The TIMP2 Membrane Type 1 Metalloproteinase “Receptor”
Regulates the Concentration and Efficient Activation of
Progelatinase A

A KINETIC STUDY

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Georgina S. Butler†, Michael J. Butler†, Susan J. Atkinson‡, Horst Will§, Tatsuya Tamura¶, Steven Schade van Westrum*†, Thomas Crabbe‡, John Clements**, Marie-Pia d’Ortho‡‡, and Gillian Murphy†††

From the §Strangeways Research Laboratory, Worts’ Causeway, Cambridge CB1 4RN and School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom, ††InVitek GmbH, Robert-Roessle-Strasse 10, D-13125 Berlin-Buch, Germany, ¶Chugai Pharmaceuticals Co. Ltd., 135 Komabado, 1 Chome, Gotemba-Shi, Shizuoka 412, Japan, ¶¶Celltech Therapeutics Ltd., 216 Bath Road, Slough SL1 4EN, United Kingdom, **British Biotech Pharmaceuticals Ltd., Watlington Road, Oxford OX4 5LY, United Kingdom, and ‡‡INSERM U296, Faculté de Médecine, Avenue du Général Sarrail, 94010 Créteil, France

We have used C-terminal domain mutants to further define the role of interactions of progelatinase A and membrane type 1 metalloproteinase (MT1 MMP) in the binding of TIMP2 and in the cell-associated activation of progelatinase A. Soluble constructs of MT1 MMP were used to demonstrate that binding with TIMP2 occurs primarily through N-terminal domain interactions, leaving the C-terminal domain free for interactions with progelatinase A. The rate of autolytic activation of progelatinase A initiated by MT1 MMP cleavage could be potentiated by concentration of the proenzyme by binding to heparin. Residues 568–631 of the progelatinase A C-terminal domain are important in formation of the heparin binding site, since replacement of this region with the corresponding stromelysin-1 sequence abolished binding to heparin and the potentiation of activation. The same region of gelatinase A was required for binding of latent and active enzyme to TIMP2, but residues 418–474 were not important. A similar pattern was seen using cell membrane-associated MT1 MMP; residues 568–631 were required for binding and activation of progelatinase A, whereas residues 418–474 were not. Neither region was required for activation in solution. The addition of TIMP2 to HT1080 membrane preparations expressing MT1 MMP, but depleted of endogenous TIMP2, resulted in potentiation of progelatinase A activation. This effect was dependent upon TIMP2 binding to MT1 MMP rather than at an independent membrane site. Together, the data suggest that TIMP2 forms a receptor with MT1 MMP that regulates the concentration and efficient generation of functionally active gelatinase A.

The activation of MMPs by sequential proteolysis of the propeptide blocking the active site cleft is regarded as one of the key levels of regulation of these proteinases (1, 2). The activation of progelatinase A (MMP2) has proved particularly enigmatic since early studies indicated that it cannot be efficiently activated by plasmin, in contrast to other MMPs including stromelysin 1, collagenase 1, and gelatinase B (3). Treatment of progelatinase A with organonemucerals initiates autolytic cleavages, leading to activation, as does proteolysis by either matriylsins or collagenase (4–6). At high concentrations, progelatinase A also self-activates in a process that is concentration-dependent, and the rate of activation can be enhanced by the presence of heparin at low ionic strength (7). Truncated forms of progelatinase A lacking the C-terminal domain are also activated by all the above mechanisms, but when no C-terminal domain is present, self-cleavages are not accelerated in the presence of heparin (7, 8). This implies a role for the C-terminal domain in the concentration of proenzyme by binding to heparin.

Searches for potential physiological activation mechanisms for progelatinase A have implicated a role for a membrane-mediated process involving certain types of activated cell (9, 10). This has led to the identification of the membrane-associated MT MMPs as potential mediators of progelatinase A activation (11–14). Many laboratories noted that activation of progelatinase A at the cell surface requires the C-terminal domain of progelatinase A (8, 15, 16). It was shown that MT1 MMP expressed at the surface of cells lacks the propeptide and

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††† To whom correspondence should be addressed: School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom. Tel.: 44-1603-456161; Fax: 44-1603-592250; E-mail: g.murphy@uea.ac.uk.

