TIMP-2 Promotes Activation of Progelatinase A by Membrane-type 1 Matrix Metalloproteinase Immobilized on Agarose Beads*

(Received for publication, October 7, 1997, and in revised form, April 13, 1998)

Takeshi Kinoshita†‡§, Hiroshi Sato†§, Akiko, Okada†, Eiko Ohuchi¶, Kazushi Imai¶, Yasunori Okada** and Motoharu Seiki††‡‡‡

From the ††Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, Japan, ‡Department of Molecular Virology and Immunology and §Department of Pathology and Immunology, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa 920, Japan, ¶Biopharmaceutical Department, Fuji Chemical Industries Ltd., Takao, Toyama 933, Japan, and **Department of Pathology, Medical School of The Keio University, Shinano-machi, Shinjyuku-ku, Tokyo 160, Japan

Membrane-type 1 matrix metalloproteinase (MT1-MMP)/MMP-14 is the activator of progelatinase A (proGelA)/proMMP-2 on the cell surface. However, it was a paradox that a tissue inhibitor of metalloproteinase-2 (TIMP-2), which is an inhibitor of MT1-MMP, is required for proGelA activation by the cells expressing MT1-MMP. In this study, a truncated MT1-MMP having a FLAG-tag sequence at the C terminus (MT1-F) was immobilized onto agarose beads (MT1-F/B) and used to analyze the role of TIMP-2. The proteolytic activity of MT1-F/B against a synthetic peptide substrate was inhibited by TIMP-2 in a dose-dependent manner. In contrast, TIMP-2 promoted the processing of proGelA by MT1-F/B at low concentrations and inhibited it at higher concentrations. TIMP-2 promoted the binding of proGelA to the MT1-F on the beads by forming a trimolecular complex, which was followed by processing of proGelA. A stimulatory effect of TIMP-2 was observed under conditions in which unoccupied MT1-F was still available. Thus, the ternary complex is thought to act as a means to concentrate the substrate to the bead surface and to present it to the neighboring free MT1-F.

However, proGelA lacks such a basic motif and therefore cannot be activated by serine proteinases (9). ProGelA had been reported to be activated by an unknown MMP-like activity on the surface of cancer and fibroblastic cells (10–15), and we identified MT1-MMP as such an activator on the cell surface (16, 17). Three other genes encoding similar enzymes that have a transmembrane domain and a short cytoplasmic tail were identified (18–20); at least two of them (MT2-MMP and MT3-MMP) activated proGelA in vitro (21).

Upon cell-mediated activation, proGelA binds to the cells through its hemopexin-like domain (HLD) (22). Using the HLD of GelA, Strongin et al. (23) isolated TIMP-2 complexed with the activated form of MT1-MMP from the cell membrane extract. We also purified a shaded fragment of MT1-MMP from the culture medium of the human breast carcinoma cell line MDA-MB-231 as a form inhibited by TIMP-2 (24). The C-terminal domain of the TIMP-2 in the complex was available for further complex formation with proGelA through its HLD (trimolecular complex). Strongin et al. also demonstrated that a small amount of TIMP-2 is an essential component for the activation of proGelA on the surface, in contrast to evidence that TIMP-2 is a well established inhibitor for all of the known MMPs. It would thus be of interest to clarify the discrepancy that TIMP-2 is an inhibitor of MT1-MMP on one hand but also a stimulator for proGelA processing on the other hand. Although TIMP-2 mediates formation of a trimolecular complex by binding both proGelA and MT1-MMP, its relevance to proGelA processing on the cell surface was not clear, because the catalytic function of MT1-MMP in the complex is inhibited by the TIMP-2.

In previous studies, the recombinant catalytic fragment of MT1-MMP expressed in Escherichia coli was sufficient to cleave the propeptide sequence of proGelA and trigger its autocatalytic activation in vitro (25–28). However, unlike the cell-mediated activation, the in vitro reaction with recombinant enzymes did not require TIMP-2 for proGelA activation. TIMP-2 inhibited the reaction in a dose-dependent manner, and no stimulation was observed at any concentration range. Also, the HLD of proGelA that was essential for cell-mediated activation was not required for this reaction. Thus, the in vitro reaction was thought to lack an important element of the cell surface event. The difference may be caused by the HLD of MT1-MMP which was deleted in the recombinant enzymes or by the transmembrane domain that links the enzyme on the cell surface, or some important factors included in the crude preparations were missing in the assay.

