived from MT1-MMP extended up to the TM domain, with only the CP domain derived from NGFR (Fig. 4A). With these two sets of chimeras, one can compare the contribution of the TM domain of MT1-MMP in its dimer formation. As shown in Fig. 4B, MT1F/NGFR<sub>LTMC</sub> and MT1FΔCat/NGFR<sub>L2TMCP</sub> showed strong phospho-tyrosine signals (lanes 2 and 3), whereas MT1FΔHpx/NGFR<sub>L2TMCP</sub> showed a minimal signal (lane 4), indicating the importance of Hpx domain for dimerization among these ecto-domains. On the other hand, all the second set of chimeras including MT1F/NGFR<sub>CP</sub> and MT1FΔCat/NGFR<sub>CP</sub>, MT1FΔHpx/NGFR<sub>CP</sub> and MT1FΔHpxΔL2F/NGFR<sub>CP</sub> showed strong phospho-tyrosine signals regardless of whether or not the Hpx domain or L2 region is present (lanes 5–8). These data strongly indicate that the TM domain can induce dimerization. Furthermore, the Hpx and TM domains can dimerize independently, i.e. neither TM-dependent dimerization nor Hpx-dependent dimerization is a prerequisite for dimerization of Hpx domain or TM-dependent dimer, respectively. When the proMMP-2 activation ability of these constructs was compared, it is clear that the catalytic domain of MT1-MMP is absolutely essential (see lanes 3 and 6) and also that the presence of MT1-MMP-derived TM domain greatly increases proMMP-2 activation (compare lanes 4, 7, and 8), supporting earlier results.

Role of Hpx-dependent Dimerization in Cell Surface Collagenolytic Activity—We have recently reported that dimerization of MT1-MMP is essential for cell surface collagenolytic activity (52). Inhibition of dimerization by co-expression of membrane-bound or soluble Hpx domain inhibited collagen degradation by MT1-MMP-expressing cells (52). Therefore, we examined which mode of dimerization plays a role in cell surface collagenolytic activity. For this purpose, we tested the collagen-degrading ability of the mutants that cannot activate proMMP-2 efficiently due to the absence of the MT1-MMP TM domain,
namely MT1F/NGFR_{TM} (Fig. 5A). As shown in Fig. 5B, expression of MT1F in COS7 cells caused degradation of a collagen film, and the activity was significantly inhibited upon co-expression of a membrane-anchored or soluble Hpx domains, MT1F-ΔCatL1 or MT1F-ΔCatL1ΔTM, respectively. Deletion of the CP domain, exchanging the TM domain with the one derived from NGFR, did not affect collagenolytic activity, and all of these were inhibited by co-expression with MT1F-NGFR_{CP} or MT1F-NGFR_{CP}/H9004, respectively. These data suggest that TM domain-dependent dimer formation is not essential for cell surface collagenolytic activity, and Hpx domain-dependent dimerization is sufficient to support the activity.

Inhibition of Hpx-dependent Dimerization Inhibits TM-dependent Dimer Formation—We have previously shown that co-expression of membrane-anchored Hpx domain (MT1F-ΔCatL1) with MT1-MMP inhibits proMMP-2 activation (31). MT1F-ΔCatL1 contains two dimer interfaces, the Hpx and the TM domains. We thus next asked whether the inhibition of Hpx domain-dependent dimerization is sufficient to cause inhibition of proMMP-2 activation. As shown in Fig. 6A, co-expression of membrane-anchored Hpx domain, MT1F-ΔCatL1, or soluble Hpx (MT1F-ΔCatL1ΔTM) with MT1F inhibits proMMP-2 activation in a dose-dependent manner, although MT1F-ΔCatL1ΔTM had weaker activity. Since soluble Hpx inhibits Hpx-dependent dimerization, we postulated that inhibition of Hpx-dependent dimerization by soluble Hpx also inhibits TM-dependent dimerization. To test this, soluble Hpx domain (MT1F-ΔCatL1ΔTM) was co-expressed with MT1F/NGFR_{CP}, which contains two dimer interfaces, the Hpx and TM domains. As shown in Fig. 6B, co-expression of either MT1F-ΔCatL1 or MT1F-ΔCatL1ΔTM with MT1F/NGFR_{CP} significantly decreased phospho-tyrosine signal in a similar manner. These data suggest that disruption of Hpx domain-dependent dimer formation also disrupts TM domain-dependent dimerization.