1 The abbreviations used are: MMP, matrix metalloproteinase; MT1 MMP, membrane type 1 matrix metalloproteinase; MT1 MMPcat, catalytic domain of MT1 MMP (Δ569–559); ΔTM MT1 MMP, MT1 MMP lacking the transmembrane and cytoplasmic domains (Δ501–559); TIMP, tissue inhibitor of metalloproteinases; N-GLA, N-terminal domain of gelatinase A (Δ418–631); C-GLA, C-terminal domain of gelatinase A (Δ1–414); N-GLC-SL, gelatinase A (residues 1–417) fused to stromelysin-1 (residues 248–460); N-G.C-SGG, gelatinase A (residues 418–631); N-G.C-SGS, gelatinase A (residues 418–631) fused to stromelysin-1 (residues 248–460); N-G.C-SGS, gelatinase A (residues 418–631) fused to stromelysin-1 (residues 248–460); N-G.C-SGS, gelatinase A (residues 418–631) fused to stromelysin-1 (residues 248–460); PAGE, polyacrylamide gel electrophoresis; HBSS, Hank’s balanced saline solution; BSA, bovine serum albumin; CHO, Chinese hamster ovary.
is largely associated with TIMP2 (17, 18). The TIMP2 has no inhibitory capacity, which is likely to be due to an interaction of the N-terminal domain with the catalytic domain of MT1 MMP. Progelatinase A can bind to TIMP2 by interactions between the C-terminal domain of the enzyme and the C-terminal three loops and charged tail of the inhibitor (19). The problem has arisen as to how to unequivocally identify this mechanism of progelatinase A binding to the cell surface as a prerequisite for its efficient activation. Data suggest that TIMP2 is critical for the cellular activation process; too little and activation is compromised, but in the presence of excess TIMP2, there is no free MT1 MMP available to initiate progelatinase A activation. It has been clearly established, however, that MT1 MMP can cleave the propeptide of progelatinase A at Asn-638, a process that is not inhibited by TIMP1 (15, 20). The efficiency of this activation process depends upon the concentration of gelatinase A present, since this effects the final cleavage at Asn-638.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were obtained from Sigma, ICN, or Pierce. Molecular biology enzymes and buffers were obtained from Promega or Stratagene. CT1746 (N1–1–8-carbamoyl-2,2-dimethylpropyl)-N4-hydroxy-2-(3-[4-chlorophenyl]propyl)succinamide) was kindly donated by Dr. A. Docherty (Celtech Ltd., Slough, United Kingdom (UK)). The quenched fluorescent peptide (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Arg-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH2 was a gift from G. Knight (Strangeways Research Laboratory, UK).

**Production of Enzymes and Inhibitors**—The catalytic domain of MT1 MMP (Δ269–559; MT1 MMP9) was prepared as described previously (20). A version of MT1 MMP with the transmembrane and cytoplasmic domains deleted (Δ501–559; ΔTMT1 MMP) was produced.2 Progelatinase A, the N-terminal domain of progelatinase A (Δ418–631; N-GLA) and N-GLA-C-SL (gelatinase A residues 1–417 fused with stromelysin-1 residues 248–460) were prepared as described previously (8, 22). The C-terminal domain of gelatinase A (Δ1–414; C-GLA) was purified as before (16). TIMP2, (Δ186–194)TIMP2, and (Δ128–194)TIMP2 were prepared as described (23). The catalytically inactive mutant of gelatinase A, E375A-progelatinase A, was prepared as detailed (24).

C-terminal stromelysin-1/gelatinase A hybrids were created as follows. The hybrid consisting of the pro and catalytic domains of gelatinase A (residues 1–417) and a C-terminal domain of stromelysin-1 (residues 248–305), gelatinase A (residues 475–631) and a C-terminal domain of stromelysin-1 (residues 248–305) was produced as described previously (23).

**Binding of Proenzymes to TIMP2**—Two additional N-GLA-SGG and encoded gelatinase A (residues 1–417), stromelysin (residues 248–305), and gelatinase A (residues 475–631).

The TIMP2 was prepared as described previously using gelatin-Sepharose (8, 22) and resulted in preparations of proenzyme which ran identically to progelatinase A on SDS-PAGE (M, 72,000). Gelatinase A and the C-terminal domain mutants are shown schematically in Fig. 1.

**Preparation of Membrane-associated Active MT1 MMP—HT1080 fibrosarcoma cells were transfected with wild-type MT1 MMP using the HCMV/gpt vector, pGWHG (26), and overexpressing clones were selected with mycophenolic acid. The highest MT1 MMP-expressing clone was expanded and cultured in the presence of 1 μM CT1746, a peptide hydroxamate inhibitor of metalloproteinases, to deplete endogenous TIMP2 binding to cell membrane-associated MT1 MMP. Crude plasma membranes were prepared as described (10), but with extra washes to remove the CT1746. Membranes were resuspended in buffer (20 mm Tris-HCl, pH 7.8, 10 mm CaCl2, 0.025% Brij 35, 0.02% sodium azide), containing protease inhibitors, phenylmethanesulfonyl fluoride (100 μM), pepstatin (1 μg/ml), and t-transepoxysuccinic acid (1 μg/ml).