In the present study, we attempted to clarify the role of TIMP-2 in the cell-mediated processing of proGelA. For this purpose, we expressed different forms of recombinant MT1-MMP/MMP-14 is the activator of progelatinase A (proGelA)/proMMP-2 on the cell surface. However, it was a paradox that a tissue inhibitor of metalloproteinase-2 (TIMP-2), which is an inhibitor of MT1-MMP, is required for proGelA activation by the cells expressing MT1-MMP. In this study, a truncated MT1-MMP having a FLAG-tag sequence at the C terminus (MT1-F) was immobilized onto agarose beads (MT1-F/B) and used to analyze the role of TIMP-2. The proteolytic activity of MT1-F/B against a synthetic peptide substrate was inhibited by TIMP-2 in a dose-dependent manner. In contrast, TIMP-2 promoted the processing of proGelA by MT1-F/B at low concentrations and inhibited it at higher concentrations. TIMP-2 promoted the binding of proGelA to the MT1-F on the beads by forming a trimolecular complex, which was followed by processing of proGelA. A stimulatory effect of TIMP-2 was observed under conditions in which unoccupied MT1-F was still available. Thus, the ternary complex is thought to act as a means to concentrate the substrate to the bead surface and to present it to the neighboring free MT1-F. However, proGelA lacks such a basic motif and therefore cannot be activated by serine proteinases (9). ProGelA had been reported to be activated by an unknown MMP-like activity on the surface of cancer and fibroblastic cells (10–15), and we identified MT1-MMP as such an activator on the cell surface (16, 17). Three other genes encoding similar enzymes that have a transmembrane domain and a short cytoplasmic tail were identified (18–20); at least two of them (MT2-MMP and MT3-MMP) activated proGelA in vitro (21).

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Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that play critical roles in the physiological and pathological turnover of extracellular matrix (ECM) by degrading the macromolecules (1–6). MMPs are produced as a zymogen (proMMP) that needs proteolytic activation by eliminating the N-terminal propeptide for the enzymes to function (7). Serine proteinases such as plasmin, neutrophil elastase, and trypsin are well known activators for proMMPs. These activators digest the propeptide sequences at the basic amino acid motifs and eventually induce autocatalytic activation (8).

* This work was supported by the Special Coordination Fund for Promoting Science and Technology from the Ministry of Science and Technology of Japan, and by a grant-in-aid for cancer research from the Ministry of Education, Science and Culture of Japan. 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.

† To whom correspondence should be addressed: Dept. of Cancer Cell Research, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan. Tel.: 81-3-5449-5255; Fax: 81-3-5449-5414; E-mail: mseiki@ims.u-tokyo.ac.jp.

‡‡‡ Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Shirokane-dai, Minato-ku, Tokyo 108, Japan, and **Department of Pathology, Medical School of The Keio University, Shinano-machi, Shinjyuaku-ku, Tokyo 160, Japan.

§ The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; APMA, p-aminophenylmercuric acetate; Mca, (7-methoxycoumarin-4-yl)acetyl; Dpa, N-3-(2,4-dinitrophenyl)-1,2,3-diaminopropanoyl; ECM, extracellular matrix; HLD, hemopexin-like domain; CTD, C-terminal domain; NTD, N-terminal domain; MT1, membrane type 1; MT1-F, FLAG-tag sequence at the C terminus of MT1; MT1-F/B, MT1 immobilized onto agarose beads.
MMP fragment with or without the HLD as fusion proteins with FLAG-tag at the C terminus that was used to immobilize the enzymes on the agarose beads through anti-FLAG antibody. The recombinant enzymes immobilized on the beads were used to mimic the cell surface situation.

Materials and Methods

Plasmids—Fragments of MT1-MMP cDNA encoding amino acid Ala$^{1-13}$-Gly$^{285}$ (MT1, Fig. 1A) or Ala$^{1-13}$-Gly$^{284}$ (MT1dH, Fig. 1) were amplified by a polymerase chain reaction using 5' and 3'-primers with additional HindIII and SalI sites at the ends, respectively. The polymerase chain reaction products were digested with these enzymes and subcloned into the pFLAG-CTC vector (IHI, New Haven, CT), which directed expression of the peptide encoded by the cloned fragment as a FLAG-tagged fusion protein in E. coli.