TM Dimer Arranges proMMP-2 Orientation—Activation of proMMP-2 by MT1-MMP involves TIMP-2 bridging MT1-MMP and proMMP-2. To address whether deletion of the Hpx domain affects TIMP-2 requirement for the activation, TIMP-2/H11002 fibroblasts were transfected with the expression plasmids for MT1F, MT1F-ΔHpx, and MT1F-ΔHpxL2F in the presence or absence of TIMP-2 (3 nM). As shown in Fig. 7A, the MT1-MMPs expressed in TIMP-2/H11002 cells were not able to activate proMMP-2, whereas they activated proMMP-2 with the addition of TIMP-2 in the medium. To test the ability of proMMP-2 to bind cell surface, COS7 cells were transfected with the same plasmids and reacted with proMMP-2 since COS7 cells express TIMP-2 endogenously (data not shown). As shown in Fig. 7B, cells expressing these MT1-MMPs bound MMP-2. Major MMP-2 species found in the cell fractions are the active form in all transfected cells. These results suggest that activation of proMMP-2 by Hpx-deleted mutants occurs with the same mechanism as wild-type MT1-MMP, and TM dimer is sufficient to arrange proMMP-2 orientation for the activation.

DISCUSSION
In this report, we have investigated modes of MT1-MMP dimerization and found that two domains of the enzyme can
A summary of the findings is depicted in Fig. 8. In the full-length enzyme, both the Hpx and the TM domains can form homodimer interfaces, and the enzyme shows both proMMP-2 activation and collagen-degrading activities on the cell surface. In the Hpx domain-deleted mutant, only a TM domain-dependent dimer can form. The mutant retains proMMP-2 activation ability but has lost collagen-degrading ability. The loss of collagen-degrading activity of this mutant is not only due to a loss of the Hpx domain as a dimerization domain but also to a loss of intrinsic collagenolytic activity due to lack of the Hpx domain (15, 53). The mutants lacking the MT1-MMP-derived TM domain, namely MT1F/NGFRΔTM, still retain the ability to form an Hpx domain-dependent dimer. This mutant does not activate proMMP-2 efficiently but retains collagenolytic activity. Formation of proMMP-2-TIMP-2-MT1-MMP complex was not affected by the lack of TM domain; thus TM dimer is likely to play an essential role to arrange proMMP-2 orientation for the activation. Taken together, TM-dependent dimerization is critical for proMMP-2 activation, but not for collagen degradation, and Hpx-dependent dimerization is critical for collagen degradation, but not essential for proMMP-2 activation.

The TM and Hpx domains appear to be able to dimerize independently. However, abrogation of Hpx-dependent dimer formation by co-expressing soluble Hpx domain also disrupts TM-dependent dimer formation, thereby inhibiting proMMP-2 activation. Although formation of a dimer by the TM domain does not rely on Hpx-dependent dimerization, it may be possible that Hpx domain-dependent dimer may influence TM-dependent dimerization. Previously, we have shown that expression of the constitutively active form of Rac1 small GTPase (Rac1CA) enhances Hpx domain-dependent dimerization using the MT1F/NGFRL2TMCP construct, which contains only the Hpx domain as a dimerizing domain (31). This suggests that Hpx-dependent dimer can be regulated by Rac1. Rac1CA also stimulated proMMP-2 activation by full-length MT1-MMP (31), suggesting that enhancement of Hpx-dependent dimerization has resulted in enhanced TM-dependent dimerization as TM dimerization dictates proMMP-2 activation. Although each mode of dimerization can occur independently and plays a distinct role, in full-length wild-type MT1-MMP, the dimerizations through these domains are likely to occur at the same time. It is possible that Hpx domain-dependent dimer may influence TM-dependent dimerization. Previously, we have shown that expression of the constitutively active form of Rac1 small GTPase (Rac1CA) enhances Hpx domain-dependent dimerization using the MT1F/NGFRL2TMCP construct, which contains only the Hpx domain as a dimerizing domain (31). This suggests that Hpx-dependent dimer can be regulated by Rac1. Rac1CA also stimulated proMMP-2 activation by full-length MT1-MMP (31), suggesting that enhancement of Hpx-dependent dimerization has resulted in enhanced TM-dependent dimerization as TM dimerization dictates proMMP-2 activation. Although each mode of dimerization can occur independently and plays a distinct role, in full-length wild-type MT1-MMP, the dimerizations through these domains are likely to occur at the same time. It is possible that regulation of Hpx-dependent dimerization is one of the mechanisms to control TM dimerization of MT1-MMP, which in turn regulates proMMP-2 activation on the cell surface.