**Results**—The TIMP2 was produced as described previously using gelatin-Sepharose (8, 22) and resulted in preparations of proenzyme which ran identically to progelatinase A on SDS-PAGE (M, 72,000). Gelatinase A and the C-terminal domain mutants are shown schematically in Fig. 1.
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sodium azide) containing 0.15 M NaCl (TCABN). Samples were then loaded onto gelatin-Sepharose, and columns were washed with the same buffer. Bound material was eluted using TCABN containing 15% v/v dimethyl sulfoxide and analyzed by SDS-PAGE with silver staining, and activity was measured using the rabbit collagenase diffuse fibrillar assay (27).

Activation of MT1 MMP—The catalytic domain of MT1 MMP (MT1 MMP<sub>cat</sub>) was activated at 30 μM/ml with 5 μM/ml trypsin (20). ATM MT1 MMP was activated with 1:20 w/w active MT1 MMP<sub>cat</sub> in FAB buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM CaCl₂, 0.05% Brij 35) for 16 h at 25 °C.

Titrations—TIMP2 preparations were titrated against gelatinase A immediately following titration of the enzyme against a TIMP1 standard of known concentration (23). All fluorimetric analyses were carried out in a Perkin Elmer LS50B spectrophotometer using 1 μM quenched fluorescent peptide substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-3-[4,2'-dinitrophenyl]-1,2,3-diaminopropionyl]-Ala-Alg-NH₂ (28). The machine was calibrated using a known concentration of the standard (7-methoxycoumarin-4-yl)acetyl-NH₂ (28).

Estimation of Association Rate Constants—Association rate constants (kₐ) were estimated from progress curves obtained after the addition of TIMP2 to the enzyme-substrate reaction with appropriate concentrations of enzyme and inhibitor at 25 °C for gelatinase and 37 °C for MT1 MMP using published equations (22, 23) and either Enzfitter (Biosoft) or Grafit (Erithacus Software). Three techniques—SDS-PAGE, Western Blots, and Zymography—These techniques were carried out as described previously (29).

Immunolocalization of Progelatinase A and C-terminal Domain Mutants on Cell Surfaces—Chinese hamster ovary (CHO) cells were transfected with the cDNA for MT1 MMP under transient expression conditions (29) and incubated with exogenous progelatinase A or the domain mutants; 18 h post-transfection, the cells were washed with serum-free HBSS and incubated with exogenous progelatinase A or the domain mutants in HBSS/5% BSA for 1 h on ice. Cell monolayers were extensively rinsed and then fixed with 4% paraformaldehyde prior to immunolocalization of surface-bound progelatinase using a sheep polyclonal antibody to gelatinase A (30). It had been previously established that this antibody recognized the mutant enzyme forms. The distribution of endogenous TIMP2 on the transfected CHO cells was assessed using a sheep polyclonal antibody to TIMP2 (31) and a monoclonal antibody specific to the C-terminal charged "tail" sequence of TIMP2 (kindly donated by Dr. Kazuhiro Iwata, Fuji Chemical Industries Ltd., Toyama, Japan).

RESULTS

Binding of Progelatinase A and C-terminal Domain Mutants to TIMP2—Progelatinase A, proN-GLA and the gelatinase A-stromelysin hybrids, proN-G.C-SGS and proN-G.C-SGG, each bound to gelatin-Sepharose. TIMP2 alone did not bind to gelatin-Sepharose; neither did TIMP2 that had been preincubated with proN-GLA or proN-G.C-SGS, nor Δ128–194/TIMP2 that was preincubated with progelatinase A. Only TIMP2 that was incubated with progelatinase A or proN-G.C-SGG was detected in the bound fraction by silver staining (not shown) and TIMP activity assays (Table I).

Association Constants for TIMP2 and Active Gelatinase A or C-terminal Domain Mutants—Fluorimetric assays demonstrated that TIMP2 bound rapidly to gelatinase A (kₐ<sub>on</sub> 38.0 × 10⁶ M⁻¹ s⁻¹; Table II). The mutant N-G.C-SGG was the only

| TIMP2 | Gelatinase A | ProN-GLA | C-GLA | ProN-GL.C-SL | ProN-G.C-SGS | ProN-G.C-SGG | Progelatinase A |
|-------|-------------|----------|-------|-------------|--------------|--------------|----------------|
| kₐ<sub>on</sub> | 38.0 × 10⁶ M⁻¹ s⁻¹ | 128–194/TIMP2 | 128–194/TIMP2 | 128–194/TIMP2 | 128–194/TIMP2 | 128–194/TIMP2 | 128–194/TIMP2 |