Plasmids that express proGelA and its mutant lacking a hemopexin-like domain (proGelA/dHLD, Fig. 1) were described previously (29, 30). The truncated form of TIMP-2 (Met$^{1-158}$, Fig. 1) was expressed in Chinese hamster ovary cells using polymerase chain reaction-amplified cDNA cloned into a mammalian expression vector, pSG5 (Stratagene, Chinese hamster ovary cells using polymerase chain reaction-amplified density 0.5 (at diluted to 1:100 and were grown at 37 °C with shaking to an optical density 0.5 (at λ$^{600}$). The culture was then treated with 0.1 mM isopropanol-1-thio-β-glactopyranoside and incubated for an additional 1 h. Cells were collected by centrifugation and suspended with TBS buffer (50 mM Tris, pH 7.5, 150 mM NaCl) containing 1% Triton X-100 and then lysed by sonication. The supernatant of the lysate was collected and incubated with anti-FLAG M2 affinity beads (IHI) and rocked for 4 h at 4 °C. The beads were then washed three times with TBS buffer containing 0.1% Lubrol (TBST) and suspended with TBST containing 50% glycerol. Aliquots were taken into microtubes and stored at −80 °C until use. The proteins were analyzed by electrophoresis in 10% polyacrylamide gels containing 0.1% SDS and by staining with Coomassie Brilliant Blue.

Cell Culture and Protein Purification—ProGelA and the truncated TIMP-2 mutant were expressed in Chinese hamster ovary cells by transfection of the genes, and purified from the culture supernatant as described previously (9). ProGelA/dH is purified from the culture supernatant of COS-1 cells transfected with an expression vector (15). Recombinant TIMP-2 was a gift from Dr. K. Iwata (Fuji Chemical Industries Ltd., Takaoka, Japan).

Determination of Enzyme Concentrations—The concentrations of MT1-F, MT1dH-F, proGelA, and proGelA/dHLD were determined by titration of their activities against recombinant TIMP-2 using Mca-ProGelA or proGelA/dHLD at 37 °C in the presence of various concentrations of TIMP-2 (in 10 μl of reaction mixture). The reaction was terminated by adding 10 μl of sample buffer (2% SDS, 10% glycerol, 62 mM Tris, pH 6.8, 0.1% bromphenol blue) and analyzed by gelatin zymography. In some cases, complex formation was carried out by incubating the reaction mixture at 0 °C, and then processing was initiated by shifting the incubation temperature up to 37 °C.

Gelatin zymography was performed as described elsewhere (16). Samples were mixed with SDS sample buffer in the absence of a reducing agent, incubated for 20 min at 37 °C, and separated on 10% polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 1 h, and then gelatinolysis was permitted by incubating the gel in 50 mM Tris-HCl, pH 7.6, containing 150 mM NaCl, 10 mM CaCl$_2$, and 0.02% NaN$_3$ at 37 °C for 24 h. The gel was stained with 0.1% Coomassie Brilliant Blue R250, and the location of gelatinolytic activity was detected as clear bands in the background of uniform staining.

Results

Expression and Purification of Recombinant MT1-MMPs—The signal and propeptide sequences of MT1-MMP were deleted to express the enzyme as an active form in E. coli, and its transmembrane and cytoplasmic domains at the C terminus were also deleted to make it a soluble form. The recombinant enzyme was expressed as a fusion with the FLAG-tag sequence at the C terminus (MT1-F) (Fig. 1). The mutant was purified using anti-FLAG antibody immobilized on agarose beads, and thus MT1-F immobilized on the beads was prepared (MT1-F/B). Another mutant enzyme that lacks the HLD of MT1-F (MT1dH-F) was similarly expressed and purified. The analysis of the purified enzymes by SDS-polyacrylamide gel electrophoresis demonstrated 48 kDa for MT1-F and 22 kDa for MT1dH-F, as shown in Fig. 2A. The 28-kDa band is the light chain of the anti-FLAG antibody.

TIMP-2 Inhibits the Recombinant MT1-MMP—As previously reported for recombinant MT1-MMP fragments containing the catalytic domain (25–28, 33), the MT1-F/B also retained the activity to cleave the synthetic peptide substrate and was inhibited by TIMP-2 in a dose-dependent manner as in Fig. 2B. Thus, TIMP-2 was not required for the proteolytic activity of MT1-F/B at all. TIMP-1 was not an effective inhibitor against MT1-MMP, as demonstrated previously (data not shown) (26, 27, 33). The activity of MT1-F/B was completely inhibited by TIMP-2 at a 1.54 molar ratio (TIMP-2/TIM1-F/B). TIMP-2 also inhibited MT1dH-F/B similarly (data not shown).