The L2 region does not seem to play a critical role in enzyme dimerization to form the correct complex configuration for proMMP-2 activation since co-expression of sol-
TM Domain Is the Second Dimer Interfaces

uble Hpx domain construct containing intact L2 region (MT1F-ΔCatL1ΔTM) does not inhibit proMMP-2 activation by MT1F-ΔHpx with intact L2 region (data not shown). Also, the MT1F-ΔHpxΔL2F mutant, which lacks the majority of the L2 region, leaving only three glycines, can activate proMMP-2 as well as MT1F-ΔHpx. On the other hand, complete deletion of the L2 region makes the enzyme inactive for proMMP-2 activation (MT1ΔHpxΔL2). Interestingly, MT1F-ΔHpxΔL2 was also inactive for F-gelatin film degradation, although the enzyme is expressed on the cell surface as a correctly processed active form. Since MT1F-ΔHpxΔL2F degraded F-gelatin efficiently, flexibility given by at least three glycine residues seems important for both proMMP-2 activation and F-gelatin film degradation. It is possible that the inflexible nature of MT1F-ΔHpxΔL2 did not allow correct positioning of the catalytic domain for proMMP-2 activation or for interaction with other molecules that may also be essential to localize the enzyme to F-gelatin attachment site of the cells. Systematic deletions of L2 region revealed that the enzyme becomes ineffective for proMMP-2 activation when acidic sequence (Glu516–Glu523) immediately upstream of hydrophobic sequence (Val524–Val529) is deleted (MT1F-ΔHpxΔL2B). Since further deletion of this hydrophobic region (MT1F-ΔHpxΔL2C) regains proMMP-2 activation activity, it may suggest that the hydrophobic sequence negatively affects proMMP-2 activation, but this was counteracted by the presence of the acidic sequence immediately upstream. This notion is also supported by comparison of MT1F-ΔHpxΔL2E and MT1F-ΔHpxΔL2I where the presence of the hydrophobic region correlates with inefficient proMMP-2 activation (Fig. 2). Taken together, although L2 region does not positively support MT1-MMP activity, it may be important to provide flexibility to the Hpx and the catalytic domains to determine their arrangement on the cell surface.

Arrangement of wild-type MT1-MMP ecto-domains may be dictated by the presence of two dimeric interactions within the molecule. Without TM-dependent dimerization, the enzyme still forms a Hpx domain-dependent dimer, but in this form, it cannot activate proMMP-2. This suggests that the molecular arrangement of the ecto-domains with and without TM dimer are different, and Hpx dimer is not enough to allow the domains to be arranged correctly for proMMP-2 activation. It is probable that correct orientation of two catalytic domains of MT1-MMP dimer is essential for proMMP-2 activation to take place. The report by Wu et al. (58) suggests this possibility where they found that O-glycosylation at hinge (L1) region is essential for proMMP-2 activation. Glycosylation at L1 region might be important to arrange the catalytic domains in the correct orientation (1). These ideas together with our present results suggest that linker-1 and -2 may form a defined structure and provide flexibilities that determine correct arrangement of the ecto-domains.

Functionalities of biologically active proteins are often regulated by interaction with other proteins or by clustering, and this feature is found in many membrane proteins including integrins, cadherins, and growth factor receptors. MT1-MMP, as a type I transmembrane protein, is another example of a membrane protein that forms hetero- and homocomplexes to exhibit its biological activities. We have identified the TM domain as a