3 M.-P. d’Ortho and R. M. Hembry, manuscript in preparation.
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Table III
Association rate constants for TIMP2 and MT1 MMP

| Association rate constants (k_{on}) were estimated from the inhibition progress curves using equations described (22, 23) and programs Enzfitter (Biosoft) or Grafit (Erithacus Software) (ND, not determined; n, number of assays). |
|---------------------------------|-----------------|-----------------|
| TIMP2                          | (Δ128–194/TIMP2) | (Δ186–194/TIMP2) |
| MT1 MMPcat                      | 4.18 ± 0.20 (n = 6) | 2.80 ± 0.45 (n = 6) | ND |
| ΔMT MT1 MMP                     | 2.98 ± 0.11 (n = 4) | 3.02 ± 0.10 (n = 4) | 3.88 ± 0.27 (n = 2) |

construct to show a comparable rate of binding (33.2 × 10^{6} \text{M}^{-1}\text{s}^{-1}). Mutants N-G.C-SGS, N-GLA, or N-GLC-SL bound to TIMP2 between 45 and 200 times more slowly than wild-type gelatinase A. \((Δ186–194/TIMP2), \text{which lacks the charged tail, interacted 5–7-fold more slowly with wild-type gelatinase A or the mutant N-G.C-SGG. Removal of the charged tail of TIMP2 had no effect on the already poor association rate of N-G.C-SGS.}

**Association Constants for TIMP2 and MT1 MMP**—The \(k_{cat}/K_m\) for ΔMT MT1 MMP cleavage of the fluorescent peptide at 37 °C and pH 7.5 was 1.92 × 10^{6} \text{M}^{-1}\text{s}^{-1}, which is comparable with the \(k_{cat}/K_m\) for MT1 MMPcat (20). Estimates of the association constant for wild type TIMP2, or mutant forms lacking the charged tail (Δ186–194) or the C-terminal domain three loops (Δ128–194) with soluble MT1 MMP (ΔTM or catalytic domain) were similar, ranging from 2.8 to 4.2 × 10^{6} \text{M}^{-1}\text{s}^{-1} (Table III).

**Activation of Progelatinase A and C-terminal Domain Mutants in Solution**—In agreement with previous data comparing the activation of wild-type progelatinase A and proN-GLA (8) or proN-GLC-SL (22), APMA activation of equimolar concentrations of progelatinase A and C-terminal domain mutants proN-G.C-SGS, proN-G.C-SGG, and proN-GLA was similar, showing rapid and immediate activation, which was complete by 60 min (Fig. 2). When gelatinase A or the domain mutants were activated under the same conditions with a 10:1 molar ratio of proenzyme:ΔMT MT1 MMP, activation occurred over a 6-h period and each of the enzymes showed a similar activation profile, with a lag phase of 1 h and then rapid activation followed by a plateau (Fig. 3). Similar results were obtained using MT1 MMPcat (data not shown).

**The Effect of Local Concentrations of MT1 MMP and Progelatinase A**—We have shown previously that the concentration of progelatinase A determines its rate of activation by MT MMPs in solution (32). Here, we investigate the effect of MT1 MMP concentration on progelatinase A activation (Fig. 4). In the absence of MT1 MMP, there was no detectable activation of the progelatinase A in the 7-h period studied. With a 6:1 molar ratio of progelatinase A:ΔMT MT1 MMP, a characteristic sigmoidal activation curve resulted, where a 50–60-min lag was followed by a phase of rapid activation and then a plateau. As the molar ratio of progelatinase A:ΔMT MT1 MMP was increased to 30:1, 60:1, and 125:1, the rate of activation decreased, so that after 7 h, the amount of substrate cleaved was only 43%, 35%, and 24% of that cleaved by gelatinase A incubated in a 6:1 molar ratio with ΔMT MT1 MMP. The rate of activation of progelatinase A was similarly dependent upon the relative concentration of MT1 MMPcat (data not shown), suggesting that C-terminal domain binding did not contribute to enzyme interactions.

Autoactivation of gelatinase A is promoted by binding of the enzyme to heparin via its C-terminal domain (7). This effect is proposed to be due to an increase in the local concentration of the enzyme. We examined the binding of our gelatinase A and MT1 MMP constructs to heparin to determine whether this system was suitable as a model to mimic the colocalization of reactants at the cell surface. The binding of pro and active forms of MT1 MMPcat and ΔMT MT1 MMP to heparin-agarose in TCAB was tested (Fig. 5). The recombinant ΔMT MT1 MMP preparation consisted of several forms, all of which bound to heparin: \(M_r\) 70,000 (pro form), \(M_r\) 64,000, \(M_r\) 62,000 (probably the active form), and a lower molecular weight doublet (\(M_r\) 33,000 and 31,000). After complete activation, the \(M_r\) 62,000 active form of ΔMT MT1 MMP still bound to heparin, as did the lower molecular weight doublet. A smaller doublet (\(M_r\) 28000–30,000) did not bind. The proform of MT1 MMPcat, \(M_r\) 31,000, also bound (a \(M_r\) 30,000 band in the preparation did not; Ref. 20), whereas the trypsin-activated form, \(M_r\) 21,000, did not bind.
Wild-type progelatinase A, the isolated C-terminal domain, C-GLA, and the mutant proN-G,C-SGG bound to the heparin matrix in low ionic strength buffer (data not shown). ProN-GLC-SL, pro-N-GLA, and proN-G,C-SGS did not bind. The same pattern was revealed by examination of the effect of heparin on the APMA-induced activation of progelatinase A and the C-terminal domain mutants. The rate of activation of wild-type progelatinase A and the mutant proN-G,C-SGG was enhanced considerably in the presence of heparin, especially over the first 2 h (data not shown). In contrast, the rate of activation of proN-GLA, proN-GLC-SL or proN-G,C-SGS was not affected by the presence of heparin.