TIMP-2 Promotes Processing of ProGelA by MT1-F/B—In the previous studies, TIMP-2 was not required for proGelA processing by the soluble forms of MT1-MMP lacking the C-terminal domains. The result was the same with MT1-F/B so far as the synthetic peptide was used as a substrate. To evaluate the effect of TIMP-2 on proGelA processing by MT1-F/B, increasing doses of TIMP-2 were added to the reaction mixture containing MT1-F/B and proGelA. Processing of proGelA was monitored by electrophoresis of the reaction mixture on gelatin-containing substrate gel (zymography). Processing of proGelA was observed without TIMP-2 when high concentra-
visualized by Coomassie Brilliant Blue staining. An aliquot of each sample was mixed with sample buffer and MT1dH-F/B purified from the cell lysate were thoroughly resuspended, and an aliquot was applied to a polyacrylamide gel containing 10% SDS. Proteins were visualized by Coomassie Brilliant Blue staining. Lane 1, MT1-F/B; lane 2, MT1dH-F/B. The bands corresponding to MT1-F (48 kDa), MT1dH-F (21 kDa), and light chain of the anti-FLAG antibody (28 kDa) are indicated. The molecular size marker is on the left. B, inhibition of MT1-F/B by TIMP-2. Quenched fluorescent peptide substrate (1 μM) was incubated with MT1-F/B (40 ng) in the presence of increasing doses of TIMP-2 (0, 0.21, 0.42, 1.1, 2.1, 4.2, 8.4, and 21 ng). Incubation was carried out for 1 h at 37 °C. The raw data of the measurement of fluorescence emissions (average of the three assays) were plotted against the molar ratio of TIMP-2/MT1-F/B.

The HLD of MT1-MMP Is Dispensable for the Stimulation Effect by TIMP-2—In previous studies, the catalytic fragment of MT1-MMP used for in vitro experiments did not contain the HLD (26–28). Thus, there is a possibility that the HLD of MT1-F/B is responsible for the stimulation of proGelA processing by TIMP-2. To examine this possibility, MT1dH-F/B, which lacks the HLD from MT1-F/B, was used. Reactions were carried out as described in the legend to Fig. 3. The addition of TIMP-2 to the reaction mixture clearly stimulated proGelA processing (Fig. 3, C and D), and the maximum effect was observed at the range of molar ratio (TIMP-2/MT1dH-F/B) between 0.008 and 0.24 (Fig. 3, lanes 4–9). TIMP-2 was inhibitory at higher concentrations (lanes 10 and 11). Fully activated GelA (F) was barely detected under these conditions. Thus, a TIMP-2 effect similar to that originally observed with the cell membrane fraction (23) was reproduced with MT1-F/B.

Domain Analysis of TIMP-2 and ProGelA—The HLD of proGelA was reported to be indispensable for the cell-mediated activation (15, 30). The HLD is known to interact with the C-terminal domain (CTD) of TIMP-2 and thus mediates the formation of proGelA/TIMP-2 complex (34, 35). The N-terminal domain (NTD) of TIMP-2, on the other hand, is the inhibitory domain (36), such that TIMP-2 can bind both MT1-MMP and proGelA at the NTD and CTD, respectively. The resulting ternary complex is thought to be the same as that identified by Strongin et al. (23).

The CTD of TIMP-2 and the HLD of proGelA were examined as to whether they affect the processing of proGelA by MT1-F/B. The NTD fragment of TIMP-2 (ΔTIMP-2) was prepared and added to the reaction mixture containing proGelA and TIM1-F/B. Inhibitor activity of the recombinant ΔTIMP-2 was confirmed using the activated form of interstitial collagenase (data not shown). In contrast to the wild type TIMP-2, ΔTIMP-2 did not stimulate proGelA processing at any concentration range, while it inhibited the processing in a dose-dependent manner (Fig. 4, A and B, lanes 3–11). The CTD of TIMP-2 was thus indispensable for the stimulatory effect on proGelA processing. Essentially the same result was obtained when MT1dH-F/B was used (data not shown).