FIGURE 7. Involvement of TIMP-2 in proMMP-2 activation. A, TIMP-2-null fibroblasts were transfected with MT1-F, MT1-F-Hpx, and MT1F-ΔHpxΔL2F as indicated. These cells were then reacted to proMMP-2 (0.5 μg/ml) in the culture medium for 24 h in the presence or absence of exogenous TIMP-2 (3 nM). ProMMP-2 activation was analyzed by zymography (upper panel), and cell lysates were analyzed for proMMP-2 (0.5 μg/ml) at room temperature for 1 h. Cells were reacted to proMMP-2 (0.5 μg/ml) in the culture medium for 24 h. Culture media were analyzed by zymography for MMP-2 activation (Fig. 7). Lower panel, supernatant. B, two sets of COS7 cells were transfected with MT1-F, MT1-F-Hpx, and MT1F-ΔHpxΔL2F as indicated. One set of cells was reacted with proMMP-2 (0.5 μg/ml) in the culture medium for 24 h. Culture media were analyzed by zymography for MMP-2 activation (top panel, Sup) and cell lysates by Western blotting using anti-FLAG M2 antibody (lower panel) for the expression of the proteins. Another set of transfected cells was reacted with proMMP-2 (2 μg/ml) at room temperature for 1 h. Cells were washed with PBS three times, and cell lysates were analyzed for proMMP-2 binding by zymography (middle panel, Cell). The arrows in the Western blot (lower panel) indicate MT1-MMP mutants expressed. Sup, supernatant.

FIGURE 8. Schematic representation of a model of MT1-MMP dimerization on the cell surface.
TM Domain Is the Second Dimer Interfaces