Since both progelatinase A and ΔTM MT1 MMP bound to heparin-agarose, the effect of heparin on progelatinase A activation by MT1 MMP was studied to assess the role of concentration and colocalization of these enzymes, as implicated during cell-surface activation of gelatinase A. The addition of heparin to a 6:1 molar ratio of progelatinase A:ΔTM MT1 MMP reduced the length of the lag phase (Fig. 6A). The rate of gelatinase A activation was increased by the addition of 10 μg/ml heparin and was maximal at 50 μg/ml. Addition of higher concentrations of heparin (up to 500 μg/ml, data not shown) did not increase the rate further. The effect of heparin was reduced by increasing the salt concentration in the activation buffer (data not shown). Even the very slow rate of activation of progelatinase A by low concentrations of MT1 MMP in solution (e.g. 125:1 progelatinase A:MT1 MMP at 37 °C, such that the intermediate form was not visible by silver-stained SDS-PAGE after 30 min. However, the conversion of the inactive mutant, E375A-progelatinase A to the intermediate form by ΔTM MT1 MMP was not affected by heparin (data not shown). Since active MT1 MMP cat did not bind to heparin-agarose, a further experiment with the catalytically inactive mutant E375A-gelatinase A was carried out to clarify which of the two steps in the activation process was affected by heparin. Heparin stimulated the conversion of 150 nM wild-type progelatinase A to the fully active form in the presence of 10:1 molar ratio of ΔTM MT1 MMP at 37 °C. The intermediate form of progelatinase A was measured. The ratios of ΔTM MT1 MMP:progelatinase A were as follows: ○, 0:1; ●, 1:125; □, 1:60; ▲, 1:30; ◊, 1:16.

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Fig. 7. Progelatinase A and C-terminal domain mutants are differentially activated by cell membrane-associated MT1 MMP. A, 7 nM proenzyme was incubated with 0, 0.5 mg/ml, or 4 mg/ml MT1 MMP-expressing NS0 membrane preparations for 18 h at 37 °C (shown in the figure from left to right). Products of the reaction were analyzed on 8% polyacrylamide-gelatin zymograms (0.5 mg/ml gelatin). Lanes 1–3, wild-type progelatinase A; lanes 4–6, proN-G.C-SGG; lanes 7–9, proN-G.C-SSS. B, 5 mg/ml membrane preparation from NS0 cells either cotransfected with MT1 MMP and TIMP2 cDNAs (closed shapes) or transfected with vector DNA (open shapes) were incubated with 140 nM progelatinase A (○), proN-G.C-SGG (×), or proN-G.C-SSS (△) at 37 °C. Aliquots were removed at intervals and diluted 1:10 into ice-cold FAB buffer. Samples were assayed in duplicate for activity against the quenched fluorescent substrate.

Immunolocalization of Progelatinase A and C-terminal Domain Mutants on Cell Membranes—The binding of wild-type progelatinase A and the C-terminal domain mutants proN-G.C-SSG and proN-G.C-SGG to CHO cells, transiently transfected with vector alone or with vector containing MT1 MMP cDNA, was studied using immunolocalization techniques (Fig. 9). CHO cells synthesize negligible amounts of progelatinase A or MT1 MMP, but they normally secrete significant levels of TIMP2 into the culture medium. No TIMP2 was bound to the surface of CHO cells transfected with vector alone. However, when CHO cells were transiently transfected with MT1 MMP (10–20% of cells were positively transfected), TIMP2 was detected bound at the cell surface, using either a polyclonal antibody against TIMP2 or a monoclonal antibody raised against a C-terminal peptide of TIMP2. When wild-type progelatinase A or the mutant proN-G.C-SGG were added to the system, they could be detected, using a polyclonal antibody to progelatinase A, on the surface of the cells expressing MT1 MMP (panels A and B), but not on cells transfected with vector alone. ProN-
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Previous studies have shown that the C-terminal domain of gelatinase A is required for binding of the proenzyme to TIMP2 (19, 22, 23, 33) and is also crucial for activation of progelatinase A by cell membranes (8, 15, 16). The C-terminal domains of gelatinase A and stromelysin-1 show >50% identity, but in stromelysin-1 this region does not interact significantly with TIMP2 (22). A mutant with the C-terminal domain of gelatinase A replaced with the corresponding region from stromelysin-1, N-GL.C-SL, was also not activated by cell membranes.4 To localize more precisely the regions of the C-terminal domain of gelatinase A involved in TIMP2 binding and cellular activation, we constructed further subdomain-exchange mutants.