To analyze the effect of the HLD of proGelA, a C-terminally truncated form of proGelA (proGelAΔHLD) was prepared and similarly tested. As shown in Fig. 4, C and D, the processing of proGelAΔHLD was not stimulated by TIMP-2 but rather was inhibited dose-dependently. This result indicates that the HLD of proGelA is indispensable for the TIMP-2-dependent stimulation of proGelA processing in the beads system. Thus, the domains required for the cell-mediated processing were also required for the beads system. The beads system is thus thought to mimic the cell-mediated reaction without requiring
additional factors.

Processing of ProGelA Occurs on the Beads—Domains of the proteins required for the TIMP-2-dependent stimulation of proGelA processing coincided with those required for ternary complex formation. We therefore tested whether the processing proceeds in solution or in the complex on the beads. The processing reaction was carried out as described in the legend for Fig. 3. The reaction mixture was then separated into beads and supernatant fractions by centrifugation and analyzed by gelatin zymography (Fig. 5A–D). GelA activity was exclusively in the supernatant fraction in the absence of TIMP-2 (Fig. 5, A and C, lanes 2–11). Following the addition of TIMP-2, GelA bound to the beads dose-dependently (Fig. 5, A and B). The GelA in the bead fraction was in the intermediate form at low concentrations of TIMP-2 (Fig. 5A, lanes 3–9) and at higher concentrations, the processing in the bead fraction was rather inhibited (Fig. 5A, lanes 10 and 11). In contrast, processing of proGelA in the supernatant fraction was not significantly affected by TIMP-2 (Fig. 5, C and D). Neither TIMP-1 nor dTIMP-2, which failed to promote proGelA processing, stimulated GelA binding to the beads (data not shown).

We then asked whether proGelA binds to the beads first or intermediate GelA binds to the beads after processing in solution. For this purpose, complex formation and processing steps were carried out separately. First, the trimolecular complex was allowed to form on the beads by incubating the reaction mixture (at 0.2 molar ratio of TIMP-2 against MT1-F) on ice. The processing activity of MT1-F was negligible at this temperature, and the complex on the beads was isolated by centrifugation and washed. Then the beads were resuspended with the reaction buffer and incubated at 37 °C. At each time point shown in Fig. 6, the supernatant and bead fractions were separated again and analyzed by gelatin zymography. At 0-min incubation, almost all the GelA activity kept on the beads was proform (Fig. 6A, lane 2), and no activity was detected in the supernatant (Fig. 6B, lane 2). Processing proceeded rapidly at 37 °C, and the processed GelA appeared on the beads even at 5 min of incubation (Fig. 6A, lane 3). A substantial amount of GelA was processed at 15 min, and the reaction was almost completed within 30 min (Fig. 6A, lanes 4 and 5). Processed GelA was not released to the supernatant until 30 min of incubation (Fig. 6B, lanes 3–5). A small amount of proGelA was released into the supernatant immediately after the temperature shift (Fig. 6B, lane 3), but it did not increase thereafter. These results indicate that proGelA binds to the MT1-F on the beads and that processing proceeds in the complex on the
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**DISCUSSION**

**TIMP-2 Stimulates ProGelA Processing by MT1-F/B—**In the previous studies, TIMP-2 inhibited the proteolytic activity of soluble forms of recombinant MT1-MMPs at all concentration ranges examined (25–28), while a titration amount of TIMP-2 was reportedly required for proGelA processing by plasma membrane preparation containing MT1-MMP (23). We thought that the TIMP-2 effect observed with the cell membrane may be reproducible when MT1-MMP was immobilized on some artificial matrix rather than in the soluble form. Immobilized enzymes are thought to be inefficient in utilizing substrates in solution compared with the soluble enzymes. Since TIMP-2 can mediate the ternary complex formation with MT1-MMP and proGelA (23, 24), TIMP-2 can recruit proGelA in the solution to the surface of the matrix after binding to the activated form of MT1-MMP. TIMP-2 is thus thought to increase the local concentration of proGelA at the enzyme site on the matrix surface. This possibility was directly assessed using recombinant MT1-F/B, proGelA and TIMP-2. Indeed, TIMP-2 promoted the proGelA binding to the MT1-F/B and its processing but only inhibited the MT1-F activity dose-dependently, as demonstrated using the synthetic peptide as a substrate. The cell surface event was thus reproduced, at least in part, using the immobilized MT1-MMP fragment and two other purified recombinant proteins, TIMP-2 and proGelA. Excessive TIMP-2 relative to MT1-F/B was inhibitory, as expected from its inhibitor nature. A maximum TIMP-2 effect was observed at the molar ratio 0.2 (TIMP-2/MT1-F/B) and 0.05 (TIMP-2/MT1dH-F/B), respectively, and the dose-response curves were bell-shaped. This indicates that the ratio of MT1-F in the complex versus the unoccupied enzyme on the beads is critical for the stimulation effect.