REFERENCES

1. Itoh, Y., and Seiki, M. (2004) Trends Biochem. Sci. 29, 285–289
2. Okada, A., Tomasetto, C., Lutz, Y., Bellocq, J. P., Rio, M. C., and Basset, P. (1997) J. Cell Biol. 137, 67–77
3. Zhou, Z., Apte, S. S., Soininen, R., Cao, Y., and Tryggvason, K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4052–4057
4. Holmbeck, K., Bianco, P., Cenastra, J., Yamada, A., Kuznetsov, S. A., Mankani, M., Robey, P. G., Poole, A. R., Pidoux, I., Ward, J. M., and Birkedal-Hansen, H. (1999) Cell 99, 81–92
5. Atkinson, J. J., Holmbeck, K., Yamada, A., Birkedal-Hansen, H., Parks, W. C., and Senior, R. M. (2005) Dev. Dyn. 232, 1079–1090
6. Oblender, S. A., Zhou, Z., Galvez, B. G., Starcher, B., Shannon, J. M., Durbeej, M., Arroyo, A. G., Tryggvason, K., and Apte, S. S. (2005) Dev. Biol. 277, 255–269
7. Hiraoka, N., Allen, E., Apel, I. J., Gyetko, M. R., and Weiss, S. J. (1998) Cell 95, 365–377
8. Chun, T. H., Seiki, M., Ota, I., Murphy, H., McDonagh, K. T., Holmbeck, K., Birkedal-Hansen, H., Allen, E. D., and Weiss, S. J. (2004) J. Cell Biol. 167, 757–767
9. Sato, H., Takino, T., Okada, Y., Cao, J., Shimagawa, A., Yamamoto, E., and Seiki, M. (1994) Nature 370, 61–65
10. Hotary, K. B., Allen, E. D., Brooks, P. C., Datta, N. S., Long, M. W., and Weiss, S. J. (2003) Cell 114, 33–45
11. Honda, S., Migit, K., Hirai, Y., Origuchi, T., Yamasaki, S., Kamachi, M., Shibatomi, K., Fukuda, T., Kita, M., Hida, A., Ida, H., Aoyagi, T., Kawakami, A., Kawabe, Y., Oizumi, K., and Eguchi, K. (2001) Clin. Exp. Immunol. 126, 131–136
12. Rajavashisth, T. B., Xu, X. P., Jovinge, S., Meisel, S., Xu, X. O., Chai, N. N., Fishbein, M. C., Kaul, S., Cerneck, B., Sharifi, B., and Shah, P. K. (1999) Circulation 99, 3103–3109
13. Lehti, K., Allen, E., Birkedal-Hansen, H., Holmbeck, K., Miyake, Y., Chun, T. H., and Weiss, S. J. (2005) Genes Dev. 19, 797–991
14. Filipov, S., Koenig, G. C., Chun, T. T., Hotary, K. B., Ota, I., Bugge, T. H., Roberts, J. D., Fay, W. P., Birkedal-Hansen, H., Holmbeck, K., Sebgh, F., Allen, E. D., and Weiss, S. J. (2005) J. Exp. Med. 202, 663–671
15. Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M., and Okada, Y. (1997) J. Biol. Chem. 272, 2446–2451
16. Fosang, A. J., Last, K., Fujii, Y., Seiki, M., and Okada, Y. (1998) FEBS Lett. 430, 186–190
17. Koshikawa, N., Giannelli, G., Cirulli, V., Miyazaki, K., and Quaranta, V. (2000) J. Cell Biol. 148, 615–624
18. Knäuper, V., Will, H., López-Otin, C., Smith, B., Atkinson, S. J., Stanton, H., Hemby, R. M., and Murphy, G. (1996) J. Biol. Chem. 271, 17124–17131
19. Kajita, M., Itoh, Y., Chiba, T., Mori, H., Okada, A., Kinoh, N., and Seiki, M. (2001) J. Cell Biol. 153, 893–904
20. Endo, K., Takino, T., Miyamori, H., Kinsen, H., Yoshizaki, T., Furukawa, M., and Sato, H. (2003) J. Biol. Chem. 278, 40764–40770
21. Takino, T., Miyamori, H., Watanabe, Y., Yoshikawa, K., Seiki, M., and Sato, H. (2004) Cancer Res. 64, 1044–1049
22. Gingras, D., Bouquot-Gagnon, N., Langlois, S., Lachambre, M. P., Anabi, B., and Beliveau, R. (2001) FEBS Lett. 507, 231–236
23. Yana, I., and Weiss, S. J. (2000) Mol. Biol. Cell 11, 2387–2401
24. Sato, H., Takino, T., Koshikawa, N., Imai, K., Okada, Y., Stetler, S. W., and Seiki, M. (1996) FEBS Lett. 385, 238–240
25. Will, H., Atkinson, S. J., Butler, G. S., Smith, B., and Murphy, G. (1996) J. Biol. Chem. 271, 17119–17123
26. Oh, J., Takahashi, R., Kondo, S., Mizoguchi, A., Adachi, E., Sasahara, R. M., Nishimura, S., Imamura, Y., Kitayama, H., Alexander, D. B., Ide, C., Horan, T. P., Arakawa, T., Yoshida, H., Nishikawa, S., Itoh, Y., Seiki, M., Itohara, S., Takahashi, C., and Noda, M. (2001) Cell 107, 789–800
27. Nakada, M., Yamada, A., Takino, T., Miyamori, H., Takahashi, T., Yamashita, J., and Sato, H. (2001) Cancer Res. 61, 8896–8902
28. Nakada, M., Miyamori, H., Yamashita, J., and Sato, H. (2003) Cancer Res. 63, 3364–3369
29. Mori, H., Tomari, T., Koshikawa, N., Kajita, M., Itoh, Y., Sato, H., Tojo, H., Yana, I., and Seiki, M. (2002) EMBO J. 21, 3949–3959
30. Sato, T., del Carmen Ovejero, M., Hou, P., Heegaard, A. M., Kumegawa, M., Foged, N. T., and Delaïse, J. M. (1997) J. Cell Sci. 110, 589–596
31. Itoh, Y., Takamura, A., Ito, N., Maru, Y., Sato, H., Sunagana, Y., Aoki, T., and Seiki, M. (2001) EMBO J. 20, 4782–4793
32. Nakahara, H., Howard, L., Thompson, E. W., Sato, H., Seiki, M., Yeh, Y., and Chen, W. T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7959–7964
33. Stanton, H., Gavrilovic, J., Atkinson, S. J., D’Ortho, M. P., Yamada, K. M., Zardi, L., and Murphy, G. (1998) J. Cell Sci. 111, 2789–2798
34. Tho, M., Hernandez-Barrantes, S., Osenkowski, P., Bernardo, M. M., Gervais, D. C., Shimura, Y., Meroueh, O., Kotra, L. P., Galvez, B. G., Arroyo, A. G., Mobashery, S., and Fridman, R. (2002) J. Biol. Chem. 277, 26340–26350
35. Lehti, K., Lohi, J., Vatanen, H., and Keski-Oja, J. (1998) Biochem. J. 334, 345–353
36. Jiang, A., Lehti, K., Wang, X., Weiss, S. J., Keski-Oja, J., and Pei, D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13693–13698
37. Uekita, T., Itoh, Y., Yana, I., Ohno, H., and Seiki, M. (2001) J. Cell Biol. 155, 1345–1356
38. Remacle, A., Murphy, G., and Roghi, C. (2003) J. Cell Sci. 116, 3905–3916
39. Anilkumar, N., Uekita, T., Couchman, J. R., Nagase, H., Seiki, M., and Itoh, Y. (2005) FASEB J. 19, 1326–1328

Acknowledgments—We thank Yohei Otake for useful discussion and Rob Visse and Linda Troebjerg for critical reading of the manuscript. We also thank Paul Soloway and Gill Murphy for a kind gift of TIMP-2-null fibroblasts.

JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 283 • NUMBER 19 • MAY 9, 2008
13061
TM Domain Is the Second Dimer Interfaces

40. Wang, X., Ma, D., Keski-Oja, J., and Pei, D. (2004) J. Biol. Chem. 279, 9331–9336
41. Takino, T., Miyamori, H., Kawaguchi, N., Uekita, T., Seiki, M., and Sato, H. (2003) Biochem. Biophys. Res. Commun. 304, 160–166
42. Itoh, Y., and Seiki. M. (2006) J. Cell. Physiol. 206, 1–8
43. Stetler-Stevenson, W. G., Aznavorian, S., and Liotta, L. A. (1993) Annu. Rev. Cell Biol. 9, 541–573
44. Taniwaki, K., Fukamachi, H., Komori, K., Ohtake, Y., Nonaka, T., Sakamoto, T., Shiomi, T., Okada, Y., Itoh, T., Itohara, S., Seiki, M., and Yana, I. (2007) Cancer Res. 67, 4311–4319
45. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995) J. Biol. Chem. 270, 5331–5338
46. Butler, G. S., Butler, M. J., Atkinson, S. J., Will, H., Tamura, T., van, W. S., Crabbe, T., Clemens, J., d’Ortho, M. P., and Murphy, G. (1998) J. Biol. Chem. 273, 871–880
47. Kinoshita, T., Sato, H., Okada, A., Ohuchi, E., Imai, K., Okada, Y., and Seiki, M. (1998) J. Biol. Chem. 273, 24360–24367
48. Lehti, K., Lohi, J., Juntunen, M. M., Pei, D., and Keski-Oja, J. (2002) J. Biol. Chem. 277, 8440–8448
49. Holmbeck, K., Bianco, P., Yamada, S., and Birkedal-Hansen, H. (2004) J. Cell. Physiol. 200, 11–19
50. Visse, R., and Nagase, H. (2003) Circ. Res. 92, 827–839
51. Morrison, C. J., and Overall, C. M. (2006) J. Biol. Chem. 281, 26528–26539
52. Itoh, Y., Ito, N., Nagase, H., Evans, R. D., Bird, S. A., and Seiki, M. (2006) Mol. Biol. Cell 17, 5390–5399
53. Wang, P., Nie, J., and Pei, D. (2004) J. Biol. Chem. 279, 51148–51155
54. Itoh, Y., Kajita, M., Kinoh, H., Mori, H., Okada, A., and Seiki, M. (1999) J. Biol. Chem. 274, 34260–34266
55. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
56. Itoh, Y., Ito, A., Iwata, K., Tanzawa, K., Mori, Y., and Nagase, H. (1998) J. Biol. Chem. 273, 24360–24367
57. Sato, H., Kinoshita, T., Takino, T., Nakayama, K., and Seiki, M. (1996) FEBS Lett. 393, 101–104
58. Wu, Y. I., Munshi, H. G., Sen, R., Snipas, S. J., Salvesen, G. S., Fridman, R., and Stack, M. S. (2004) J. Biol. Chem. 279, 8278–8289
59. Huber, O., Kemler, R., and Langosch, D. (1999) J. Cell Sci. 112, 4415–4423
60. Mendrola, J. M., Berger, M. B., King, M. C., and Lemmon, M. A. (2002) J. Biol. Chem. 277, 4704–4712
61. Noordeen, N. A., Carafoli, F., Hohenester, E., Horton, M. A., and Leitinger, B. (2006) J. Biol. Chem. 281, 22744–22751
62. Kubatzky, K. F., Ruan, W., Gurezka, R., Cohen, J., Ketteler, R., Watowich, S. S., Neumann, D., Langosch, D., and Klingmuller, U. (2001) Curr. Biol. 11, 110–115

13062 JOURNAL OF BIOLOGICAL CHEMISTRY