The binding of TIMP2 to progelatinase A required the C-terminal region of both the inhibitor and proenzyme as demonstrated previously (8) and substitution of the C-terminal domain of gelatinase A with that of stromelysin-1 abolished binding of proN-GL.C-SL to TIMP2. TIMP2 was able to bind to proN-G.C-SGG, where residues 418–474 of gelatinase A have been replaced with stromelysin-1 sequence, but was unable to bind to proN-G.C-SGS, where there is an additional replacement of residues 568–631. A similar effect of these C-terminal domain mutations was seen on the rate of association of TIMP2 with the active enzymes, i.e. only N-G.C-SGG showed a rate of binding to TIMP2 comparable to that for wild-type gelatinase A.

Our results agree with those of Fridman et al. (33), who made deletion mutants of gelatinase A (Δ426–631 and Δ455–631). The proform of these mutants did not bind TIMP2 and the active enzyme showed decreased inhibition by TIMP2. However, our domain-swap mutants not only preserve the C-terminal disulfide bond (data not shown), which is thought to be essential for integrity (16), but also allow us to more precisely define the site of interaction with TIMP2. Our results suggest that the region between residue 568 and the C-terminal residue 631 is required for formation of the TIMP2 binding site, whereas residues 418–474 do not interact significantly with TIMP2. The x-ray crystal structure of the C-terminal domain of gelatinase A predicts a disc-shaped four-bladed propeller (34, 35). The region implicated here (residues 568–631) consists of part of blade 3 and all of blade 4. Electrostatic interactions between the C-terminal domain of TIMP2 and that of gelatinase A are believed to be responsible for an initial docking, which aligns the inhibitory domain of TIMP2 with the catalytic domain of the enzymes, increasing the rate of inhibition (22, 23). A surface patch of positively charged residues around lysine residues 566, 567, and 568 (the latter is replaced in mutant N-G.C-SGS by a serine residue) may be involved in binding TIMP2, in particular, the negatively charged C-terminal tail. Our immunolocalization studies support this proposal, since the binding of gelatinase A to cell-bound TIMP2 blocked binding of the anti-TIMP2 C-terminal peptide monoclonal antibody.

4 M. Cockett and G. Murphy, unpublished observation.
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FIG. 9. C-terminal domain mutants of gelatinase A show differential binding to MT1 MMP on CHO cells. CHO cells were transiently transfected with MT1 MMP cDNA and incubated with exogenous proenzymes. Panels A, D, and G, wild-type gelatinase A; panels B, E, and H, proN-G-C-SGG; panels C, F, and I, proN-G-C-SGS. Bound gelatinases were detected using a sheep polyclonal antibody to human gelatinase A (panels A–C), and the distribution of endogenous TIMP2 was examined using either a sheep polyclonal antibody against TIMP2 (panels D–F) or a monoclonal antibody specific to the C terminus of TIMP2 (panels G–I).

We then compared the association of ΔTM MT1 MMP and MT1 MMP\textsuperscript{cat} with TIMP2 and C-terminally truncated forms of TIMP2, to determine which interactions are likely to occur in vivo. The similarity of the association rates for all combinations of full-length and truncated proteins suggests that only N-terminal domain interactions occur between soluble MT1 MMP and TIMP2. Other evidence suggests that this interaction is important at the cell surface; binding of TIMP2 to HT1080 cell membranes is abolished by excess N-terminal domain of TIMP2, and the activation of gelatinase A is abolished by antibodies against the N-terminal domain of TIMP2 (17, 36). The role of the C-terminal domain of MT1 MMP is unclear; however, in vitro, it has been shown to be important for specific cleavage of type I collagen. This domain appears to be responsible for the binding of active ΔTM MT1 MMP to heparin.

The data presented here suggest that interactions occur between the C-terminal domain of TIMP2 and the C-terminal domain of gelatinase A and between the N-terminal domain of MT1 MMP and the N-terminal domain of TIMP2. This is consistent with the theory that a ternary complex between MT1 MMP, TIMP2, and gelatinase A can form on the cell surface (17, 18, 37). In agreement with this, an E375A-progelatinase A-TIMP2 complex was able to inhibit MT1 MMP\textsuperscript{cat} in a fluorimetric assay, presumably due to the formation of a ternary complex as described by Kolkenbrock and colleagues (37–39).