Since the active site of the MT1-MMP in the trimolecular complex is inhibited by TIMP-2, MT1-MMP in the complex is thought to be inactive. Indeed, when the trimolecular complex was formed in the presence of an excessive amount of TIMP-2 (in this condition the majority of MT1-F/B is thought to form a complex), the processing of proGelA did not occur efficiently even after excess TIMP-2 was washed out and after prolonged incubation (data not shown). Thus, the proGelA in the trimolecular complex is thought to be processed by the neighboring free MT1-F. This idea is in agreement with the bell-shaped curve obtained for the TIMP-2 effect (Figs. 3 and 4). The requirement of domains of each component to obtain TIMP-2 stimulation also supports the above idea, because they are also the domains required for the trimolecular complex.

**Clustering of MT1-MMP on the Cell Surface May Be a Regulatory Step for ProGelA Activation—**At least two MT1-MMP molecules are thought to be required for proGelA processing; one functions as a substrate presentation complex and the other as a processing enzyme. These two components should be close in distance to each other for efficient proGelA processing to take place on the cell surface. In our beads system, this situation is thought to be accomplished by the divalent anti-FLAG antibody that was used to immobilize MT1-F on the beads. The MT1-F molecules on the two arms are adjacent to each other and are thought to facilitate proGelA processing when either one of the molecules formed a trimolecular complex and the other remained free.

The processing of proGelA by MT1-MMP generates the intermediate form, and it is eventually converted into the mature form by intermolecular autocatalytic processing (29, 37). For this reaction, two trimolecular complexes which hold the intermediate form of GelA should be close together. This situation may not be achieved easily with our beads system because of the lack of fluidity of the beads surface. Presumably this is why processing of the intermediate form to the fully activated form was very slow.

As discussed above, at least two MT1-MMP molecules must get together for proGelA activation on the cell surface. The clustering of MT1-MMP on the cell surface is therefore thought to be an important factor regulating the rate of proGelA processing. There are many reports that concanavalin A, a multivalent lectin-like molecule, increases cell’s ability to cause the activation of proGelA (10, 12, 38–41). The concanavalin A effect may be mediated at least partly by the clustering of MT1-MMP on the surface, because the effect was observed with cells that express MT1-MMP, and the difference of the expression levels before and after the treatment was not enough in some cases to account for the dramatic induction of proGelA activation.

Questions remain as to how the clustering of MT1-MMP is accomplished on the cell surface and how this event is regulated in the cells. Cells may localize the enzyme to a restricted area on the surface during ECM degradation. For example, invasive cells are reported to form protrusions to degrade ECM at the attachment side. Such protrusions, called invadopodia, contain proteolytic enzymes at the edge where active proteolysis is taking place (6). Both activated GelA and MT1-MMP were found to co-localize at the invadopodia (42) and the transmembrane/cytoplasmic portion of MT1-MMP was responsible for the invadopodial localization and degradation of the ECM at the cell’s attachment site. It will be interesting to clarify how cell surface events are regulated by intracellular apparatus and signals through the cytoplasmic domain of MT1-MMP.

**Involvement of Other Cell Surface Molecules—**The proposed mechanism does not exclude the possibility that other cell surface molecules participate in the proGelA processing by MT1-MMP. For example, αvβ3 integrin was proposed to be a receptor for proGelA in melanoma cells (43). It is possible that the αvβ3 in melanoma cells facilitates the formation of the trimolecular complex on the surface, or that αvβ3 itself may
form a substrate presentation complex in addition to the one mediated by MT1-MMP. It is also possible that if putative TIMP-2 binding proteins exist on the cell surface and act as sites for proGelA binding after binding TIMP-2, they would also provide another substrate presentation complex.

Different types of integrins or other ECM receptors may associate with MT1-MMP at the invadopodia. These associations are thought to make it easy for cells to detect the appropriate substrates in the ECM and hold them for degradation by the neighboring enzymes. These possibilities are also an open area for further study.

Acknowledgments—We thank Dr. G. I. Goldberg for critical discussion and G. G. Gregorio for participation in this project at Kanazawa University. We also thank Dr. E. Thompson for valuable discussion and preparation of the manuscript.

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