In these experiments, we attempt to establish whether TIMP2 is involved in the activation of progelatinase A at the cell surface by MT1 MMP. In solution, the activation of progelatinase A and C-terminal domain mutants by APMA was virtually identical. This is in accordance with previous studies, which suggest that the C-terminal domain of gelatinase A is not involved in catalysis (16, 22, 33). Similarly, progelatinase A and the mutants were activated equally well by soluble ΔTM MT1 MMP or MT1 MMP\textsuperscript{cat}. Thus, in solution, the activation of progelatinase A by MT1 MMP does not require the C-terminal domain of the proenzyme or of MT1 MMP and is efficient even in the absence of TIMP2.

The heparin binding site in the C-terminal domain of gelatinase A has not previously been identified (7, 40). Other than C-GLA and progelatinase A, N-G-C-SGG was the only mutant that bound to heparin-agarose, suggesting that N-G-C-SGG retains the heparin binding domain, whereas it is absent or non-functional in the other mutants, N-G-C-SGS, N-GLA, and N-GL-C-SL. Hence removal of gelatinase A residues 568–631 disrupts the heparin binding site. ΔTM MT1 MMP was previously purified on heparin-Sepharose (21); in this study, pro and active ΔTM MT1 MMP bound to heparin-agarose, whereas active MT1 MMP\textsuperscript{cat} did not. Hence, there appears to be a heparin binding site in the hemopexin domain of MT1 MMP.

We have previously demonstrated that activation of progelatinase A by MT1 MMP is dependent upon the concentration of the proenzyme (32). In vitro, the rate of activation of progelatinase A was also dependent upon the concentration of MT1 MMP. At low ratios of MT1 MMP:progelatinase A, e.g. 1:125, activation in solution was very slow, but as the MT1 MMP concentration was increased, the activation rate increased. The rate of activation of progelatinase A by MT1 MMP was increased by heparin. Although both ΔTM MT1 MMP and gelatinase A bind to heparin, experiments with MT1 MMP\textsuperscript{cat} and E375A-gelatinase A suggest that the major effect of heparin was exerted on the autolytic step. Hence, the two-step activation process described by Will et al. (20) is likely to be critically dependent upon the concentration and colocalization of both components.

In contrast to the activation of progelatinase A and the C-terminal domain mutants in solution, there were differences when the mutant proenzymes were exposed to membrane preparations from NS0 or HT1080 cells displaying MT1 MMP. Wild-type progelatinase A and proN-G-C-SGG were efficiently activated by these membrane preparations, whereas the mutant proN-G-C-SGS was not. An identical amount of membranes from NS0 cells transfected with vector DNA did not activate any of the proenzymes, indicating a requirement for MT1 MMP for activation of progelatinase A. We have demonstrated by immunolocalization that TIMP2 is bound to CHO cells transiently expressing MT1 MMP, but not to those transfected with vector DNA alone, suggesting that TIMP2 binds to MT1 MMP in transiently expressing cells. Progelatinase A and proN-G-C-SGG, but not proN-G-C-SGS, can bind to CHO cells that are transfected with MT1 MMP, whereas none of the proenzymes bind to cells transfected with vector alone. The pattern of binding of the gelatinase A C-terminal domain mutants to CHO cells expressing MT1 MMP and their activation by NS0 and HT1080 cell membrane preparations containing MT1 MMP correspond to their propensity to bind to TIMP2, i.e. the region between residues 568 and 631 in gelatinase A is required, whereas residues 418–474 do not play a significant role. Thus, it is likely that the proenzymes are localized on the cell surface via TIMP2. These results indicate that, although TIMP2 in the HT1080 membranes was below the level of detection by Western blotting, sufficient TIMP2 remained to ac-

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...for the differences in the efficiency of activation of the mutants. It is also possible that activation occurs in the absence of TIMP2 due to the presence of TIMP3 (which can bind to both MT1 MMP (20) and progelatinase A) or heparan sulfate proteoglycans which might localize progelatinase A at the cell surface. The integrin, αvβ3, has also been reported to act as a receptor for gelatinase A (41), although in our laboratory no significant interaction has been detected in the cellular systems under study.7 Progelatinase A binding to and activation by membrane preparations or cells that were transfected with vector DNA rather than MT1 MMP did not occur, suggesting that MT1 MMP is the only membrane protein required for the interaction with TIMP2 and progelatinase A. The involvement of both TIMP2 and MT1 MMP in the activation process is further substantiated by the potentiation of the activation of progelatinase A after the addition of TIMP2 to TIMP2-depleted membranes. The addition of increasing amounts of TIMP2 to a fixed concentration of MT1 MMP in a membrane preparation derived from HT1080 cells overexpressing MT1 MMP resulted in potentiation at low levels of TIMP2, but then inhibition of progelatinase A activation at the highest concentrations of TIMP2. We propose that this is due to the formation of additional receptors for progelatinase A composed of MT1 MMP and TIMP2 and that activation is inhibited when enough TIMP2 is added to bind and inhibit all the free MT1 MMP. Membranes from HT1080 cells transfected with vector alone did contain some endogenous MT1 MMP but the level was 10 times lower than the MT1 MMP overexpressing cells. Progelatinase A activation was inhibited in all cases by the same concentrations of TIMP2, which were able to potentiate activation in the membranes from cells transfected with MT1 MMP. Since the same concentrations of membrane proteins were present in each experiment, the difference in progelatinase A activation can be attributed directly to the difference in TIMP2 concentration. (Δ128–194)TIMP2 was inhibitory at all concentrations, possibly reflecting its ability to inhibit MT1 MMP and active gelatinase A but its failure to form a progelatinase A “receptor.” Since the balance between the amount of “free” MT1 MMP and MT1 MMP-TIMP2 complex appeared to determine the degree of activation of gelatinase A, we carried out a further experiment where the level of free MT1 MMP remained constant and increasing levels of MT1 MMP membranes:TIMP2 were added. As expected, molar ratios of TIMP2:MT1 MMP up to 3:2 resulted in a potentiation of progelatinase A activation consistent with the idea of extra receptors being formed. As a consequence, more progelatinase A is concentrated in the vicinity of the free MT1 MMP; therefore, the two-step cleavage of progelatinase A, initiated by MT1 MMP (15, 20), is enhanced. When higher molar ratios of TIMP2:MT1 MMP membranes:TIMP2 were added to MT1 MMP, no potentiation was observed. In this case, many of these receptors may be distant from active MT1 MMP molecules so that an inactive ternary complex with progelatinase A persists; this is in accordance with the findings of Strongin et al. (17).

In contrast to previous studies that have been analyzed by zymography, this kinetic study has allowed us to precisely measure the amount of gelatinase A activity generated. The results described in this paper suggest that a crucial step in the cellular activation of progelatinase A is its concentration. Here, we have shown that the activation of progelatinase A by membrane-bound MT1 MMP is dependent upon binding of the C-terminal domain of the proenzyme to TIMP2. TIMP2 is able to bind to the N-terminal domain of MT1 MMP, suggesting that

G. Butler and G. Murphy, unpublished observation.

A. J. Messent, G. Murphy, and J. Gavrilovic, unpublished observations.
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30. Hipps, D. S., Hembry, R. M., Docherty, A. J. P., Reynolds, J. J., and Murphy, G. (1991) Biol. Chem. Hoppe-Seyler 372, 287–296
31. Ward, R. V., Hembry, R. M., Reynolds, J. J., and Murphy, G. (1991) Biochem. J. 278, 179–187
32. Butler, G. S., Will, H., Atkinson, S. J., and Murphy, G. (1997) Eur. J. Biochem. 244, 653–657
33. Fridman, R., Fuerst, T. R., Bird, R. E., Hoyhtya, M., Oelkuct, M., Kraus, S., Komarek, D., Liotta, L. A., Berman, M. L., and Stetler-Stevenson, W. G. (1992) J. Biol. Chem. 267, 15398–15405
34. Libson, A. M., Gittis, A. G., Collier, I. E., Marmer, B. L., Goldberg, G. I., and Lattman, E. E. (1995) Nat. Struct. Biol. 2, 938–942
35. Gohlie, U., Gomis-Ruth, F. X., Crabbe, T., Murphy, G., Docherty, A. J. P., and Bode, W. (1996) FEBS Lett. 378, 126–130
36. Emmert-Buck, M. R., Emonard, H. P., Corcoran, M. L., Krutzsch, H. C., Foidart, J. M., and Stetler-Stevenson, W. G. (1995) FEBS Lett. 364, 28–32
37. Kolkenbrock, H., Hecker-Kia, A., Orgel, D., Ruppitsch, W., and Ulbrich, N. (1994) Biol. Chem. Hoppe-Seyler 375, 589–593
38. Lichte, A., Kolkenbrock, H., and Tchesche, H. (1996) FEBS Lett. 397, 277–282
39. Kolkenbrock, H., Hecker-Kia, A., Orgel, D., Unbrich, N., and Will, H. (1997) Biol. Chem. Hoppe-Seyler 378, 71–76
40. Wallon, U. M., and Overall, C. M. (1997) J. Biol. Chem. 272, 7473–7481
41. Brooks, P. C., Strumblad, S., Sanders, L. C. von Schalscha, T. L., Aimes, R. T., Stetler-Stevenson, W. G., Quigley, J. P., and Cheresh, D. A. (1996) Cell 85, 683–693
42. Stetler-Stevenson, W. G. (1995) Invasion Metastasis 14, 259–268